The expression of the human apolipoprotein genes and their regulation by PPARs

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M.Sc. Thesis
Biochemistry
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June 2008

Abstract

The expression of the human apolipoprotein genes and their regulation by PPARs.
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June 2008

Keywords: nuclear receptors; peroxisome proliferator-activated receptor; PPAR response element; apolipoprotein; lipid metabolism; high density lipoprotein; low density lipoprotein.

Lipids are any fat-soluble, naturally-occurring molecules and one of their main biological functions is energy storage. Lipoproteins carry hydrophobic lipids in the water and salt-based blood environment for processing and energy supply in liver and other organs. In this study, the genomic area around the apolipoprotein genes was scanned *in silico* for PPAR response elements (PPREs) using the *in vitro* data-based computer program. Several new putative REs were found in surroundings of multiple lipoprotein genes. The responsiveness of those apolipoprotein genes to the PPAR ligands GW501516, rosiglitazone and GW7647 in the HepG2, HEK293 and THP-1 cell lines were tested with real-time PCR. The *APOA1, APOA2, APOB, APOD, APOE, APOF, APOL1, APOL3, APOL5* and *APOL6* genes were found to be regulated by PPARs in direct or secondary manners. Those results provide new insights in the understanding of lipid metabolism and so many lifestyle diseases like atherosclerosis, type 2 diabetes, heart disease and stroke.

Acknowledgements

I would like to thank Prof. Carsten Carlberg and Dr. Merja Heinäniemi for being my mentors and introducing me to the world of science. Thanks also to the whole research group of "Gene regulation by nuclear hormones and their receptors" (Department of Biosciences) for sharing knowledge and creating such a humane atmosphere. Especially I would like to thank Dr. Thomas W. Dunlop and Tatjana Degenhardt for inspiring discussions. I am very grateful also to Maija Hiltunen for preparing cells. Thanks also to all the people in the research group of Docent Sami Väisänen for your help and opinions.

Finally, I would like to thank my family and Irina for supporting me during this project.

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Abbreviations

APO	apolipoprotein
ARP0	acidic riboprotein P0
ChIP	chromatin immunoprecipitation
Chr	chromosome
CoA	coactivator
CoR	corepressor
DBD	DNA-binding domain
DMSO	dimethylsulfoxide
DR	direct repeat
ER	everted repeat
FBS	fetal bovine serum
Gln	glutamine
GO	gene ontology
GW501516	2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)1,3thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid
GW7647	2-(4-(2-(1-cyclohexanebutylcyclohexyllureido)ethyl)phenylthio)-2-methylpropionicacid
HDL	high density lipoprotein
HEK293	human embryonic kidney epithelia cell line
HepG2	human hepatoma derived cell line
IDL	intermediate density lipoproteins
IGF	insulin-like growth factor
IgG	immunoglobulin G
IR	inverted repeat
kB	kilo base pairs
K_d	dissociation constant
kDa	kilo Dalton
LBD	ligand-binding domain

LBP	ligand-binding pocket
LDL	low density lipoprotein
LIPE	hormone sensitive lipase
LPL	lipoprotein lipase
LRP	lipoprotein receptor-related protein
LXR	liver X receptor
NR	nuclear receptor
PPAR	peroxisome proliferator-activated receptor
p-PolII	phosphorylated RNA polymerase II
PPRE	PPAR response element
RAR	retinoic acid receptor
RE	response element
Rosiglitazone	5-[4-[N-methyl-N-(2pyridyl)amino)ethoxy]benzyl]thiazolidine-2,4-dione
RXR	retinoid X receptor
TF	transcription factor
Trap220	thyroid receptor-interacting protein 2
TSS	transcription start site
VDR	vitamin D receptor
VLDL	very low-density lipoprotein

1. Introduction

1.1 Nuclear receptors in general – an overview

Human nuclear receptors (NRs) form a superfamily of 48 members. NRs are transcription factors (TF), many of which are ligand-activated and regulate the expression of specific target genes involved in many essential processes, such as reproduction, development and general metabolism (Chawla et al., 2001). Their ligands are either high-affinity hormonal lipids or low-affinity dietary lipids, all which are able to penetrate cellular membranes and reach their receptors in the nucleus or cytosol directly. Also some unexpected ligands have been suggested for NRs, such as glucose for the liver X receptor and PPARs (Mitro et al., 2007, Hostetler et al., 2008). Like other TFs, NRs work in a concert with coactivators (CoAs) and corepressors (CoRs) to achieve primary chromatin remodeling events, which lead to activation or suppression of target gene expression (Glass and Rosenfeld, 2000).

When NRs are in the nucleus and bound to DNA, they modulate transcription by recruiting coregulators and components of the basal transcriptional machinery. When the receptor has no ligand, it recruits transcription-repressing factors known as CoRs to the target gene promoters (Bowen et al., 2004). CoRs, such as nuclear corepressor (NCoR), recruit histone deacetylases (HDACs). ATP-dependent remodeling complexes (ADCRs), such as the nucleosome remodelling and histone deacetylase complex (NURD), are also recruited by NRs in their repressive state to silence transcription by affecting chromatin structure in an ATP-dependent manner (Xue et al., 1998). Together these factors create a chromatin environment that actively reduces transcription. When a specific ligand binds the receptor, it undergoes a conformational change, which leads to CoR abolishment and CoA recruitment (Glass and Rosenfeld, 2000). CoAs can affect in different ways. Certain CoAs form a protein bridge between TFs and the basal transcription machinery enabling the communication between the distal enhancer elements and the proximal promoter (Lewis and Reinberg, 2003). Thus, TFs play an important role in gene expression.

The similar structural organisation of NRs (Figure 1) defines the superfamily and reflects their function as ligand-regulated TFs (Mangelsdorf et al., 1995). The variable N-terminal domain contains in most NRs a ligand-independent transcription activation function (AF-1) (Folkertsma et al., 2004). Moreover, NRs contain a highly conserved DNA-binding domain (DBD) of approximately 70 amino acid residues and also conserved ligand-binding domain (LBD) of approximately 250 amino acid residues. In general, the N-terminal and linker areas of NR proteins are not conserved. The interaction with CoAs and CoRs is controlled via helix 12 (AF-2).

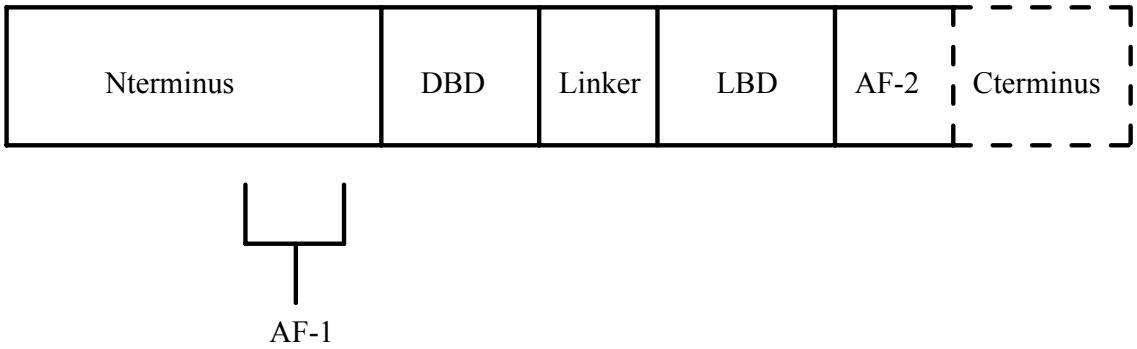

Figure 1. Schematic representation of general structure of a NR (according to Folkertsma et al., 2004). NRs contain five domains. The variable N-terminal domain contains the ligand-independent activation function 1 (AF-1). The conserved DBD recognizes response elements (REs) in target genes. The short variable linker area connects the DBD to the structurally conserved LBD, which is responsible for the ligand specific functions. The activation function 2 (AF-2) –domain (helix 12) is ligand-dependently recruiting coregulators.

The NR superfamily can be divided into three district groups (Table 1) (Chawla et al., 2001, Germain et al., 2006). The first group consists of the classical endocrine receptors, which include the receptors for 3,5,3'triiodothyronine (thyroid hormone receptor, TR), all-*trans* retinoic acid (retinoic acid receptor, RAR), 1α,25dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) (vitamin D receptor, VDR), 17β–estradiol (estrogen receptor α and β, ER), cortisol (glucocorticoid receptor, GR), aldosterone (mineralocorticoid receptor, MR), progesterone (progesterone receptor, PR) and dihydrotestosterone (androgen receptor, AR). Those classical endocrine receptors bind their ligands at high affinity with a dissociation constant (K_d) of 0.1 to 1 nM (Chawla et al., 2001). For adopted orphan NRs ligands have been subsequently identified. Members of this group include receptors for fatty acids (peroxisome proliferator–activated receptors, PPARs), oxysterols (liver X receptor, LXR), bile acids (farnesoid X receptor, FXR) and xenobiotics (pregnane X receptor, PXR and constitutive androstane receptor, CAR). The ligands are low-affinity dietary lipids like cholesterol derivatives and bile acids with a K_d up to 10 μM (Chawla et al., 2001). Adopted orphans preferably bind DNA as heterodimers with retinoid X receptor (RXR), which itself is a member of this second subgroup. For the remaining NRs (21), the so-called true orphan, no ligand has yet been identified. Phylogenic analyses of the NR superfamily indicate that the first NRs may have been true orphan and a ligand has been adapted at a later step in evolution (Escriva et al., 2000). This observation suggests that some of the present orphan NRs have still not acquired a ligand or there are undiscovered ligands for many present orphans (Ingraham et al., 2005). The true orphan NRs prefer to bind DNA as homodimers, some as RXR-heterodimers and some, like the estrogen-related receptor α (ERRα), as monomers (Giguère, 1999). The physiological significance of many orphan receptors is poorly known, but they are definitely active bodies in development and adult physiology and they often regulate the action of classic liganded receptors (Benoit et al., 2006).

Table 1. The human NRs and their ligands (adapted from Germain et al., 2006).

Subfamily	Name	Ligand(s)
Endocrine receptors, receptors with high affinity for ligand	GR	Cortisol, dexamethasone, RU486
	MR	Aldosterone, spirolactone
	PR	Progesterone, medroxyprogesterone acetate, RU486
	AR	Testosterone, flutamide
	ERα	Estradiol-17, tamoxifen, raloxifene
	ERβ	Estradiol-17, various synthetic compounds
	RARα,β,γ	All-*trans* retinoic acid
	TRα,β	Thyroid hormones
	VDR	Vitamin D, lithocholic acid
Adopted orphan receptors, receptors with low affinity for ligands	RXRα,β,γ	9-*cis*retinoic acid
	PPARα	Fatty acids, leukotriene B4, fibrates
	PPARβ	Fatty acids
	PPARγ	Fatty acids, prostaglandin J2, thiazolidinediones
	LXRα,β	Oxysterols, T0901317, GW3965
	FXR	Bile acids, fexaramine
	PXR	Xenobiotics, 16 -cyanopregnenolone
	CAR	Xenobiotics, phenobarbital
	ERRβ,γ	DES, 4-OH tamoxifen
	RORα	Cholesterol, cholesteryl sulfate
	RORβ	Retinoic acid
Orphan receptors, receptors without ligand	SF-1	
	LRH-1	
	DAX-1	
	SHP	
	TLX	
	PNR	
	GCNF	
	HNF-4	
	TR2, 4	
	NGF1-Bα,β,γ	
	RORγ	
	RVRα,β,γ	
	ERRα	
	COUP-TFα,β,γ	

NRs bind DNA through zinc-finger motifs (zinc-finger C4-type) in their DBD. The DBD of all NRs contains two α-helices perpendicular to each other (Shaffer and Gewirth, 2002; Carlberg, 2004). One of these α-helices is located behind the zinc finger and so is inserted into the major groove of a hexameric DNA sequence. Since the recognition helix is highly conserved throughout the NR family, almost all NRs recognize common DNA sequences. These sequences, mostly formed by two hexamers, are called REs. The hexameric REs are distinguished into three different configurations: direct repeats (DRs) inverted repeats (IRs) and everted repeats (ER) (Figure 2). The general consensus NR RE sequence is RGKTSA (R = A or G, K = G or T, S = C or G) (Carlberg, 1995). This is, however, not true for AR, GR, MR and PR NRs, which bind the sequence RGAACA (Germain et al., 2006). In general, most members of the NR superfamily bind DNA either as homo- or intra-familial heterodimeric complexes on REs that are found by two hexameric binding complexes spaced by different numbers of nucleotides (Mangelsdorf and Evans, 1995).

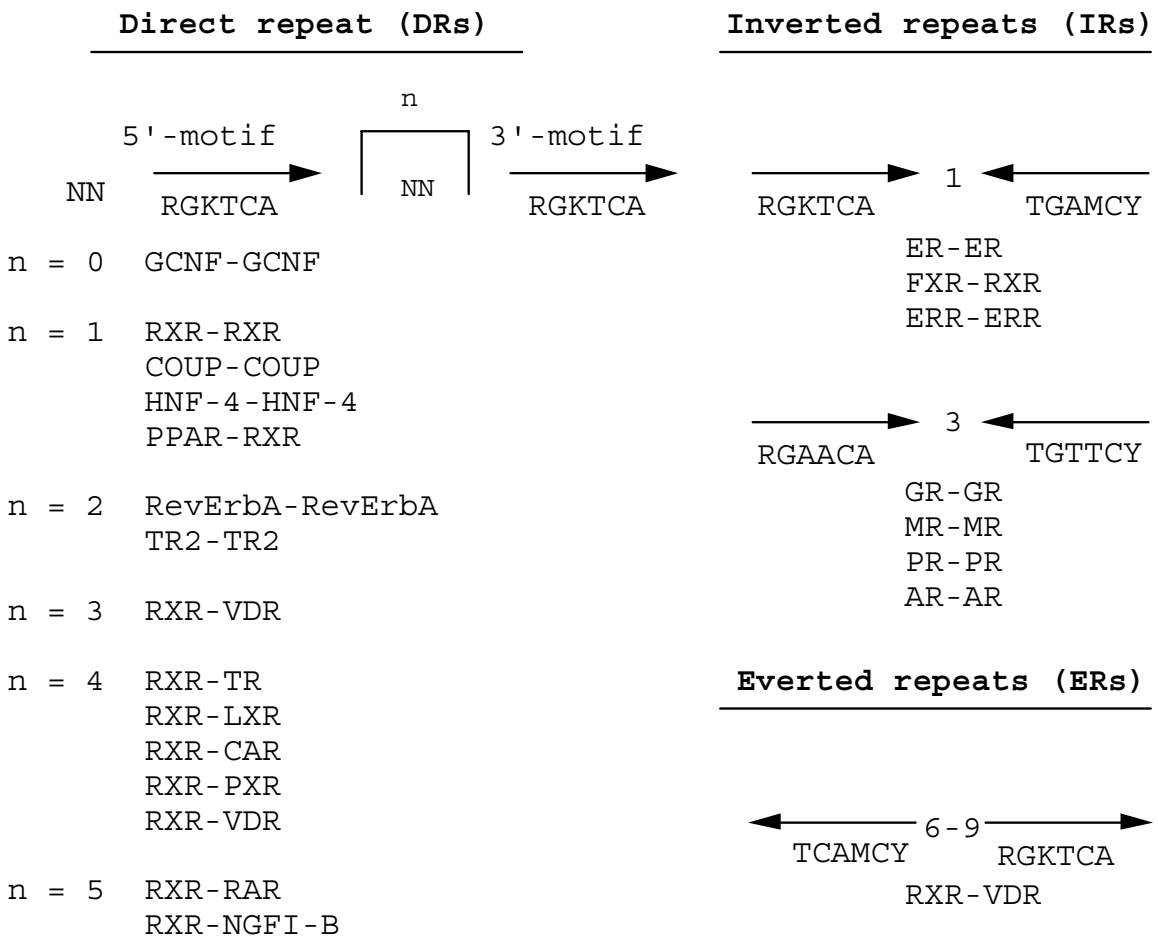

Figure 2. The types of REs (according to Carlberg et al., 2004). The most preferred RE for each type of homo- or heterodimeric NR complex is shown. "NN" represents 5'-flanking sequences and "n" amount of nucleotides between the hexameric NR bidning sequences.

1.2 Screening of TFs binding sites

Fields such as genomics and systems biology are built on the synergism between computational and experimental techniques. This type of synergism is especially important in accomplishing goals like identifying all functional transcription factor binding sites in vertebrate genomes. Positional information on TF binding sites in whole genomes is useful to identify target genes of specific TFs. Furthermore, such information is helpful to generate models on the regulation of genes that are investigated. Since the completion of the first draft sequence of the humangenome in 2001, *in silico* methods have been used to find TFs binding sites.

The most obvious application of finding TFs is the weight matrix (WM). WM, where known, experimentally defined binding sites collected from publications are aligned and used to form a matrix that represents the frequencies of four DNA bases in TFs binding sites (Stormo, 2000). They are easy to use and so then are come really popular. WM is easy to generate but then the problem is to determine whether the sequence is likely to be sampled from the matrix or not. This is done by use of threshold value which is crucial to the false detection level of TF binding sites.

Recently, alternatives methods that exploit experimental binding data set were developed (Hallikas and Taipale, 2006). Those scoring matrices perform well in the detection of near consensus REs variants and also approximates, to a certain extant their relative binding strengths. This is essential, when are talking about NRs. Without elegant fine adjustment of *in silico* screening it is easy to lose or mix up interesting findings. There is still a clear need to incorporate biological knowledge and principles into the methods in order to elucidate the findings of the *in silico* screening.

1.3 Peroxisome proliferator-activated receptors

In the early 1990s the PPARs were cloned. They were the first NRs that mediate effects of synthetic compounds. The first member, PPARα, was found responsive to peroxisome proliferators, in liver of rodents (Issemann and Greese. 1990). Three PPAR subtypes have been identified, α, γ and δ/β, which show a tissue-specific expression pattern. PPARα is highly expressed in brown adipose tissue and liver and to a lesser extend in kidney, heart and skeletal muscle. PPARγ is mainly expressed in adipose tissue and plays an important role in adipocyte differentiation and lipid storage. PPARγ is present at low levels in skeletal muscle; it can be also detected in macrophages, where it induces their differentiation and where it is involved in lipid efflux. PPARδ/β is found in many tissues, but it shows the highest expression in the gut, kidney and heart (Desvergne and Wahli, 1999). All three PPARs are also involved in different aspects of inflammation (Kostadinova et al., 2005). In general, the functions of all three PPARs are overlapping to each other and even with that of other NRs.

PPARs belong to the adopted orphan receptor group of the NR superfamily. Like the other members of the family, PPARs are considered to be ligand-dependent TFs, meaning that activation of the target gene depends on the binding of the ligand to the receptor. However, recently evidence for a ligand-independent association with cofactors was shown (Molnár et al., 2005). Some ligands are shared between the three subtypes, such as polyunsaturated fatty acids and oxidized fatty acids (Kersten et al., 2000a). An alternative activation pathway of PPAR-RXR heterodimers occurs through ligand binding to RXR. The natural RXR ligand, 9-*cis* retinoic acid, as well as synthetic RXR-selective compounds can activate a PPRE– driven reporter gene in a PPAR-RXR-dependent manner (Desvergne and Wahli, 1999, Feige et al., 2005).

1.3.1 PPREs

PPREs are comprised of a direct repeat of two core recognition motifs, AGGTCA, spaced by one nucleotide, so-called DR1-type REs (Kliewer et al., 1992). The first natural PPRE was found in the promoter of the *acyl-CoA oxidase* gene and all subsequently identified natural PPREs fulfil this DR1 criterion (Dreyer et al., 1992; Tugwood et al., 1992). However, this definition has specified later on: an extended 5' – half site, an imperfect core DR1 and an adenine as the spacing nucleotide between the two hexamers have been proposed as further determinants of PPREs (IJpenberg et al., 1997).

PPARs regulate transcription as heterodimers with RXRs. The receptor dimer binds the REs with PPARs occupying the 5'-hexamer (Glass, 1994). This is the abnormal binding order of NR-RXR heterodimers. For different NRs an influence of the 5'-flanking sequence is discussed. The 5'-flanking effect has been shown on constitutive androstane receptor (CAR) (Frank et al., 2003). Also for PPARs, especially for PPARα, it looks that an extended 5'–flanking sequence of seven nucleotides influences DNA binding, and thus may contribute to the subtype specificity (Juge-Aubry et al., 1997). Some PPAR target genes with known PPREs and *in silico* defined binding strength are listed in table 2.

Table 2. List of the some known human PPAR target genes with their PPREs. REs location to the TSS and relative strength to the consensus sequence where consensus is 100 and receptor is PPARγ. Adapted from (Heinäniemi et al., 2007).

Gene	Sequence	Location	REs class and relative strength
ACBP	GGGACAGAGGTCC	-957	None
ACOX1	AGGTCACTGGTCA	-1918	None
ADRP	AGGTGAAAGGGCG	-2357	Strong (40)
APOA1	GGGGCAGGGGTCA	-214	None
APOA2	AGGGTAAAGGTTG	-734	Weak (1)
APOA5	AGGTTAAAGGTCA	-271	Strong (70)
APOC3	TGGGCAAAGTTCA	-87	Weak (5)
APOE	AGGGGAAGGGTCA	5655	Strong (40)
CPT1β	AGGGAAAAGGTCA	-774	Strong (40)
CPT2	GTGCTGAAGGTCA	-97	None
CYP1A1	GGGGCAGAGGTCA, AGCCCTGAGGTCA	-931, -531	Weak (3), None
CYP27A1	CGCCCAGAGTTCA	-291	None
FIAF	AGGGGAAAGGTCG	3270	Strong (40)
FXR	AGGTCAAGTGCCA, TGTCCATGAGGCA	-239, -80	Weak (5), None
GSTA2	AGGTCATCACCGA, GCAGGAAGGATCA AGGACAAAGATTA	-792, -746, -549	None, None, None
I-BABP	GCCAGCAGGGTCA	-198	None
I-BAT	AGGCCAGAGGTCA	-1577	Weak (3)
Insig-I	AGCCAGAAGGTCA	-757	None
LXRα	CGTACAAAGTTCA	-2216	None
P16	AGGAGACAGGACA	-1023	None
PEX11A	GGGTGAGAGGTGA	-8400	Weak (3)
PLTP	GGGTCAGTGACCCA	-339	None
PXR	AGGACAGAGCTCT	-1350	None
Resistin	GAGGAGAAAGTTC	-2102	None
SR-B1	AGGAGAAAGGGGA	-472	Weak (5)
SULT2A1	AGGTGAAAGGTAA	-5949	Strong (70)
Transferrin	AGGTCAAGATTG	-76	None
UCP3	GGTTTCAGGTCAG, TGACCTTTGGACT	-67, -281	None, Strong (20)
UGT1A9	AAATCAGAGGTGA	-719	None
UGT2B4	AGATTAAAGTTCA	-1193	None

1.3.2 PPARα

Several compounds bind with high affinity to PPARα, including long-chain unsaturated fatty acids, such as linoleic acid, branched, conjugated and oxidized fatty acids, such as phytanic acid and conjugated linoleic acid, and eicosanoids, such as leukotriene B4 (Desvergne and Wahli, 1999). Also, synthetic ligands from the fibrate family, such as GW7647 (2-(4-(2-(1-Cyclohexanebutyl-3-cyclohexylureido)ethyl)phenylthio)-2- methylpropionic acid), activate the receptor.

Much of the function of PPARs can be extrapolated from the identity of their target genes, most of which belong to lipid transport and metabolism pathways (Planavila et al., 2006). PPARα has mostly been studied in the context of liver. The target genes of PPARα are a relative homologous group of genes that participate in aspects of lipid catabolism, such as fatty acid uptake through membranes, fatty acid binding in cell, fatty acid oxidation (in microsomes, peroxisomes and mitochondria) and lipoprotein assembly and transport (Kersten et al., 2000b). However, extensive lists of direct targets, for which binding sites have been identified, exist only for mouse PPARα, but not in human (Mandard et al., 2004). To use fatty acids in energy production requires the uptake of free fatty acids carried by lipoproteins. In the mouse liver, PPARα up-regulates the *fatty-acid transport protein-1*. Also the *CD36* gene (APO receptor) has been described as a target of PPARα in human and mouse (Frohnert et al., 1999; Sato et al., 2002).

The *long-chain fatty acyl-CoAsynthetase* gene is described as target of PPARα in rat (Schoonjans., et al 1996). This gene is responsible of fatty acid translocation in mitochondria. PPARα is increasing β–oxidation by up-regulating *acyl-CoA-oxidase* (*ACOX1*), which has an orthologous PPAR-regulated human gene (Tugwood et al., 1992; Varanasi et al., 1996). So PPARα not only stimulates energy production, but also shortens long-chain fatty acids thus preventing lipid accumulation and toxicity. Moreover, PPARα up-regulates the apolipoprotein (APO) genes *APOAI* and *APOA2*, which are the major compound of high density lipoprotein (HDL) (Vu-Dac et al., 1994; Vu-Dac et al., 1995). PPARα is also up-regulating HDLs surface protein *phospholipid transfer protein* (*LPL*) and receptor, which is located in liver cells outer membrane *scavenger receptor-class B type I* (*SR-BI*) (Malerød et al., 2003). PPARα is down-regulating *APOC3,* which is an elevating triglyceride level by secondary effects (Hertz et al., 1995; Li et al., 1995). Finally, the human *APOA5* gene, which is involved the maintenance of normal triglyceride level, is a direct PPAR target (Prieur et al., 2003).

1.3.3 PPARγ

PPARγ is activated by ligands of the glitazone class, like troglitazone, pioglitazone and rosiglitazone (5-((4-(2-(methyl-2-pyridinylamino) ethoxy)phenyl)methyl)- 2,4-thiazolidinedione), which are used for the treatment of type 2 diabetes (Kallen and Lazar, 1996; Maeda et al., 2001). Natural PPARγ ligands are 15-deoxy-Δ12,14-prostaglandine J_2 and also linoleic acid acts as an activator for this receptor (Krey et al., 1997).

In humans there are two isoforms of PPARγ. The longer isoform PPARγ$_2$ has 28 additional amino acids in the N-terminus and has been characterized as the master regulator of the formation of fat cells and their normal function (Barak et al., 1999; Rosen et al., 1999). PPARγ$_2$ influences mainly the storage of fatty acids in the adipose tissue to gether with the CCAAT/enhancer binding proteins (C/EBPs), especially C/EBPα. PPARγ$_2$ is part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into fat cells (Farmer, 2005, Darlington et al., 1995, Tontonoz et al., 1995a; Tontonoz et al., 1994b). Most of the PPARγ target genes in adipose tissue are directly implicated in lipogenic pathways.

PPARγ promotes insulin sensitivity through altering the communication between adipocytes, muscle and liver cells by inducing the expression of the insulin-sensitizing factor *adiponectin*, but PPARγ is also linked to insulin resistance by lipotoxicity hypothesis (Kershaw and Flier, 2004). It states that abnormal accumulation of triglycerides and fatty acyl-CoA in both muscle and liver cells can result in insulin resistance (Shulman, 2000). The system by which insulin is regulated and fine-tuned by PPARγ is not fully understood.

The immune system and inflammation reactions play a critical role in development of atherosclerosis. Recently, PPARγ agonists have been found to improve the symptoms of the disease. The main impact of PPARγ is the prevention of the transformation of macrophages into foam cells. Oxidized low-density lipoproteins (ox-LDL) are cumulating in macrophages, which causes transformation into foam cells. Foam cells secrete cytokines that promote inflammatory reaction and smooth muscle cell proliferation. PPARγ has been shown to counteract these processes (Frohnert et al., 1999; Chawla et al., 2001a; Sato et al., 2002).

1.3.4 PPARδ/β

PPARδ/β binds both unsaturated and saturated fatty acids, but with lower affinity than PPARα. Also for this receptor synthetic ligands exist. One example is GW501516 (2-Methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid).

PPARδ/β is required for placental development and is involved in the control of lipid metabolism. The best-characterized function of PPARδ/β is the role in the control of cell proliferation, differentiation and survival, especially in keratinocytes (Tan et al., 2001; Tan et al., 2002). The receptor exerts its anti-apoptotic functions through increased expression of the *integrin like kinase* (*ILK*) and the *pyruvate dehydrogenase kinase 1* (*PDK1*) genes, which are important in signalling pathways that control cell adhesion, proliferation and survival (Yin et al., 2006; Burdick et al., 2007). ILK and PDK1 phosphorylate and activate the survival factor AKT1 (Di-Poï et al., 2002). Because all of these proteins are ubiquitously expressed, it is likely that PPARδ/β participates in the regulation of many cell functions that are involved in the development of tumors, when uncontrolled. In addition, PPARδ/β has a role in skin and wound healing (Wahli, 2002).

1.4 Lipids and lipoproteins

Lipids are fat-soluble, naturally-occurring molecules, such as fats, oils, waxes, cholesterol, sterols, fat-soluble vitamins (vitamins A, D, E and K), monoglycerides, diglycerides, phospholipids and others. The main biological functions of lipids include energy storage, acting as structural components of cell membranes, and being important signalling molecules. Lipids also encompass fatty acids and their derivatives as well as other sterol-containing metabolites like cholesterol. Lipids play diverse and important roles in nutrition and health. Many lipids are vital for life. However, there is also considerable awareness that abnormal levels of certain lipids are risk factors for many common diseases in industrial countries. High fat intake contributes to increased risk of obesity and so risk of metabolic syndrome, diabetes and atherosclerosis.

A lipoprotein is a biochemical assembly that contains both proteins and lipids. The lipids or their derivatives may be covalently or non-covalently bound to the proteins. Many enzymes, transporters, structural proteins, antigens, adhesins and toxins are lipoproteins. Examples include the high density and low density lipoproteins of the blood, the transmembrane proteins of the mitochondrion and the chloroplast. Lipoproteins in the circulation carry hydrophobic lipids in the water/salt-based blood environment. The protein particles have charged groups on their surface and non-polar groups inward, so that triglyceride-fats and cholesterol are carried internally, shielded by the protein particle from the water.

1.4.1 Lipoprotein metabolism

Lipids that are absorbed from food by the intestine are dissolved in body fluids and then transported all over the body (Figure 3). In the exogenous pathway, dietary esterified triacylglycerols and cholesterol are combined with apolipoprotein B-48 (APOB-48), phospholipid and free cholesterol to produce nascent chylomicrons in the enterocyte (Karpe at al., 1996). Chylomicron particles (CM) are then secreted into lymphatic vessels and flow into the circulation, where they acquire apolipoprotein E (APOE) and apolipoprotein C2 (APOC2) from other lipoproteins (Redgrave, 1983). Lipoprotein lipase (LPL) is activated by APOC2, which causes triacylglycerols to break from chylomicrons. After chylomicrions enzymatic processing chylomicron remnants (CR) remain and they are then removed from the circulation by hepatic APOE/APOB receptors (LRP (LDL-related protein), LDL-R (LDL receptor)).

In the endogenous pathway very low-density lipoproteins (VLDLs) transport hepatically synthesized triacylglycerols and cholesterol in the circulation to extrahepatic tissues (Mahley et al., 1984). Nascent VLDLs circulate in blood and pick up APOC2 and APOE donated from high-density lipoproteins (HDLs). At this point, the nascent VLDL becomes a mature VLDL. Nascent VLDLs contain one molecule of apolipoprotein B-100 (APOB-100) per particle. VLDLs differ in size according to their triacylglycerol content. The larger VLDL pieces are hydrolyzed by LPL, which is activated by APOC2 and inhibited by apolipoprotein C3 (APOC3), hydrolysis of the VLDL particle releases glycerol and fatty acids (Shachter, 2001). Most of the remnant particles formed by this process are removed directly by the liver. Some larger VLDL species are, however, subjected to further LPL-mediated lipolysis, resulting in the formation of small dense LDL particles. The hydrolyzed VLDL particles are now called intermediate density lipoproteins (IDLs) or VLDL remnants. IDLs can circulate and, via an interaction between APOE and the LDL receptor and/or LRP, be absorbed by the liver, or they can be further hydrolyzed by hepatic lipase converting to IDL remnants, which are also called low density lipoproteins (LDLs) (Chappell and Medh, 1998). LDLs are the major carrier of cholesterol in the circulation. The cellular uptake and degradation of LDL particles is facilitated by a saturating mechanism involving the interaction of the APOB-100 component of LDL with LDL receptors.

In humans, excess cholesterol from peripheral tissue is transported back to the liver by a process called reverse cholesterol transport (RCT) (von Eckardstein et al., 2001). Apolipoprotein A1 (APOA1) discs are produced by enterocytes and hepatocytes or dissociate from triacylglycerol-rich lipoproteins following lipolysis by LPL. The lipid-poor APOA1-containing particles interact with peripheral cells and acquire phospholipids and free cholesterol (FC) through ATP-binding cassette protein A (ABCA1), and possibly by scavenger receptor B1 (SR-B1) (Bewer, 2004). Phospholipid transfer protein (PLTP) also facilitates the first step of RCT by transferring phospholipids from cellularmembranes and lipoprotein surfaces to form pre-HDLs (Fielding and Fielding, 1995). Once associated with nascent HDL, free cholesterol is esterified by the enzyme lecithin:cholesterol

acyltransferas (LCAT). The nascent HDL (pre-HDLs) particles are converted into spherical small dense HDL_3. The maturation of HDL_3 particles requires further acquisition of free cholesterol and subsequent cholesterol esterification converts HDL_3 into larger, less dense cholesterol-rich HDL_2 particles. The majority of free cholesterol in HDL can be taken up selectively by the liver through the action of SR-B1. Cholesteryl ester can also be selectively transferred to APOB-containing lipoprotein in exchange for triacylglycerols through the action of cholesteryl ester transfer protein (CETP).

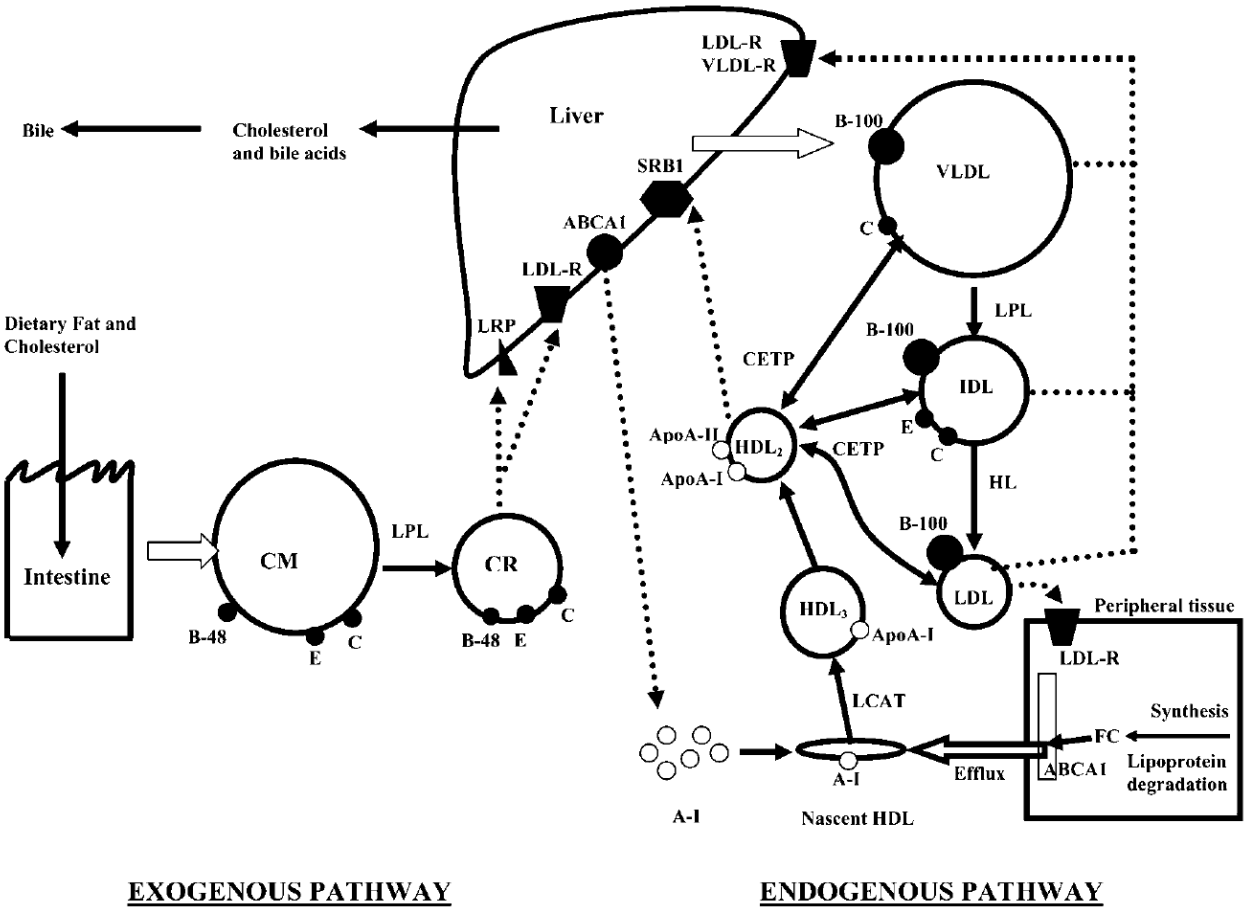

Figure 3. Lipoprotein metabolism adapted from (Dick et al., 2004). Abbreviations: CM, chylomicron; CR, chylomicron remnant; FC, free cholesterol; HL, hepatic lipase; LDL-R, LDL receptor; VLDL-R, VLDL receptor.

1.4.2 Apolipoproteins

Apolipoproteins are a homologous gene family that is mostly expressed in liver tissue (Duchateau et al. 2001; Dayhoff, 1976). They are the major components of HDLs and LDLs so they are responsible for transporting fatty acids and cholesterol in the blood circulation and they bind receptors, which control the intake of the lipoproteins. Many apolipoprotein gene family members are located in gene clusters on different chromosomes (Myklebost and Rogne 1988; Karathanasis, 1985). There are eight well-characterized apolipoproteins: *APOA1*, *APOA2*, *APOA5*, *APOB*, *APOC1*, *APOC2*, *APOC3*, and *APOE*. Some of them are already identified as PPAR target genes. Not so well known apolipoproteins are: *APOA*, *APOC4*, *APOD*, *APOH*, *APOL* (*1-6*), *APOM*, *APOO* and *APOOL*. Apolipoproteins and their lipoprotein particles are listed in table 3.

Table 3. Apolipoproteins location, lipoprotein particles and regulation.

Gene	Chr	PPAR targed (established)	Lipoprotein particle
APOA (*LPA*)	6	No	LDL
APOA1	11	Yes	HDL
APOA2	2	Yes	HDL
APOA4	11	Yes	chylomicron, HDL
APOA5	11	Yes	chylomicron, VLDL, HDL
APOB	2	No	chylomicron, CM, LDL, IDL
APOC1	19	No	VLDL, HDL, LDL
APOC2	19	No	chylomicron, CR, IDL, VLDL
APOC3	11	Yes	VLDL
APOC4	19	No	unknown
APOD	3	No	HDL
APOE	19	Yes	chylomicron, CR, IDL, VLDL
APOF	12	No	HDL
APOH	17	No	cytoplasm
APOL (1-6)	22	No	HDL
APOM	17	No	HDL, LDL, TGRLP
APOO	X	No	unknown
APOOL	X	No	unknown

APOA (LPA)

APOA is a highly polymorphic protein: its size varies over range of approximately 500 kDa due to number of tandem repeats (Kamboh et al. 1991). APOA links to LDL forming one disulfide bond to APOB-100 surface protein forming Lp(a) lipoprotein. Elevated plasma levels of Lp(a) are associated with increased risk for atherosclerosis and its manifestations: myocardial infarction, stroke and restenosis (Mancini et al., 1995).

APOA family

Apolipoprotein A1 is the major apolipoprotein of HDL and is a relatively abundant plasma protein. It is a single polypeptide chain with 243 amino acids (Brewer et al., 1978). The protein promotes cholesterol efflux from tissues to the liver for excretion (Zhang et al., 2003). It is a cofactor for lecithin cholesterolacyltransferase (LCAT), which is responsible for the formation of most plasma cholesteryl esters (Jonas, 2000). APOA1 is also isolated from prostacyclin stabilizing factor (PGI2), and thus may have an anti-clotting effect. Defects in the gene encoding *APOA1* are associated with HDL deficiencies. Also APOA1 mimetics are suggested to substantially increase HDL cholesterol levels, which can be beneficial on cardiovascular events (Toth, 2007). Like apolipoprotein A1, APOA2 is a major apolipoprotein in HDLs. APOA2 controls the levels of free fatty acids in plasma. Mouse studies indicate that APOA2 plays a complex role in lipoprotein metabolism, with some antiatherogenic properties such as the maintenance of a stable HDL pool, and other proatherogenic properties, such as decreasing clearance of atherogenic lipoprotein remnants and promotion of insulin resistance (Weng and Breslow, 1996, Blanco-Vaca et al., 2001). Apolipoprotein A4 (APOA4) is a component of chylomicrons and high-density lipoproteins. APOA4 protects against atherosclerosis by inducing cholesterol transportation from tissues to liver for elimination (Duverger et al., 1996). Apolipoprotein A5 (APOA5) is an important determinant of plasma triglyceride levels in an age-independent manner (Martin et al. 2003). It is a component of several lipoprotein fractions including VLDL, HDL and chylomicrons. It is believed that APOA5 affects lipoprotein metabolism by interacting with *LDL-R* gene family receptors. Notably, whole APOA gene family is referenced as PPAR targets (Vu-Dac et al., 1994; Vu-Dac et al., 1995, Prieur et al., 2003, Nagasawa et al., 2007).

APOC family

Apolipoprotein C1 (APOC1) is a specific inhibitor of cholesteryl ester transfer protein (CETP) (Gautier et al., 2002). CETP is a plasma protein that facilitates the transport of cholesteryl esters and triglycerides between the lipoproteins. It collects triglycerides from VLDL or LDL and exchanges them for cholesteryl esters from HDL (and vice versa). Apolipoprotein C2 (APOC2) is a necessary cofactor for the activation of lipoprotein lipase, the enzyme that hydrolyzes triglycerides in plasma and transfers the fatty acids to tissues. Apolipoprotein C3 (APOC3) is a VLDL protein. APOC3 inhibits lipoprotein lipase and hepatic lipase; it is thought to delay catabolism of triglyceride-rich particles. It is up-regulated by the NRs REV-ERBα and RORα and down-regulated by PPARs (Coste and Rodriguez, 2002). Moreover, the *APOA1*, *APOC3* and *APOA4* genes are closely linked in both rat and human genomes and transcription is controlled by a common enhancer (Zannis et al., 2001). Increase in *APOC3* gene expression is associated development of hypertriglyceridemia (Vu-Dac et al., 1998).

APOB

Apolipoprotein B (APOB) is the primary apolipoprotein of chylomicrons and LDLs, but is also present in IDLs. It occurs in the plasma in two main forms, APOB-48 and APOB-100. The first is synthesized exclusively by the gut, the second by the liver. APOB-100 is present in LDLs and there it activates the LDL receptor, which starts LDL endocytosis. APOB-48 is generated, when a stop codon (UAA) is created by RNA editing as a result of the human APOB mRNA editing protein (BEDP). APOB-100 and APOB-48 share a common N-terminal sequence, but APOB-48 lacks APOB-100's C-termin. It is well established that high APOB-100 levels are associated with coronary heart disease, and are even a better predictor than the LDL level (McCormick et al., 1996, Farese et al., 1995).

APOD

Apolipoprotein D (APOD) is an atypical apolipoprotein and, based on its primary structure is a member of the alpha (2 mu)-microglobulin protein family of carrier proteins, also known as lipocalins (Pervaiz and Brew, 1987). APOD can bind cholesterol, progesterone, pregnenolone, bilirubin and arachidonic acid, but it is unclear if anyof these represent its physiological ligands. APOD is a protein component of HDLs in human plasma, comprising about 5 % of total HDL (Fielding and Fielding, 1980). APOD is closely associated with the enzyme LCAT (Jonas, 2000). The APOD gene is expressed in many tissues, with high levels of expression in spleen, testes and brain. It is associated with increased risk of breast cancer. It also accumulates at sites of regenerating peripheral nerves and in the cerebrospinal fluid of patients with Alzheimer's disease. APOD may, therefore, participate in maintenance and repair within the central and peripheral

nervous systems. APOD is likely to be a multi-ligand, multi-functional transporter. There are evidences that APOD could transport a ligand from one cell to another within an organ, scavenge a ligand within an organ for transport to the blood or transport a ligand from the circulation to specific cells within a tissue (Rassart et al., 2000).

APOE

Apolipoprotein E (APOE) is a main apopliporotein of chylomicrons and VLDLs. Chylomicron remnants and VLDL remnants are rapidly removed from the circulation by receptor-mediated endocytosis in the liver and peripheral cells. There are seven currently identified mammalian receptors for APOE, which belong to the evolutionarily conserved LDL receptor gene family. APOE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. The *APOE* gene is mapped to chromosome 19 in a cluster with *APOC1* and *APOC2* genes. More recently, it has been studied for its role in several biological processes not directly related to lipoprotein transporting, including Alzheimer's disease, immunoregulation and cognition (Saunders et al., 1993; Corder et al., 1993; Van den Elzen et al., 2005).

APOF

Apolipoprotein F (APOF) is one of the minor apolipoproteins in human plasma (Olofsson et al., 1978). It is part of HDL and its subfractions. Cholesterol ester transfer protein (CETP) moves triglyceride and cholesteryl ester between lipoproteins. APOF adjusts the action of CETP by controling CETP-facilitated lipid flux among HDL subfractions. APOF inhibits VLDL to HDL_2 transfer to one-half of the rate of VLDL to LDL and it stimulates the VLDL to HDL_3 transfer. Moreover, APOF directs CETP-mediated remodelling of HDL_3 and HDL_2 particles in subclass-specific ways, so APOF is important regulator of HDL metabolism (Paromov and Morton, 2003).

APOH

Apolipoprotein H (*APOH*), previously known as *β_2-glycoprotein I* or *beta-2 glycoprotein I*, is a multifunctional apolipoprotein (Nakaya et al. 1980). One of its functions is to bind cardiolipin in mitochondrial inner membrane. When bound the structure of cardiolipin and APOH both undergo large changes in structure (Borchman et al., 1995). APOH has been implicated in a variety of physiologic pathways including lipoprotein metabolism, coagulation, inhibition of serotonine release and the production of anti-phospholipid autoantibodies (Shi et al., 2004, Sanghera et al. 1997, Nimpf et al., 1985).

APOL 1-6

Apolipoprotein L (APOL) proteins belong to the HDL family, which plays a central role in cholesterol transport. The six *APOL* genes are clustered on chromosome 22q12.3 and are the result of tandem gene duplication, whereas *APOL5* and *APOL6* are more distantly located. The *APOL1-APOL4* cluster might contribute to the substantial differences in the lipid metabolism of humans and mice (Monajemi et al., 2002). Also there are evidences anti-parasite effects of APOL1 protein (Shiflett et al., 2005).

APOM

Apolipoprotein M (APOM) is a minor component of HDLs and LDLs as well as triglyceride-rich lipoproteins (TGRLP) (Xu and Dahlbäck 1999). It has two transcript variants. APOM is important for the formation of pre-beta-HDL and cholesterol efflux to HDL and thereby inhibits formation of atherosclerotic lesions (Wolfrum et al., 2005). APOM is also associated with Alzheimer's disease (Kabbara et al., 2004). *APOM* gene is a direct target of liver receptor homolog-1 (NR5A2), which is an orphan NR (Venteclef et al., 2008).

2. Aims of the present study

Information regarding PPAR binding-site specificity is complete, but it has not been extensively tested in practise (Heinäniemi et al., 2007). Testing of this *in silico* method at the example of the regulation of the apolipoprotein gene family by PPARs is the focus of this study. The specific aims are:

1. Collect precise and more extensive data for comparison of the *in silico* screening and *in vitro* methods.

2. Perform an *in silico* screening to identify new and established PPREs from apolipoproteins genes.

3. Measure the expression level of all 23 apolipoproteins by quantitative real-time PCR in response to different PPAR agonist in different human cell lines.

4. Perform chromatin immunoprecipitation (ChIP) assay, in order to study the association of PPAR and RXR with the apolipoproteins gene TSS and their association with other factors, such as CoAs, mediators and p-Pol II

3. Material and methods

3.1 Materials

Ligands

NR ligands used in the studies were GW7647 [2-(4-(2-(1 cyclohexanebutylcyclohexyllureido)ethyl)phenylthio)-2 methylpropionicacid] (Alexis Biochemicals, San Diego, CA, USA), rosiglitazone [5-[4-[N-methyl-N-(2pyridyl)amino)ethoxy]benzyl]thiazolidine-2,4-dione] (Dr. M.W. Madsen, LEO Pharma, Ballerup, Denmark) and GW501516 [2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)1,3thiazol-5-yl)-methylsulfanyl)phenoxy-acetic acid] (Alexis Biochemicals, San Diego, CA, USA). All these ligands were diluted in DMSO.

Cell culture

HEK293, THP-1 and HepG2 cell lines were grown in medium that was supplemented with 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Cells were grown in humidified 95 % air / 5 % CO_2 incubator. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containig 10 % fetal bovine serum (FBS). THP-1 and HepG2 were cultured in Roswell Park Memorial Institute's medium (RPMI 1640) containing 10 % FBS. When split for experiment, the cells were grown overnight in phenol-red free DMEM supplemented with 5 % charcoal-stripped fetal bovine serum (FBS). FBS was stripped of lipophilic compounds by stirring it with 5 % (w/v) activated charcoal (Sigma-Aldrich) for 3 h at room temperature (RT). Charcoal was then removed by centrifugation and sterile filtration.

3.2 Methods

3.2.1 *Ex vivo* methods

RNA extraction

Cells were seeded into 6-well plates and grown overnight to reach a density of 60–70 %. The cells were stimulated with either GW7647, rosiglitazone, GW501516 or DMSO at a concentration of 100 nM. Total RNA was isolated from the cells using RNAeasy kit (ZymoReaserch, HiSS Diagnostics GmbH, Freiburg, Germany) as instructed by the manufacturer. Total RNA amount was quantified (NanoDrop ND-1000, NanoDrop, USA).

cDNA synthesis

cDNA was synthesized using 1 µg of total RNA as template. The primer used was 100 pmol of oligodT_{18}. cDNA was synthesized with 40 U of MMLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and RNA degradation was prevented with 40 U of RNase inhibitor (Fermentas). The reaction mixture included also 4 µl 5 mM dNTPs, 2 µl 0,1 M DTT, 8 µl 5 x reverse transcriptase

buffer (250 mM Tris-Hcl (pH 8,3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) and the reaction volume was adjusted to 40 µl with RNase-free water. The reaction mixture was placed in 37 °C for 1 h. After reaction was complete, the mixture was heated to 85 ºC at 10 min. The synthesized cDNA was diluted with sterile water to a volume of 400 µl.

Real-time quantitative PCR

Real-time quantitative PCR was performed in a IQ-Cycler 4 (BioRad) using the dye SybrGreen (Molecular Probes, Leiden, Netherlands) and the following reaction set up:

2 µl 10 x buffer (HotStart PCR buffer (Fermentas))

2.4 µl 25 mM $MgCl_2$

0.4 µl dNTPs (10 nM)

1.2 µl SybrGreen (1:2500 dilution from stock)

0.15 µl HotStart DNA polymerase (5 U/µl)

4.8 µl H_2O

4 µl template cDNA

5 µl primer mix (0.8 µM)

For PCR the following program was used:

1. Denaturation for 5 min at 95 ºC
2. PCR amplification repeated for 40 cycles
   - Denaturation for 30 s at 95 ºC
   - Annealing for 30 s at primer-specific annealing temperature
   - Elongation for 40 s at 72 ºC
3. Final elongation for 10 min at 72 ºC
4. Denaturation for 1 min at 95 ºC
5. Melt curve analysis with 0.5 ºC decrease in 70 temperature steps

The control gene used was *acidic riboprotein P0* (*ARP0*, also known as *36B4*). Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where ($\Delta\Delta Ct$) is the $\Delta Ct_{(treatment)}$-$\Delta Ct_{(solvent)}$, ΔCt is $Ct_{(gene)}$-$Ct_{(ARP0)}$ and Ct is the cycle, at which the threshold is crossed. PCR product quality was monitored using a post-PCR curve analysis at the end of amplification cycles. PCR-primer and used temperatures are listed in table 4.

Table 4. Primers and temperatures for real-time PCR

gene	Primer pairs (5'-3')	Temperature (°C)	Product size (bp)
APOA	CAGGACTGAATGTTACATCAC CTTCTCGAAGCAAACCAGAG	56	123
APOA1	GTGCTCAGATGCTCGGTGG AGAAGAAGTGGCAGGAGGAG	60	273
APOA2	CTCCATCAGGTCCTTGCCATA GCAACTGTGCTACTCCTCACC	60	135
APOA4	CTCACCCAGCAACTCAATG GAGCTCCTCCACGTTCTG	58	214
APOA5	CACCCATACGCCGAGAGC CCTTCCTCAGTCCCAGTGC	60	326
APOB	CATGGATATGGATGAAGATGAC CACTCAGCATTGTTCTGCAG	58	245
APOC1	CAGACGTCTCCAGTGCCTTG GTCCTCATGAGTCAATCTTGAG	60	152
APOC2	GAACCTGTACGAGAAGACATAC GAGGAGGATGCAAGAGCTAC	60	320
APOC3	CATGCAGGGTTACATGAAGCAC GTAGGAGAGCACTGAGAATAC	60	325
APOC4	CAGAAATGTCCCTCCTCAG CATCATCCTACCTCAGCCTC	60	113
APOD	AATCAAATCGAAGGTGAAGCCA ACGAGGGCATAGTTCTCATAGT	60	131
APOE	GCAAGCGGTGGAGACAGAG CCTCAGTTCCTGGGTGACC	60	156
APOF	TGGTCATCAGAAGGTCATATCCC AGAGGACTGTGAGAATGAGAAGG	60	182
APOH	AACGTAGGTATGGATGGTGGA CTGAATGGCGCTGATTCTGC	60	104
APOL1	GCTTTTGATGACCAGGTCGTG CAGCCTTGTACTCTTGGAACC	60	133
APOL2	GTCAGAGGAAGATCCCTTG CAGTCAGCAGTTGTAGCAG	60	224
APOL3	GCAAGGGACATGATGCCAGA AAGAGTTTCCCCAAGTCAAGAGG	62	151
APOL4	GTCAGTGCTGGTTGCAGTC CAGAGGAAGATCCCTTGGAG	60	289
APOL5	AGCTCAAACTTGTGCAAAGGAA TGATGAGGCTGGTATGCTGTC	60	135
APOL6	CAGATTTGCTGCCACAGAG GTGACATAGTCTGCCTTCTC	62	198
APOM	GAGCACAGATCTCAGAACTG CTCTTGATTCCTAGGAGTC	58	225
APOO	TTCGATTGACCCTCAGGAACT GCTTGCTCACCTTCAAAGTCT	60	109
APOOL	CTTGCCAACAGAACTCAGCTC CTTCTACCTAAGTCTCCTCATC	60	201
RPLP0	AGATGCAGCAGATCCGCAT GTGGTGATACCTAAAGCCTG	56-62	318

siRNA transfection

HepG2 cells were seeded into 6-well plates and grown overnight to reach a density of 30–40 %. 1.1 µl of each siRNA subtype (6 µg/ml) (Eurogentec, Liege, Belgium) or 2 µl siRNA control (20 µg/ml) (stealth siRNA) (Eurogentec) was mixed with medium (DMEM, 1 % glutamine) to get total volume of 250 µl. Transfection reagent (Mix Lipofectamin™, RNAiMAX, Invitrogen) (10 µl) were mixed with medium (DMEM, 1% glutamine) (490 µl) and incubated 5 min at RT. Transfection reagent mixed with medium were mixed with PPAR siRNA and control siRNA to get a total volume of 500 µl and incubated 20 min at RT. Medium was removed from the cells and fresh medium (DMEM, 1 % glutamine) was added (1.5 ml). PPAR siRNA and control siRNA medium mixes were added to the cells (500 µl).Total siRNA supply was 200 pmol/well. Medium containing serum (20 % FC, DMEM, 1 % glutamine) was added after 6 h of incubation (500 µl).

RNA extraction, cDNA synthes and PCR performed as described earlier. Fold inductions were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where ($\Delta\Delta Ct$) is the $\Delta Ct_{(treatment)}$-$\Delta Ct_{(solvent)}$, ΔCt is $Ct_{(gene)}$-$Ct_{(ARP0)}$ and Ct is the cycle at which the threshold is crossed. PCR product quality was monitored using a post-PCR curve analysis at the end of amplification cycles.

Chromatin immunoprecipitation assay

Cells were seeded into bottle and grown overnight to reach a density of 60–70 %. At the start of the experiment the medium in the bottles was reduced to 10 ml. The cells were stimulated with GW7647 ligand at a final concentration of 100 nM.

Cross-linking of proteins and DNA

Formaldehyde was added to culture medium to cross-link proteins bound to DNA as follows:

270 µl formaldehyde (final concentration of 1 %), incubating for 5 min at RT with 1.5 ml lysine (1 M), incubating for 5 min in RT (to stop cross-linking).

The medium was removed and the cells washed twice with ice-cold PBS. Subsequently, the cells were scraped two times into ice-cold PBS (5 ml) and centrifuged for 5 min at 700 g at 4 ºC.

Lysis of cells and sonication

After centrifugation the cell pellet was resuspended in 1 ml of lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) supplemented with a protease inhibitor cocktail (Roche). Lysis was performed for 10 min at RT. The lysate were sonicated to result in DNA fragments ranging from 200 to 400 bp in lengh (Diagenode Bioruptor, Liege, Belgium). Cellular debris was removed by centrifugation from 15 min at 20 000 g at 4 ºC.

Immunocollection

The lysates were diluted 1:10. The following incubation was carried out in presence of specific antibodies:

100 µl of undiluted lysate

5 µl (Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) 200 µg/ml MED1, PPARα, RXRα, PCG-1α, p-Pol II) antibody

or

1 µl (Normal Rabbit IgG, Upstate Biotechnology Inc. (Lake Placid, New York, USA) 1000 µg/ml,

IgG) antibody

2.4 µl sonicated salmon's sperm (10 mg/ml)

500 µl ChIP Dilution Buffer (0.01 % SDS, 1.1 % Triton-X 100. 1.2 mM EDTA, 16.1 mM NaCl,

freshly added protease inhibitors (Roche), 50 mM Tris-HCl, pH 8.1)

Incubation overnight on a rocking platform at 4 ºC.

For input sample, 25 µl of undiluted lysate was diluted with 475 µl ChIP dilution buffer and the processing of these samples was continued with reverse cross-linking and DNA extraction as described below.

Collection of the immunocomplexes

For blocking of Protein A magnetic beads, their buffer was replaced of equal volume of Dilution Buffer and 100 µg/ml of sonicated salmon sperm DNA. They were incubated overnight rotating platform at RT. The immune complexes were collected with 25 µl of blocked MagaCell® Protein A magnetic beads (Fitzgerald Industries International, Concord, Maine, USA).

Elution and reversal of cross-linking

Elution of immunocomplexes was performed twice with 250 µl of elution buffer (25 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS) for first elution Protein A magnetic beads were incubated at 65 ºC for 30 min. A second elution was performed for 2 min at RT. Cross-linking was reversed by an over night incubation at 64 ºC with 2.5 µl of Proteinase K (900 U/ml; Fermentas).

DNA extraction

The DNA was extracted by adding 500 µl of 25/24/1 phenol/chloroform/isomyl alcohol followed by 5 min a centrifugation at 20 000 g at RT. DNA was recovered from the aqueous phase using pipette. DNA was precipitated with 0.1 volumes of 3 M sodium acetate, pH 5.2 and 2 volumes of ethanol (ice cold) using 1 µl of glycogen (20 mg/ml, Fermentas) as carrier. Samples were centrifuged 20 min at 14 000 rpm at RT. Supernatant was removed and pellets were washed with 500 µl of 70 % ice cold ethanol followed by 10 min centrifugation at 14 000 rpm at RT. DNA pellets were air dried and resuspented in water (input sample: 200 µl, output sample: 50 µl).

PCR of chromatin templates

PCR conditions for the different primers are described in table 5. PCR reactions were performed in an IQ-Cycler 4 using the dye SybrGreen. The following reaction was set up:

2 µl 10 x buffer (HotStart PCR buffer (Fermentas).

2.4 µl 25 mM $MgCl_2$

0.5 µl dNTPs (10 nM)

1 µl SybrGreen (1:2500 dilution from stock)

0.3 µl HotStart DNA polymerase (5 U/µl)

7.5 µl H_2O

4 µl template cDNA

5 µl primer mix (0.8 µM)

The PCR program performed as described earlier. Products were resolved on 2 % TAE (200 mM Tris-HCl pH 7.5, 100 mM acetate, 5 mM EDTA) gel. The gels were imaged with Fuji FLA3000 reader using ImageGauge software. The melt curve analysis combined with gel imaging allowed the detection of specific primer-dimer-free PCR products that were used for calculation from Ct-values the quantitative data relative to controls. Output samples were first normalized to their inputs and subsequently the fold change relative to non-specific IgG background was calculated. The fold inductions were calculated using $2^{(\Delta Ct)}$, were ΔCt is Ct(specific antibody)- Ct(IgG control antibody) and Ct is the cycle at which the threshold is crossed. Relative association level were calculated using $2^{-(10\text{-}Ct(output\text{-}input))}$.

Table 5. PCR primers and temperatures for ChIP RT-PCR

gene	Primer pairs (5'-3')	Temperature (°C)	Product size (bp)
APOA	CTTGTGTTCTACAGCCTCAC GACTAACTTTGGTCTCTTGATC	58	276
APOA1	CACATTGCCAGGACCAGTG CACTCATTGCAGCCAGGTG	58	241
APOA2	CATCACCATGAGTCTTCCATG CATTCCAACCTGGCTCTCTC	60	262
APOA4	GATCTGCTGTCAGCTTCCAC CAGGAGTGCCATCCAAAGAC	60	346
APOC2	CTGTCACTTGAGAGAAGGTTC CACAGTCATGGTTCCAACAC	58	311
APOE	GAGGGTGTCTGTATTACTGG GCTCTCCTGAGACTACCTG	58	353
APOF	CATAGAGGTTGAGTGTGTGAC CAAAGTGATAGGCTTCCAGATG	60	361
APOM	GTACTATGGAGTGGTTGCATC CAGAGTTGTCAGTTGACTGTG	60	288

3.2.2 *In silico* methods

In silico screening of putative PPREs

Genomic sequences spanning +/-10 kB around the TSS of the gene analyzed were extracted from the current database release (NCBI build 36, Ensembl release 48) of the human and mouse genome. Conservation of putative REs between human, mouse, rat, and dog was checked using the Vertebrate Multiz Alignment and Conservation track available from UCSC (NCBI releases: human 36.1, mouse 36) (Blanchette et al., 2004). A PPRE was marked conserved, when the sequence was 75% conserved in alignment location. This conservation definition allows a change or a gap in three nucleotides from native sequence. Conservation of surrounding sequence was checked 50 bp up- and downstream of the TSS: two occurrence of a continuous stretch of in minimum five matching bp was required to label the PPREs to be located within conserved surrounding sequence. The human and mouse sequences were screened from weak to strong putative PPREs using a classifier. The classifier was based on experimentally collected data from *in vitro* PPAR-RXR binding to DNA. Collected data was converted to simplified tables, which were used for *in silico* screening (Table 6) (Heinäniemi et al., 2007).

Table 6. PPARγ's RE variants (according to Heinäniemi et al., 2007). First row shows REs sequence number from 5'- start. Second row is the consensus sequence for PPARγ. Rows three to five shows departure from consensus sequence and theirs effect to the binding strength.

% binding		1	2	3	4	5	6	7	8	9	10	11	12	13
cons.	(90-100)	A/G	G	G	T	C/G	A	A	A	G	G	T	C	A
class I	(60-90)				C/G	A/T		T	G		T	C/G	A/G/T	G
class II	(30-60)	C/T	A/T	T	A			C	C	A/C/T				C/T
class III	(0-30)		C	A/C			C/G/T	G	T		A/C	A		

4. Results

4.1 *In silico* analysis of the apolipoprotein gene family

In order to find PPAR-regulated genes from the apolipoprotein gene family, we performed *in silico* screening to find putative PPREs. We analysed each gene in human and mouse at 10 kB of the genomic sequence up- and downstream of their respective TSS. *In silico* screening resulted in various numbers of putative PPREs. Therefore, we organized the genes according to strength and level of conservation of PPREs found (strong and conserved (SC), strong and conserved weak (S-CW), strong (S) and weak (W)). Results are shown in figures 4 and 5.

The six genes *APOA1*, *APOA2*, *APOA4*, *APOA5*, *APOC3* and *APOM* have strong and conserved PPREs. *APOA1*, *APOA2*, *APOA4, APOA5* and *APOC3* are already known PPAR target genes. *APOA4* has no mouse ortholog. *APOM* has the two conserved strong elements and several weak conserved elements.

The strong and conserved weak element category has the four genes (*APOA*, *APOC1*, *APOE* and *APOF*), of which one (*APOE*) is an established PPAR target gene. *APOA* has no mouse ortholog. *APOA* and *APOF* have one strong and one conserved weak putative PPRE. *APOC1* and *APOE* have the two strong and one conserved weak putative PPRE. There fore, *APOC1* is a good candidate for a PPAR target gene. *APOE* gene's strong element is already known as an established PPRE.

The nine genes *APOD, APOL1, APOL2, APOL3, APOL4, APOL5, APOL6, APOO* and *APOOL* are categorized in a strong element category. *APOL4, APOL3* and *APOL5* have no mouse ortholog. *APOL2* has the four putative strong PPREs, but the mouse ortholog does not look too convincing. *APOL1* has the three putative strong PPREs and the same pattern can be found in its mouse ortholog. *APOL4* and *APOO* have the two strong putative REs. The remaining five genes (*APOD*, *APOL3, APOL5, APOL6* and *APOOL*) each have the one strong putative PPRE.

We found four genes (*APOB, APOC2, APOC4* and *APOH*) in the weak category. *APOB* has three conserved PPREs and is so the most promising gene in this group. *APOC2* and *APOC4* are located very close in human genome and have only two conserved weak elements. *APOH*, which gene product is located usually in cytoplasm, has no conserved putative PPREs.

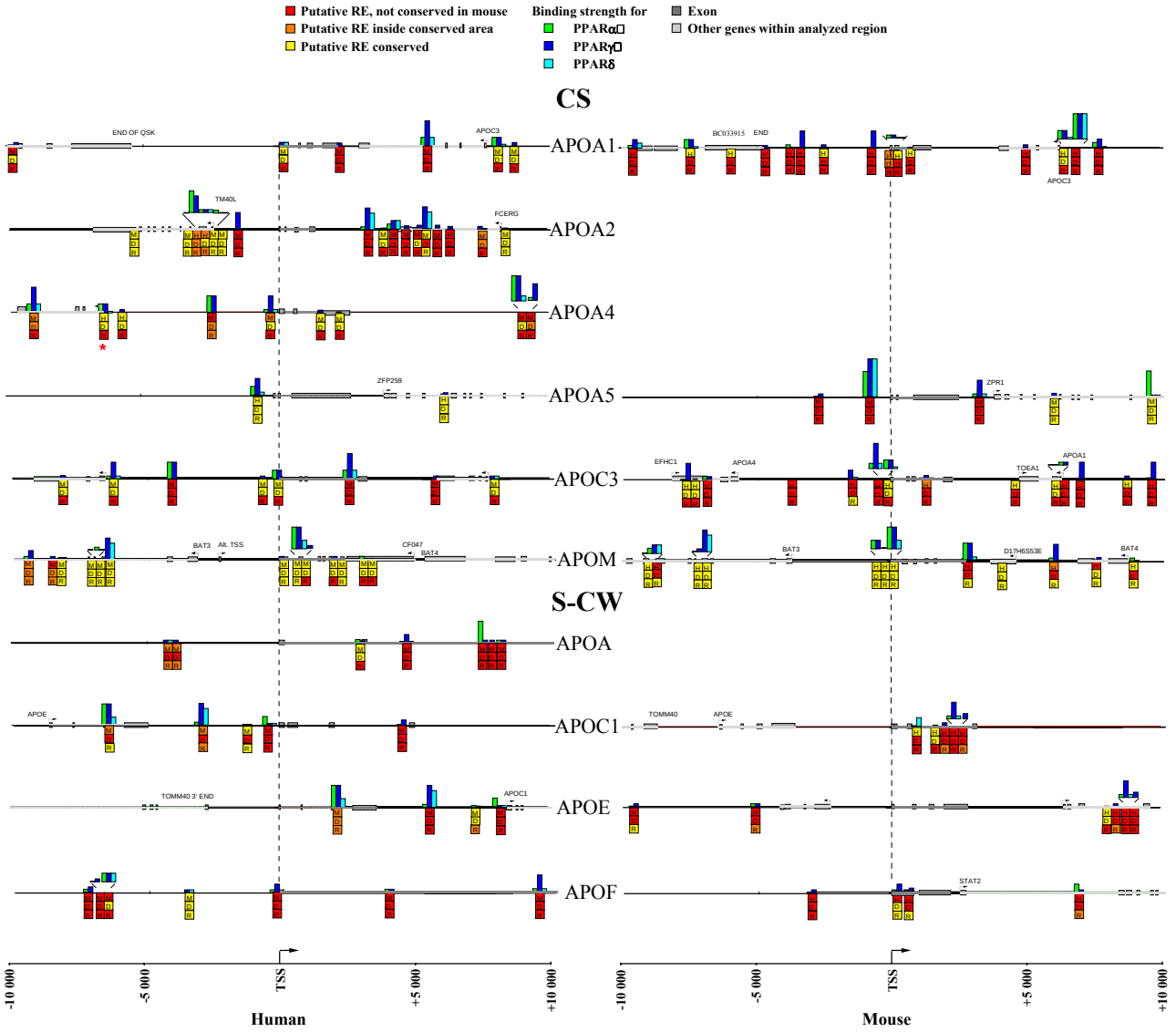

Figure 4. Overview of the genomic organization of the human and mouse genes *APOA1, APOA2, APOA4, APOC3, APOM, APOA, APOC1, APOE* and *APOF* (10 kB up- and downstream of their TSS) indicating putative PPREs and theirs relative strength (green, dark blue and light blue). Colored boxes are indicating the conservation degree of putative REs (yellow (conserved), orange (inside conserved area) and red (not conserved)) and letters species (M = *Mus musculus*, D = *Canis familiaris*, R = *Rattus norvegicus* and H = *Homo sapiens*). Genes are organised two groups: the first group has at least one conserved strong (CS) putative RE and second group at least one strong and conserved weak (S-CW) putative RE.

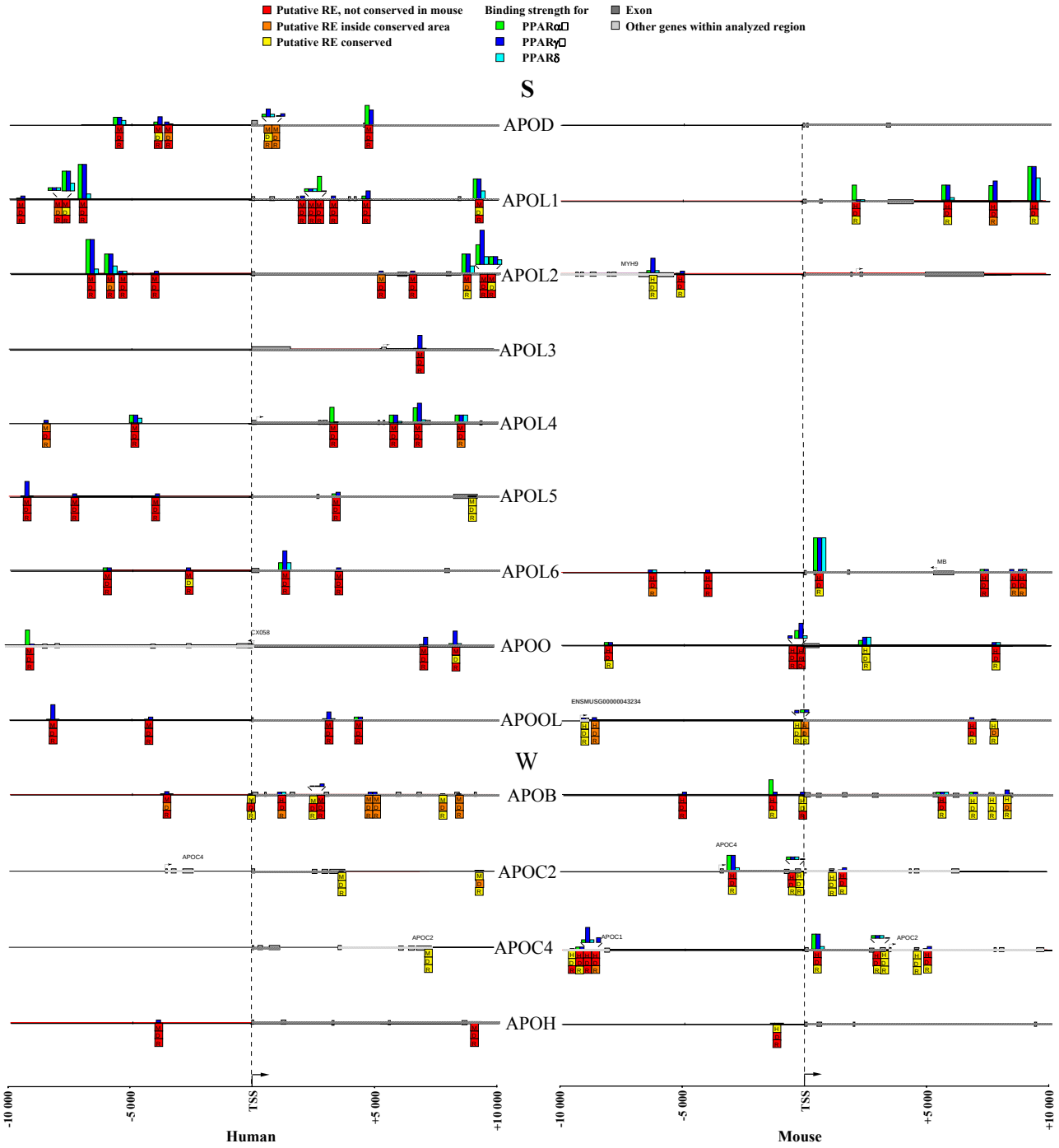

Figure 5. Overview of the genomic organization of the human and mouse genes *APOD, APOL1, APOL2, APOL4, APOL5, APOL6, APOO, APOOL, APOB, APOC2, APOC4* and *APOH* (10 kB up- and downstream of their TSS) indicating putative PPREs and theirs relative strength (green, dark blue and light blue). Colored boxes are indicating the conservation degree of putative REs (yellow (conserved), orange (inside conserved area) and red (not conserved)) and letters species (M = *Mus musculus*, D = *Canis familiaris*, R = *Rattus norvegicus* and H = *Homo sapiens*). Genes are organised two groups: the first group has at least one strong (S) putative RE and second group has only weak (W) putative REs.

4.2 RT-PCR results

HepG2, HEK239 or THP-1 cells were treated for 2, 4, 6 and 24 h with 100 nM of either the PPARα agonist GW7647, the PPARγ agonist rosiglitazone or the PPARβ/δ agonist GW501516, and RNA was extracted. The relative fold inductions of the mRNA amounts of the apolipoprotein genes and of known PPAR target genes (*CPTI* and *PDK4*) were determined by real-time quantitative PCR. Results are shown in figures 6, 7, 8, 9A, 9B and 10. For HepG2 and HEK293 cells we used established PPAR target gene *CPT1A* gene as a positive control and for THP-1 cells we selected the *PDK4* gene.

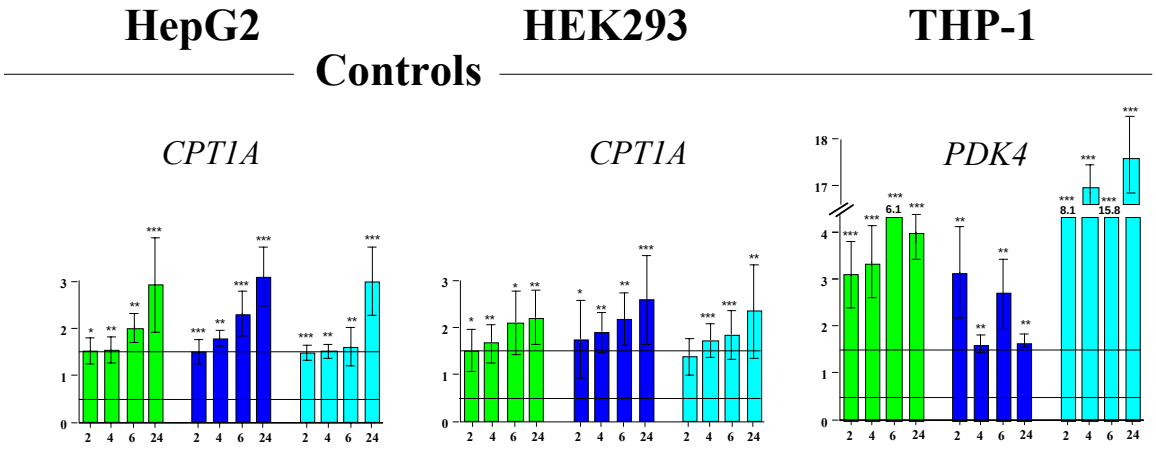

Figure 6. Expression of the control genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the mRNA levels of the control genes (established PPAR target genes *CPT1* and *PDK4*) in our three cellular models (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6, and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist, light blue). Columns represent the means of at least three independent treatment and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

CPT1A shows an approximately 1.5-fold induction after 2 and 4 h with PPARα ligand in HepG2 and HEK293 cells. In HepG2 cells the induction has the same strength and timing also with PPARδ/β ligand. The induction of gene expression with PPARγ ligand starts already at the first time point (1.5-fold) and continues up to a 3.3-fold change after 24 h. *CPT1A* in HEK293 cells with PPARγ ligand has a same trend as in HepG2 cells, but the induction is not so obvious. *PDK4* in THP-1 cell line shows strong inductions. With PPARα ligand the fold induction is 3.1 to 6.1. *PDK4* induction with PPARγ ligand is less prominent, but an almost up to 3.0-fold induction can be seen after 2 and 6 h. With PPARδ/β ligand inductions after 2 h are already 8.1, 17.1-fold after 4 h and 15.8-fold after 6 h.

Next we analyzed the apolipoprotein genes. Results from the apolipoprotein genes are sorted according to their classification in the *in silico* screening. First are the genes, which contain conserved strong (CS) putative PPREs (Figure 7). The second apolipoprotein group has strong and conserved weak (S-CW) genes (Figure 8). Third group has genes, which we establish one or more strong (S) PPRE (Figures 9A and 9B). The last and the smallest group have weak (W) PPREs containing genes (Figure 10).

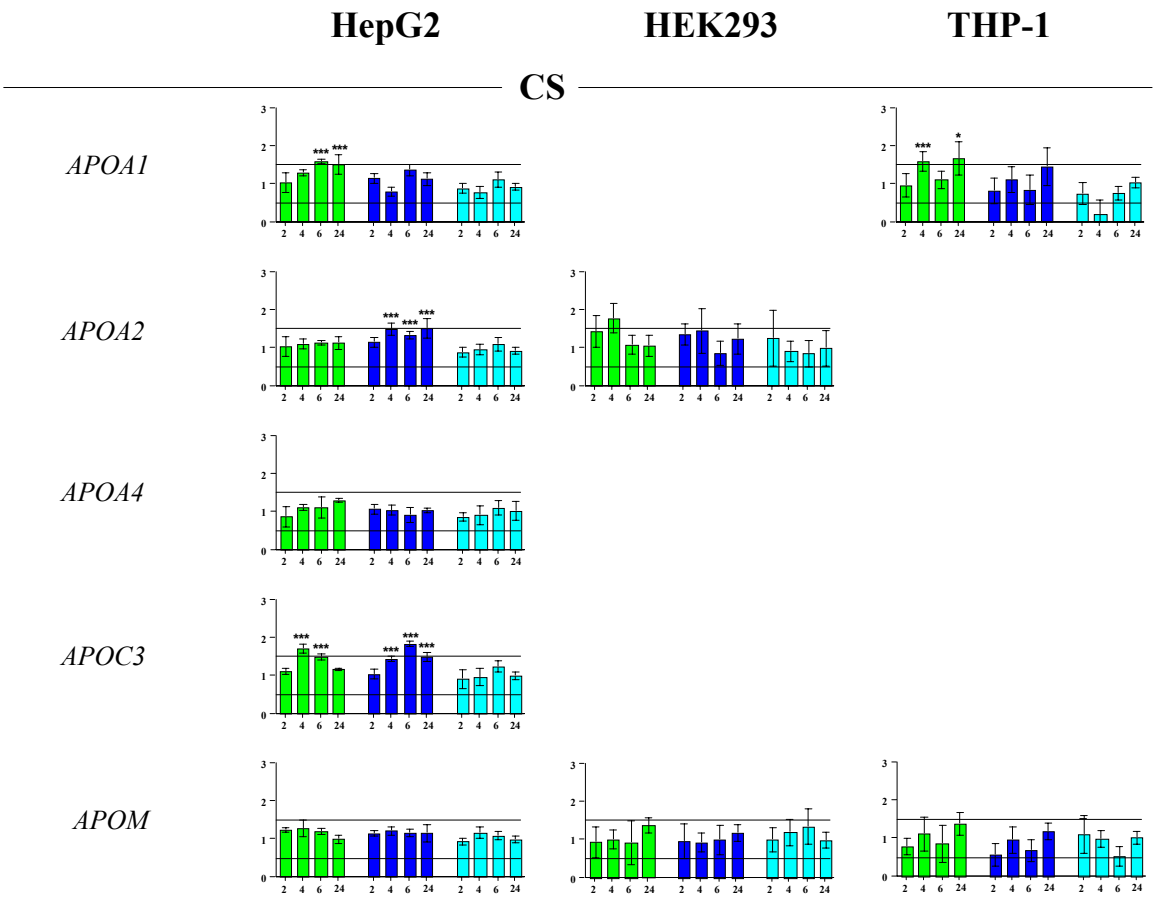

Figure 7. Expression of the CS apolipoproteins genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the mRNA levels of the apolipoproteins (*APOA1, APOA2, APOA4, APOC3, APOM*,) in three cell line (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6, and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist light blue). Columns represent the means of at least three independent treatment and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

The *APOA1* gene is expressed in HepG2 cells and THP-1 cells. It has a significant fold induction with PPARα ligand, which is over 1.5-fold in HepG2 cells after 6 and 24 h and in THP-1 cells after 4 and 24 h. *APOA2,* is expressed in HepG2 and HEK293 cells. It has a significant induction (over 1.5-fold) with PPARγ ligand in HepG2 cell line after 4 and 24 h. *APOA4* is expressed only in HepG2 cells and has no significant induction with any ligand. *APOC3* is up-regulated with PPARα ligand and PPARγ ligand. *APOC3* responds after 4 and 6 h to all PPAR ligands and in addition after 24 h to PPARγ ligand. *APOM* is not responding to any ligand in any cell line.

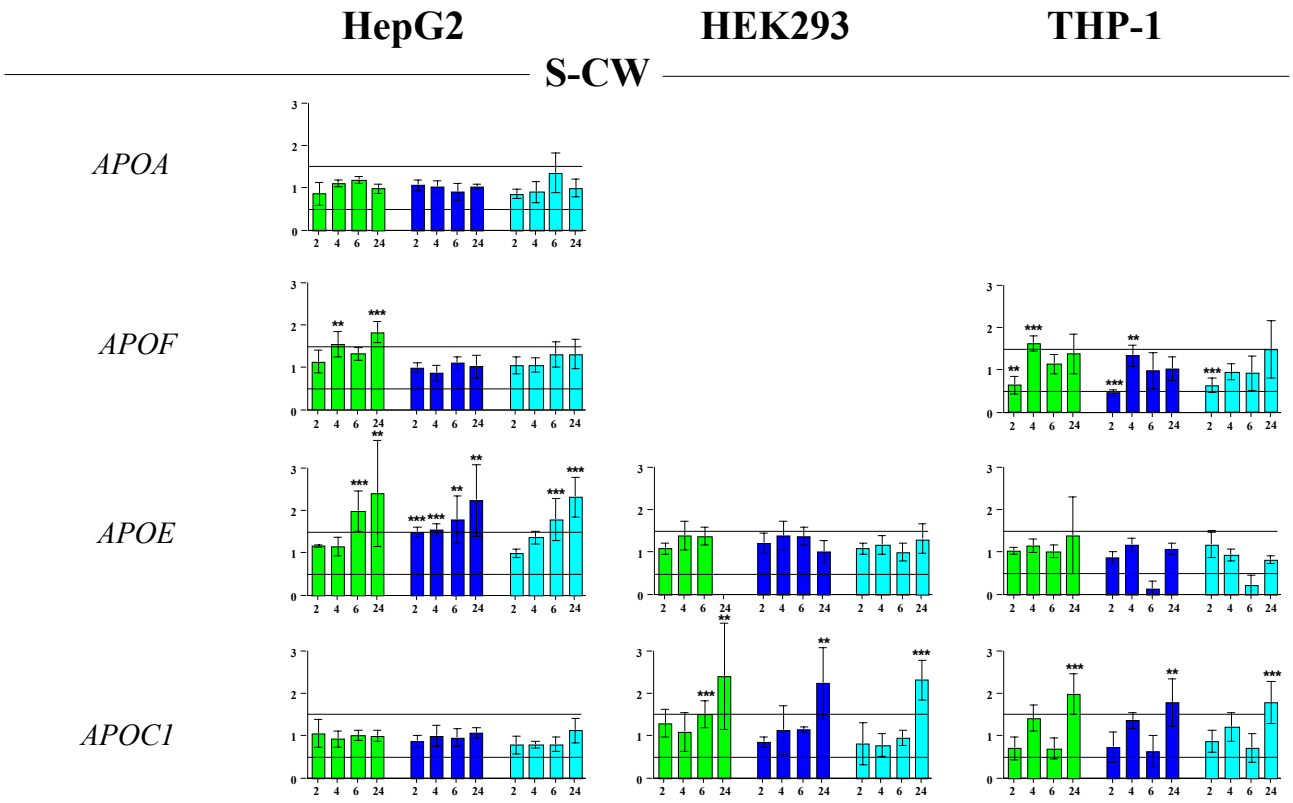

Figure 8. Expression of the CS apolipoproteins genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the real time mRNA levels of the apolipoproteins (*APOA, APOF, APOE, APOC1,*) in three cell lines (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6, and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist light blue). Columns represent the means of at least three independent treatments and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

APOA is not responding to any ligand in any cell line. *APOF* is up-regulated with PPARα ligand in HepG2 and THP-1 cell lines and down-regulated with all ligands in THP-1 cell line. Up-regulation is seen after 4 and 24 h in HepG2 cell line and after 4 h in THP-1 cells. In THP-1 cells *APOF* is down-regulated 0.3-fold with all ligands after 2 h. *APOE* is responding to all ligands in HepG2 cells but has no response in HEK293 or THP-1 cell lines. With PPARα and PPARδ/β ligands *APOE* is up-regulated approximately 2-fold after 6 and 24 h. *APOE* is also responding to PPARγ ligand in all time points between 1.5- and 2.3-fold induction. *APOC1* is responding in HEK293 and THP-1 cells but not in HepG2 cells. In both responsive cell lines *APOC1* gets approximately 2.0-fold induced with all ligands after 24 h.

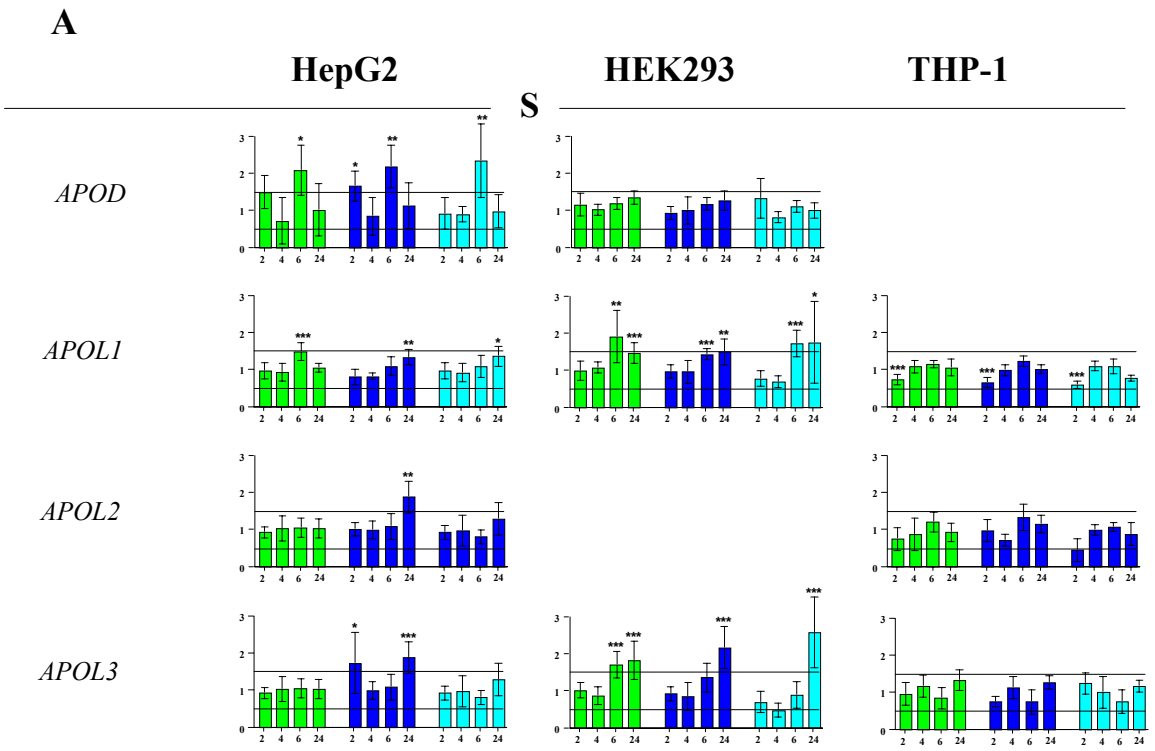

Figure 9A. Expression of the strong (S) apolipoproteins genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the mRNA levels of the apolipoproteins (*APOD, APOL 1-3)* in three cell lines (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6, and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist light blue). Columns represent the means of at least three independent treatments and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

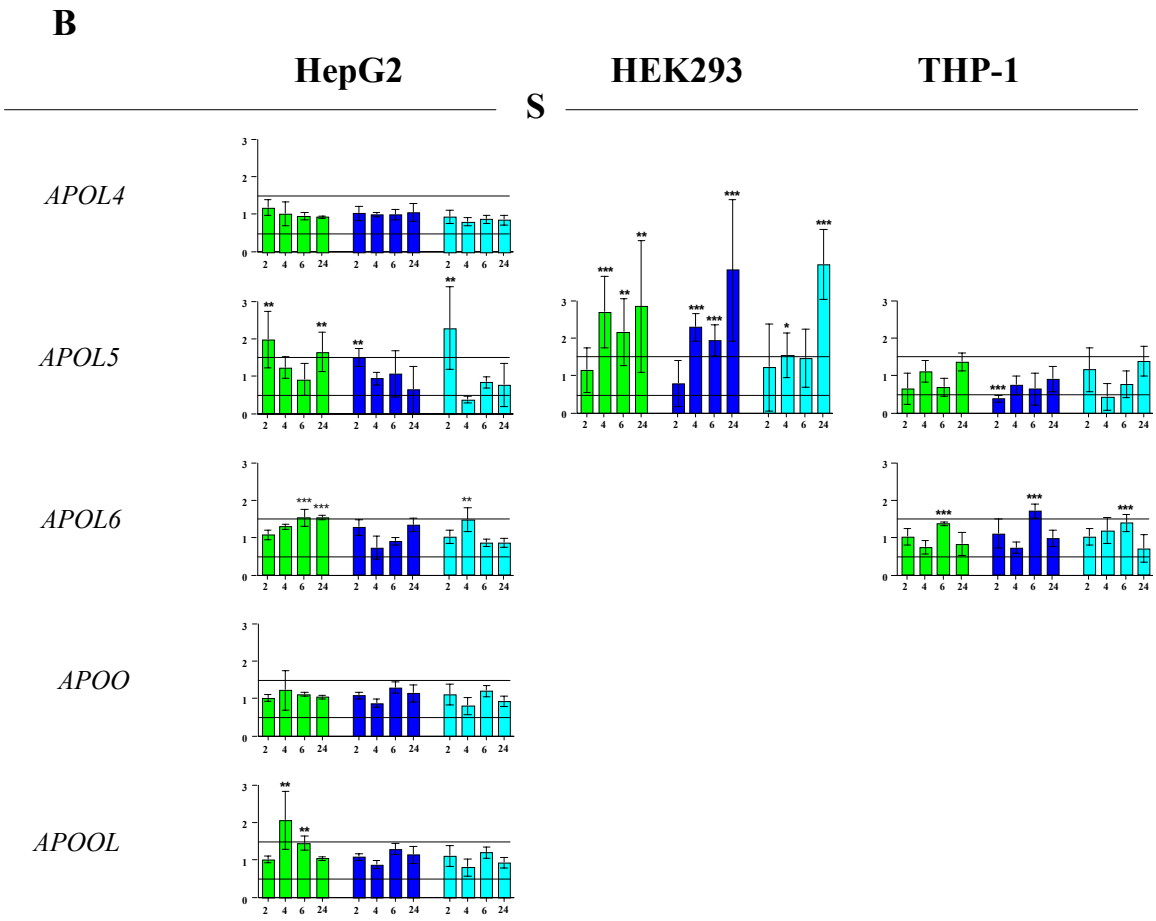

Figure 9B. Expression of the strong (S) apolipoproteins genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the mRNA levels of the apolipoproteins (*APOL 4-6, APOO, APOOL*) in three cell lines (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6 and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist light blue). Columns represent the means of at least three independent treatment and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

APOD is expressed in HepG2 and HEK293 cells. A significant expression level is detected in HepG2 cells with all ligands. All ligands provide a 2.0-fold induction after 6 h. *APOL1* is expressed in all three cell lines. In HepG2 cells with PPARα ligand *APOL1* gets induced more than 1.5-fold. In HEK293 cells same kind of induction can be seen with all three ligands after 6 and 24 h. Down-regulation is seen after 2 h in THP-1 cells. *APOL2* shows only with PPARγ ligand in HepG2 cells after 24 h. *APOL3* is expressed in all three cell lines. In HepG2 cells there is a 2-fold induction after 2 and 24 h. The gene is induced also in HEK293 cells, but not with all ligands. PPARα ligand gives an approximately 2.0-fold induction after 6 and 24 h with the *APOL3* gene. PPARγ ligand and PPARδ/β ligand shows the same induction after 24 h. *APOL4* is only expressed in HepG2 cells and

is not ligand responsive. *APOL5* is expressed in all three cell lines. In HepG2 cells there is a 2.0-fold induction with PPARα and PPARδ/β ligand. There are also ligand effects with PPARγ ligand but only a 1.5-fold induction is observed. In HEK293 cells up-regulation can be seen with all three ligands. With PPARα and PPARγ ligand has earliest effect after 4 h and stays active through the other time points. With PPARδ/β ligand induction can be seen only after 24 h. In THP-1 cell line, there is a 0.6-fold down-regulation after 2 h with PPARγ ligand. *APOL6* is expressed in HepG2 and THP-1 cells. In HepG2 cells only PPARα and PPARδ/β agonist treatment exceeds the1.5 threshold line (with PPARα ligand after 6 and 24 h and with PPARδ/β after 4 h). Approximately 1.7-fold up-regulation can be also detected in THP-1 cells by PPARγ ligand. *APOO* and *APOOL* are expressed only in HepG2 cell line. *APOO* gene expression is not affected by PPAR ligands. *APOOL* is induced after 4 and 6 h with PPARα ligand.

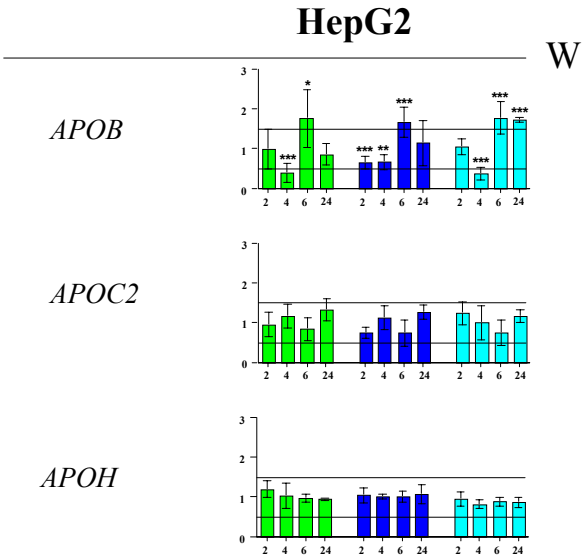

Figure 10. Expression of the CS apolipoproteins genes in HepG2, HEK239 and THP-1 cells. Real-time quantitative PCR was used to determine the mRNA levels of the apolipoproteins (*APOB, APOC2* and *APOH*) in three cell lines (HepG2, HEK239 and THP-1). Cells were stimulated for 2, 4, 6 and 24 h with 100 nM GW7647 (PPARα agonist, green), 100 nM rosiglitazone (PPARγ agonist, dark blue) or 100 nM GW501516 (PPARδ/β agonist light blue). Columns represent the means of at least three independent treatments and the bars represent standard deviations. Solid lines indicate the threshold of 1.5-fold up-regulation or 0.5-fold down-regulation. Two-tailed Student's t-test was performed to determine the significance the mRNA induction by PPAR agonist in reference to solvent controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

APOB is expressed only in HepG2 cells, where it shows a strange expression pattern. Afer 4 h the gene is down-regulated and up-regulated after 6 h with all ligands. *APOC2* genes expression is not affected by PPAR ligands at any time point. *APOH* is expressed in HepG2 cell line but was not ligand responsive. *APOC4* should be analysed in this category but expression was not detected in any of the cell lines. This same problem was encountered also in siRNA experiment.

4.3 siRNA results

Next we made a siRNA experiment, where we silenced PPARα, PPARγ and PPARβ/δ in HepG2 cells. After 48 h RNA was collected and real-time PCR experiments were performed for receptor genes (*PPARα, PPARγ* and *PPARβ/δ*) and the known target gene (*CPT1A*) (Figure 11) and apolipoproteins results are seen in Figure 12.

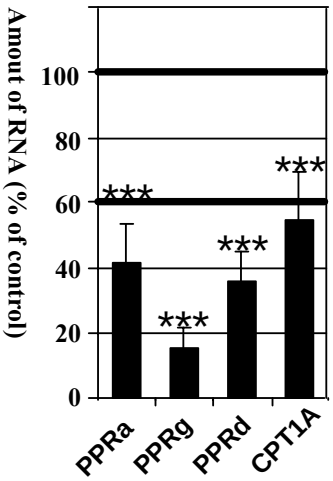

Figure 11. Effects of silencing *PPARα*, *PPARγ* and *PPARβ/δ* on control genes in HepG2 cell line. *PPARα, PPARγ* and *PPARβ/δ* were silenced from HepG2 cells with siRNA while cells transfected with non-specific siRNA were used as a control. Treatment was performed for 48h. Real-time quantitative PCR was used to determine the mRNA levels of the *PPARα, PPARγ, PPARβ/δ* and *CPT1A* genes. Columns represent the means of at least three independent treatments and the bars represent standard deviations. Solid broad lines indicate 100 and 60 %. Two-tailed Student's t-test was performed to determine the significance the mRNA chance by PPAR knockdown in reference to random RNA controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

PPARα gene expression after siRNA treatment is 40 %, that of *PPARγ* only 15 % and that of *PPARβ/δ* is approximately 30 %. The remaining expression of the control gene (*CPT1A*) is 50 %.

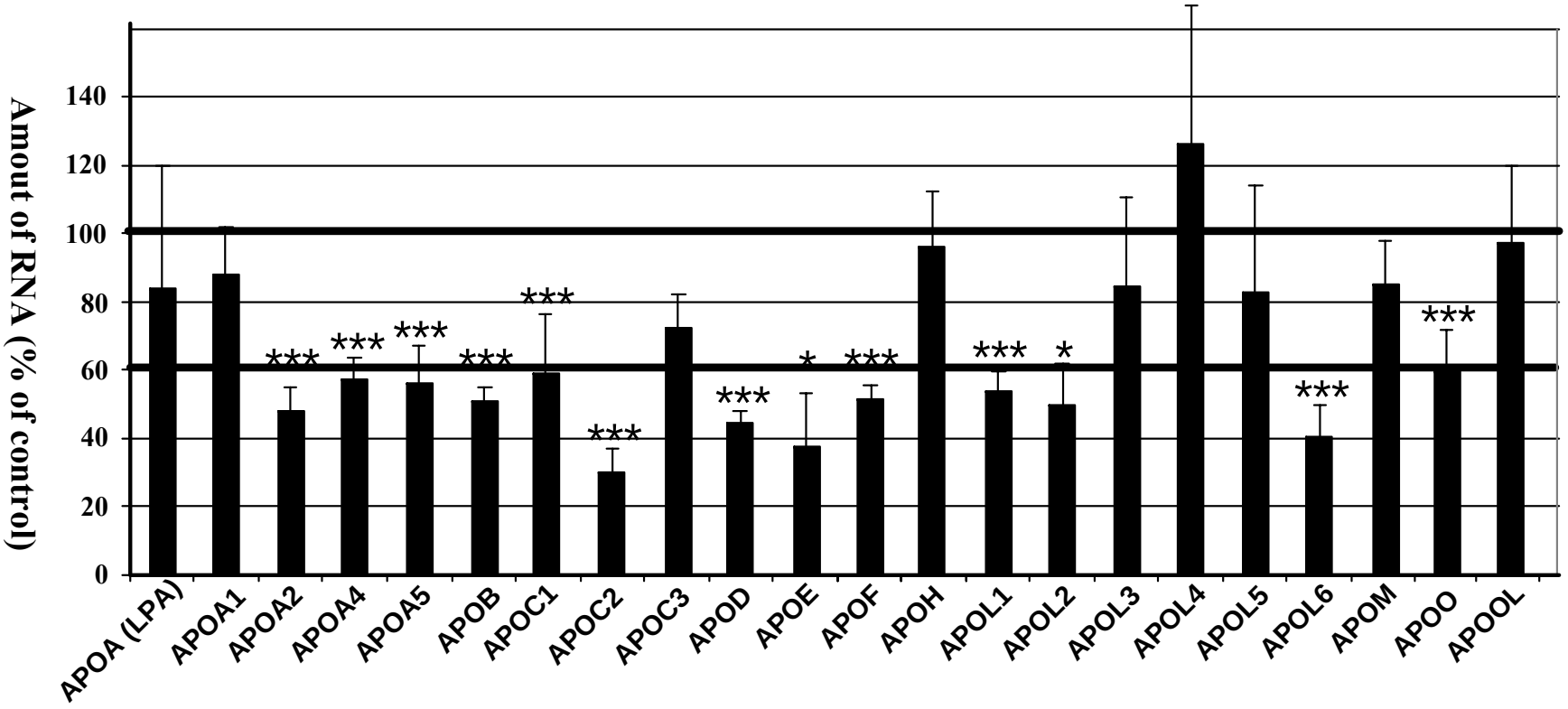

Figure 12. Effects of silencing *PPARα*, *PPARγ* and *PPARβ/δ* on apolipoproteins in HepG2 cell line. *PPARα*, *PPARγ* and *PPARβ/δ* were silenced in HepG2 cells with siRNA, while cells transfected with nonspecific siRNA were used as a control. Treatment was performed for 48 h. Real-time PCR was used to determine the mRNA levels of the apolipoproteins. Columns represent the means of at least three independent treatments and the bars represent standard deviations. Solid broad lines indicate 100 and 60 %. Two-tailed Student's t-test was performed to determine the significance the mRNA chance by PPAR knockdown in reference to random RNA controls (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

APOA gene expression seems to be unchanged after siRNA treatment. The expression of *APOA* family is generally going down with the exception of *APOA1,* which expression level is almost unchanged. *APOA2, APOA4* and *APOA5* are clearly going down by 60 %. In the *APOC* family *APOC3* is not reacting to siRNA treatment but *APOC2* goes down to 30 %. *APOC1* is between of *APOC3* and *APOC2* and is down-regulated to 60 %. Also *APOB* and *APOD* expression goes down approximately to 50 %. The *APOE* gene expression is down to 40 %. *APOF* expression went to down to approximately 40 %. One good example of genes, whose expression remains unchanged, is *APOH.* The reaction of siRNA treatment in the *APOL* family is variable. *APOL1*, *APOL2* and *APOL6* are going down nearly 50 % but *APOL4* genes expression is going up to 120 %. *APOL3* and *APOL5* expression is unchanged. The same observation was made for *APOM* and *APOOL* genes. Surprisingly, *APOO* gene goes down 40 %.

4.4 ChIP results

Next we examined in HepG2 cells, whether PPARα, RXRα, p-Pol II, the mediator proteins TRAP220 or the CoA PGC-1α were located to the TSS regions of selected genes (*APOA*, *APOA1, APOA4, APOA2, APOC2, APOF, APOE* and *APOM*). *APOA1, APOA4, APOA2* and *APOE* were serving as positive control because they are already established PPAR target genes. *APOM* and *APOA* genes were serving as negative control because expression chances were not detected during quantitative PCR or siRNA experiments. The *APOF* gene was selected from conclusion of promising quantitative PCR results and its evident importance in HDL metabolism. The *APOC2* gene was selected from conclusion of promising siRNA results and its role as cofactor of LPL. Chromatin was extracted from HepG2 cells, which had been treated for 0 min, 120 min or 240 min with 100 nM GW7647 and then cross-linked in the presence of formaldehyde. ChIP experiments were performed with anti-PPARα, anti-RXRα, anti-p-Pol II, anti-TRAP220, anti-PGC-1 antibody and IgG was used as negative control. The genomic DNA fragments that were recovered from reverse-cross-linked chromatin served as templates for PCR reactions with primers specific for the TSS containing the PPRE responsive TSS and control TSS's. There was several problems with the ChIP protocol so results are unreliable (data is not shown).

5. Discussion

This is the first study of the responsiveness of all apolipoproteins to PPAR ligands in HepG2, HEK239 and THP-1 cells. In addition, we investigated the effect of silencing PPARs with siRNA on human hepatocellular cells. Also, we performed ChIP assay with selected genes. We used already established PPAR target genes as a positive control.

In silico analysis

In this study an *in silico* screening for putative PPREs around the apolipoprotein genes was performed (Figures 4 and 5). Traditionally, REs have been searched from the promotor regions relatively near the TSS and in the 5'-flanking regions of target genes, but recently also more distal functional REs have been discovered (Kim et al., 2006). Also the 3'-untranslated regions and introns cannot be neglected in the search for REs (Prieur et al., 2003). Present understanding is that functional PPREs can be located several tens or even hundreds of thousands base pairs away from the target gene's TSS and either on the up- or downstream side. Recent discoveries have also proven that even interchromosomal interactions in NR-mediated gene regulation are possible and necessary for the transcription of certain genes (Nunez et al., 2008).

A normal weight matrix analysis usually gives strong background of false positive predictions (Stormo, 2000). Therefore, we used a more sophisticated search method, which is based on *in vitro* data of PPAR-RXR binding to PPREs. This sensitive method decreases the number of false positive PPREs. Whole genome ChIP-on-chip experiments have shown that strong NR bindig sites can be up- or downstream of the TSS and are usually located 10 kb region from TSS (Liu et al., 2007). Based on these facts we selected our *in silico* screening parameters. We searched up- and downstream from the TSS (10 kb) using an *in vitro* based *in silico* screen. The parameters have been proven to be effective in earlier research (Heinäniemi et al., 2007).

In silico screening showed that already established PPAR responsive genes have at least one strong PPRE. We also found established PPREs in our *in silico* screening, such as the PPREs of *APOA5* and *APOE* genes, which were defined strong. We could also define established PPREs of *APOC3* and *APOA2* genes, which were defined weak, but we were unable to confirm the PPRE of the *APOA1* gene (Vu-Dac et al., 1994, Vu-Dac et al., 1995, Krey et al., 1993, Prieur et al., 2003, Galetto et al., 2001). Furthermore, findings in strong and weak element categories were promising. At least the amount and quality of elements found and genes reference to metabolism correlates generally well. In strong RE category there were three from five genes responding to PPAR ligands. Strong and conserved weak category genes had two from four genes and strong category five from nine genes responding to the PPAR ligands treatments. Surprisingly there was also one gene from three in weak category which was responding to ligands. A trend can be detected where number and quality of found putative PPREs correlate with the number of responsive genes in the groups (CS, S+CW, S and W) in the following order W<CS<S+CW<S. CS genes category result do not fit in this pattern. Possible explanation would be competition between NRs for REs, which are in this

group quite near to consensus sequence. Strong category also probably beats other categories being the larges groups in this study.

We found several conserved strong PPREs in *APOM* gene, but it was unresponsive to PPAR ligands. *APOM* gene is an established target of liver receptor homolog-1 (LRH-1) and it is possible there is competition for the REs between PPAR and LRH-1 (Venteclef et al., 2008). However, established LRH-1 REs are not overlapping with putative PPREs, which we found, and it is not uncommon that gene is regulated by several NRs. For example, *APOA1* gene transcription has been show to be modulated by various NRs, such as ARP-1, HNF-4α, PPARα and LRH-1 (Ladias and Karathanasis, 1991, Vu-Dac et al., 1994, Sladek et al., 1990, Delerive et al., 2004). Nevertheless, there can also be competition for the REs between NRs. This competitive or cooperative regulation is possible at least between PPARs and HNF-4α (Prieur et al., 2005). This kind of regulation is still impossible to predict from *in silico* screening results. Experimental methods, like quantitative PCR and ChIP, have to confirm these REs.

Real-time PCR

Real-time PCR experiments were performed in three cell lines. Cell lines were selected according to representativeness of key tissues in apoliprotein physiology. Human hepatocellular cells (HepG2) are representing liver tissue. Liver is considered the major tissue where apolipoproteins are expressed. Assembly and necessary modifications of the major lipoproteins of blood like HDL and VDL takes place in liver, also many receptors, which are responsible for uptake of chylomicron remnants and HDL are present there. The regulation of the receptor genes would also be an interesting research project. HEK293 cell line, which is derived from human embryonic kidney cells represents kidney tissue in this study. This cell line is not a cancer cell line and so it rather resembles normal tissue. PPARδ/β is highly expressed in kidney, so that it may be the dominant PPAR subtype responsible for the regulation of apolipoprotein expression. Although apolipoproteins should not be expressed in kidney, we found for the genes *APOL5* and *APOC1* an interesting up-regulation. The human acute monocytic leukemia cell line (THP-1) represents a pre-stage of macrophages and therefore cells of the immune system. Generally, detected expression levels were weak in this cell line. When we observed up-regulation in HepG2 or HEK293 cells there was usually a down-regulation in THP-1 cells. Using those three cell lines we were looking for tissue wide expression regulation of PPARs.

From the real-time PCR results in the conserved and strong category, it was not so evident that any established PPAR target gene is regulated by PPARs (Figure 7). Best results are seen with *APOA1* and *APOA2* genes, where up-regulation is seen after 6 and 24 h. However, there are also several controversial time points, where standard deviations were notable high. THP-1 cell line seems to provide most unreliable results. The best situation would be, to use primary cells, in order to avoid possible problems, which are typical for cancer cell lines. Furthermore, the statistically significant

up-regulation of the *APOC3* gene is in contrast with the literature, where it is referred to be down-regulated by PPARα (Hertz et al., 1995).

Real-time PCR results with the *APOE* gene from the strong and conserved weak category are evident. This gene is directly regulated by PPARs (Figure 8). Another interesting gene is *APOF*, which is down-regulated in THP-1 cells. But we also detect expression cycling in *APOF* gene, which we cannot explain. There is down-regulation after 2 h with all ligands and then significant up-regulation after 4 h. This is an unusual expression pattern. There is also observed up-regulation with PPARα agonist in HepG2 cells. It would be worth to repeat the experiment with higher resolution, for example with more time points and shorter intervals. This would verify, whether PPAR ligands have any real effect on *APOF* gene expression. As mentioned earlier in the literature review, APOF is a regulator of HDL metabolism (Paromov and Morton, 2003). Using PPAR antagonist it would be possible to up-regulate the *APOF* gene and so amount of HDL. This could help patients with high cholesterol or atherosclerosis. Another interesting gene in this group is *APOC1*. APOC1 is a specific inhibitor of CETP (Gautier et al., 2002). Therefore, like the *APOF* gene, also *APOC1* is controlling metabolism of lipids and so it is logical to think that *APOC1* could be a target of PPARs. However, in the results *APOC1* gene up-regulation occurred very late so it is possible that this is not a direct effect of PPARs.

In the group of strong genes, there are five very interesting candidates (Figures 9A and 9B). The *APOD* gene, which is not a typical apolipoprotein since it rather belongs to lipocalins carrier proteins family, is one of them (Pervaiz and Brew, 1987). APOD is clearly up-regulated after 6 h. It is not clear, if this is direct effect of PPAR or secondary effect. It would be interesting to repeat the experiment with higher resolution to see when up-regulation really peaks. The *APOL1* gene is the only member of the APOL gene family member, which has a known function. There is strong evidence of APOL1 protein being involved in anti-parasitic effects (Shiflett et al., 2005). Anti-parasite effects, however, refer more to immune defence than to metabolism. Anyway, we observed PPAR regulation with all three cell lines. APOL1 and other specific surface proteins of HDL are the principle anti-microbial molecules. It has been shown that HDLs can serve as platform for the assembly of multiple synergistic proteins, which may play a critical role in the evolution of primate-specific innate immune system. *APOL3, APOL5* and *APOL6* gene expression pattern is really close to *APOL1*. Unfortunately, a specific function for *APOL3*, *APOL5* or either *APOL6* genes has not been found. Nevertheless, similarity of expression patterns strengthens the assumption of shared TF structures of APOL gene family (Philippe et al., 2001). Maybe whole gene family is more related to the sophisticated immune defence of primates than to lipid metabolism but verifying that would need further experiments.

In the group of weak genes, there were two very interesting genes (Figure 10). *APOB* gene which expression pattern first shows a significant down-regulation and then almost instantly up-regulation should be interpreted with reservation. Usually this kind of result is seen, when there is RNA degradation, already low expression levels, bad primers or reaction mixture problems. All those

factors impacts the efficacy of PCR reaction. Another interesting gene in this group is *APOC2*. APOC2, functioning as a cofactor of LPL and therefore as a regulator of fatty acid uptake in the liver, would be a logical PPAR activated gene whose expression is stimulated by dietary ligands. However, when looking at *APOC2* genes expression pattern, there is unfortunately no observed ligand effect. It would be interesting to test if this gene is regulated by another NRs like, for example, apolipoprotein regulatory protein-1 (NR2F2).

siRNA

Known PPAR up-regulated genes (*APOA2, APOA4, APOA5* and *APOE*) were significantly down-regulated with siRNA against PPARs (Figure 12). However, *APOA1* and *APOC3* expression remains unchanged. *APOC3* gene expression is up-regulated by REV-ERBα and RORα and down-regulation is seen with PPARα agonist (Coste and Rodriguez, 2002). In siRNA experiments no ligands were used, so it is possible that up-regulation was not seen for this reason. *APOA1* gene is the major apolipoprotein and is promoting cholesterol efflux from tissues to the liver for excretion (Zhang et al., 2003, Jonas, 2000). It seems that *APOA1* expression is safeguarded against effects of missing PPARs. Indeed, after knock-down of PPARs there are left three NRs (ARP-1, HNF-4α and LRH-1) to regulate *APOA1* gene and even one of those binds DR1 (HNF-4α) elements (Ladias and Karathanasis, 1991, Sladek et al., 1990, Delerive et al., 2004, Prieur et al., 2005). Also the RNA extraction time is long, 48 h, so there is plenty of time to activate and compensate lack of PPARs. *APOM* expression level is not moving. This is further supporting that the PPREs found are not for PPARs at all. A good candidate would be the NR HNF-4α, which binds DR1 type REs (Prieur et al., 2005).

Many interesting genes were significantly down-regulated (*APOB, APOC1, APOD, APOC2* and *APOL1,2,6*) which provides evidence of PPAR regulation by direct or secondary effects. *APOB* gene, which was giving strange but statically significant results from quantitative PCR, is now clearly down-regulated. Also same kind effects are seen interestingly for *APOC1*, *APOD* and *APOC2* genes. Unfortunately, this experiment is not giving any more proof that *APOC2* is a direct target of PPARs, whereas the regulation of the *APOL* genes family members by PPARs gained more confirmation from the siRNA experiment. After siRNA treatment there are three genes affected and one more almost affected. *APOL1, APOL2* and *APOL6* genes are going down statistically significantly. *APOL4* gene seems down-regulated by PPARs but unfortunately the effect is not statistically significant. This effect could be checked by using some PPAR agonist combined with a siRNA experiment.

The *APOO* gene seems affected by siRNA treatment, but we can only presume that this is a secondary affect. *Fibroblast growth factor 21* (*FGF21*) is direct target of PPARα. Its function is to stimulate lipolysis in white adipose tissue. FGF21 has been reported to be the key regulator of lipids and their efflux (Inagaki et al., 2007, Micheal et al 2007). This gene is affecting whole lipid metabolism pathway and so it also might mediate the expression levels of apolipoproteins and

create artefacts in the siRNA experiment. The siRNA results do not proof a direct regulation by PPARs. However, when results are combined with the real-time PCR results, we can see short time depended regulation there is strong evidence to suggest direct regulation by PPARs.

ChIP

We performed chromatin immunoprecipitation (ChIP) assay in human hepatocellular cells which we believed to represent well liver tissue. Unfortunately, we had difficulties with the ChIP assay. We wanted to study the association of PPAR and RXR with the *APOA1, APOA2, APOA4, APOE, APOA, APOC2, APOF, APOL6* and *APOM* TSS and in addition the association with other factors, such as CoAs, mediators and p-Pol II. Binding of those factors in the TSS was not confirmed by ChIP assays. Either the antibodies used were not specific enough or the conditions of the protocol were not ideal for their function. Also it is possible that some mistakes were made during the protocol causing errors in results. To improve reliability of ChIP protocol more controls would be needed, for example adding at least one certain positive and negative region. Also use of technical duplicates or triplicates would improve reliability of the results. There are also plenty of antibodies on the market from different companies. It would be worth to try some other antibodies like Upstate Biotechnology's or Abcam's. Because the basic ChIP protocol did not work, it was not worth to even try chromatin conformation capture (3C) assays. This assay would be needed to see, whether PPREs really a loop to the TSSs and what is the kinetics of this possible looping. The differences in general signal strength between different TSS regions shows a trend, but without statistical significance no clear conclusions can be drawn.

In summary

In summary, the *APOA1, APOA2, APOB, APOD, APOE, APOF, APOL1, APOL3, APOL5* and *APOL6* genes were found to be regulated by PPARs in direct or secondary manners. Moreover, a trend can be detected where number and quality of found putative PPREs correlate with the number of responsive genes. In addition, results have provided new insights into the understanding of lipid metabolism on gene regulation level that apply to many typical diseases of industrial countries.

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