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Διδακτορική Διατριβή

ΜΗΧΑΝΙΣΜΟΙ ΜΕΤΑΓΡΑΦΙΚΗΣ ΡΥΘΜΙΣΗΣ ΤΩΝ ΓΟΝΙΔΙΩΝ ΤΩΝ ΑΠΟΛΙΠΟΠΡΩΤΕΪΝΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ IN VIVO

Ιωάννα Μόσιαλου

Επιβλέπων Καθηγητής: Δ. Καρδάσης

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Ευχαριστίες

Χωρίς την παρουσία και υποστήριξη κάποιων ανθρώπων δε θα ήταν δυνατή η υλοποίηση της διδακτορικής διατριβής.

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Abstract

Numerous epidemiological and clinical trials have established that High Density Lipoprotein (HDL) is a strong and independent risk factor for the development of atherosclerosis and Coronary heart disease (CHD) which is the leading cause of morbidity and mortality in Western countries. These studies showed that there is an inverse correlation between plasma HDL cholesterol levels and incidence of acute cardiovascular events. On the other hand, it has been well documented in recent years that the concentration of plasma HDL is not by itself a good biomarker for CHD risk prediction but the functionality of HDL particles is also important. HDL has multiple atheroprotective functions which include the removal of excess cholesterol from peripheral cells and protective functions on the vascular endothelium. These functions are disturbed in subjects with mutations in key genes of the HDL biogenesis/maturation pathway as well as in patients with chronic inflammatory diseases. HDL levels are regulated by genetic and environmental factors as well as by drugs. Existing drugs raise HDL levels to a minor extent suggesting that novel strategies are needed.

In the present study, we investigated in detail the molecular mechanisms that control the expression of genes which are involved in lipoprotein metabolism. More specifically:

In ***Parts I and II***, we explored the mechanisms that regulate the expression of the gene encoding apolipoprotein M (apoM) in hepatic cells. ApoM is a recently described apolipoprotein that plays an essential role in HDL maturation in plasma. We analyzed the human apoM promoter and we identified a novel Hormone Response Element (HRE) that serves as a binding site for various members of the hormone nuclear receptor superfamily such as HNF-4 and heterodimers of Retinoid X Receptor (RXR) with receptors for oxysterols (LXR), thyroid hormone (TRβ1) and fibrates (PPAR). This element was further characterized by site directed mutagenesis and was found to mediate the response of the apoM promoter to the above agonists. In a region adjacent to this novel HRE, we identified and characterized a dual specificity regulatory element that binds HNF-1 and

members of the AP-1 family of transcription factors (c-Jun and JunB). Using in vitro and in vivo assays we established that activation of PKC by phosrbol esters leads to the activation of Jun proteins which bind to the dual specificity response element and displace HNF-1 leading to transcription repression. Based on these findings, we propose a model that could account for the downregulation of apoM gene expression during infection and inflammation. We also found that a similar mechanism could apply to a second HDL-related gene which encodes for apolipoprotein A-II.

In ***Part III***, we investigated the mechanism of downregulation of the gene endocing the ATP Binding Cassettee Transporter A1 (ABCA1) by inflammatory factors. Similar to ApoM, we found that the proximal ABCA1 promoter contains an AP-1 element that binds c-Jun. Activation of c-Jun by Protein Kinase C was found to inhibit ABCA1 promoter activity in hepatic cells.

In ***Parts IV and V*** we focused on the role of miRNAs in the regulation of the expression of genes that are involved in lipoprotein metabolism. The focus was on the role of let-7b in the regulation of apoE gene expression in macrophages (Part IV) and on the role of miR-122 in the expression of several lipoprotein-related genes in hepatic cells (Part V). We found that the apoE gene, which plays several atheroprotective roles including the biogenesis of HDL, is a direct target of let-7b which binds to apoE 3' UTR and downregulates its expression. The let-7b target site in apoE was verified by in vitro mutagenesis and functional assays. We also found that let-7b plays a role in the inhibition of apoE gene expression by lipopolysaccharide (LPS, model of bacterial infection) in macrophages. Finally, we found that overexpression of miR122 in hepatic cells was associated with a marked increase in the expression of genes that are involved in lipid metabolism including apoB, ABCA1 and Sterol regulatory Element Binding Proteins (SREBPs). Our findings are in line with previous overexpression and knockdown studies in mice which had shown a positive association between miR-122 expression levels and plasma cholesterol and triglyceride levels, hepatic cholesterol levels and lipid accumulation, as well as fatty acid and cholesterol synthesis.

In conclusion, understanding the mechanisms of HDL physiology and regulation is very important for developing novel strategies to increase HDL levels in plasma, to correct its functional abnormalities and to effectively treat patients with CHD.

Περίληψη

Επιδημιολογικές και κλινικές μελέτες έχουν δείξει ότι τα επίπεδα της λιποπρωτείνης υψηλής πυκνότητας (High Density Lipoprotein, HDL) αποτελούν ανεξάρτητο παράγοντα κινδύνου για την ανάπτυξη αθηροσκλήρωσης και στεφανιαίας νόσου η οποία αποτελεί την κύρια αιτία θνησιμοτητας στις ανεπτυγμένες βιομηχανικά χώρες. Από τις μελέτες αυτές προκύπτει μία αντίστροφη σχέση μεταξύ των επιπέδων της HDL στο πλάσμα και της εμφάνισης καρδιαγγειακών παθήσεων. Ωστόσο, τα ανεβασμένα επίπεδα της HDL δεν αρκούν για τη προστασία από τις καρδιαγγειακές παθήσεις διότι η λειτουργικότητα της HDL είναι εξίσου σημαντική. Η αντι-αθηρογενετική δράση της HDL οφείλεται στις πλειοτροπικές της ιδιότητες που περιλαμβάνουν την εκροή της περίσσειας χοληστερόλης από τα περιφερικά κύτταρα και τη διατήρηση της ακεραιότητας των ενδοθηλιακών κυττάρων. Αυτές οι δράσεις της HDL δεν λειτουργούν σωστά σε άτομα με μεταλλάξεις σε γονίδια που εμπλέκονται στη βιογένεση ή το μεταβολισμό της HDL καθώς και ασθενείς με χρόνια φλεγμονή. Τα επίπεδα της HDL στο πλάσμα καθορίζονται από γενετικούς και περιβαλλοντικούς παράγοντες καθώς επίσης και από φαρμακευτική αγωγή. Τα διαθέσιμα θεραπευτικά μέσα οδηγούν σε μικρή αύξηση των επιπέδων της HDL καθιστώντας αναγκαία την ανάπτυξη νέων θεραπευτικών στρατηγικών.

Ο σκοπός της παρούσας διατριβής ήταν η μελέτη των μοριακών μηχανισμών που ρυθμίζουν την έκφραση γονιδίων που συμμετεχουν στο μεταβολισμό των λιποπρωτεϊνών του ανθρώπου. Πιο συγκεκριμένα:

Στις ***Ενότητες I και II***, μελετήσαμε τους μηχανισμούς που ρυθμίζουν την έκφραση του γονιδίου που κωδικοποιεί την απολιποπρωτεϊνη M (apoM) στα ηπατικά κύτταρα. Η apoM είναι μια σχετικά καινούργια απολιπο πρωτεΐνη που παίζει ουσιαστικό ρόλο στην ωρίμανση της HDL στο πλάσμα. Αναλύσαμε τον υποκινητή της apoM του ανθρώπου και ταυτοποιήσαμε ένα καινούργιο στοιχείο απόκρισης σε στεροειδείς ορμόνες (Hormone Response Element, HRE) το οποίο αποτελεί θέση πρόσδεσης για πολλά μέλη της οικογένειας των πυρηνικών υποδοχέων ορμονών, όπως ο HNF-4 και τα

ετεροδιμερή του υποδοχέα του 9-cis ρετινοϊκού οξέος (RXR) με τους πυρηνικούς υποδοχείς των οξυστερολών (LXR), της ορμόνης του θυρεοειδούς (TRβ1) και των φιμπρατών (PPAR). Η μεταλλαξιγένεση αυτής της θέσης έδειξε ότι η περιοχή αυτή είναι υπεύθυνη για την την απόκριση του υποκινητή του γονιδίου της αποΜ στους συνδέτες για τους παραπάνω πυρηνικους υποδοχείς. Κοντά σ'αυτό το καινούργιο HRE, ταυτοποιήσαμε και χαρακτηρίσαμε ένα ρυθμιστικό στοιχείο πρόσδεσης τόσο του παράγοντα HNF-1 όσο και μελών της οικογένειας μεταγραφικών παραγόντων AP-1 (c-Jun και JunB). Χρησιμοποιώντας αναλύσεις *in vitro* και *in vivo*, δείξαμε ότι η ενεργοποίηση της πρωτεϊνικής κινάσης C (PKC) από τους εστέρες της φορβόλης (PMA) οδηγεί σε ενεργοποίηση των πρωτεϊνών Jun που προσδένονται σ΄αυτό το ρυθμιστικό στοιχείο εκτοπίζοντας τον HNF-1 με αποτέλεσμα την μεταγραφική καταστολή του γονιδίου της apoM. Με βάση αυτά τα ευρήματα, προτείνουμε ένα μοντέλο για την αρνητική ρύθμιση της έκφρασης του γονιδίου της αποΜ κατά τη διάρκεια φλεγμονής. Ένας παρόμοιος μηχανισμός βρήκαμε να ισχύει για ένα άλλο γονίδιο που σχετίζεται με την HDL, το γονίδιο που κωδικοποιεί για την απολι πρωτεΐνη A-II.

Στην ***Ενότητα III***, διερευνήσαμε το μηχανισμό αρνητικής ρύθμισης του γονιδίου που κωδικοποιεί τον μεμβρανικό μεταφορέα λιπιδίων ATP Binding Cassette Transporter A1 (ABCA1) από παράγοντες φλεγμονής. Ομοίως με την apoM, βρήκαμε ότι ο εγγύς υποκινητής του γονιδίου ABCA1 έχει ένα ρυθμιστικό στοιχείο AP-1 στο οποίο προσδένεται ο μεταγαφικός παράγοντας c-Jun. Ενεργοποίηση του c-Jun από την PKC καταστέλλει την ενεργότητα του υποκινητή του ABCA1 σε ηπατικά κύτταρα.

Τέλος, στις **Ενότητες IV και V**, μελετήσαμε στο ρόλο των miRNAs στη ρύθμιση της έκφρασης γονιδίων που συμμετέχουν στο μεταβολισμό των λιποπρωτεϊνών του ανθρώπου. Επικεντρωθήκαμε στο ρόλο του let-7b στη ρύθμιση της έκφρασης του γονιδίου της αποΕ στα μακροφάγα (Ενότητα IV) και στο ρόλο του miR-122 στη ρύθμιση της έκφρασης γονιδίων που εμπλέκονται στο μεταβολισμό των λιποπρωτεϊνών σε ηπατικά κύτταρα (Ενότητα V). Βρήκαμε ότι το γονίδιο που κωδικοποιεί για την αποΕ, η οποία έχει πολλαπλή αθηροπροταστευτική δράση, περιλαμβανομένης της βιογένεσης της HDL, αποτελεί άμεσο στόχο του let-7b το οποίο προσδένεται στο 3'UTR της apoE και

καταστέλλει την έκφρασή της. Η θέση πρόσδεσης του let-7b στην αποΕ επιβεβαιώθηκε με *in vitro* μεταλλαξιγένεση. Επιπλέον βρήκαμε ότι το let-7b παίζει ρόλο στην καταστολή της έκφρασης του γονιδίου της αποΕ από λιποπολυσαχαρίτες (LPS) στα μακροφάγα. Τέλος, βρήκαμε ότι υπερέκφραση του miR-122 σε ηπατικά κύτταρα σχετίζεται με αυξημένη έκφραση γονιδίων που συμμετέχουν στο μεταβολισμό των λιπιδίων όπως η αποΒ, ο ABCA1 και μέλη της οικογένειας των μεταγραφικών παραγόντων SREBP (Sterol Regulatory Element Binding Protein). Τα ευρήματα αυτά συμφωνούν με προηγούμενες μελέτες υπερέκφρασης και αποσιώπισης σε ποντίκια που έχουν δείξει ότι τα επίπεδα του miR-122 σχετίζονται θετικά με τα επίπεδα της χοληστερόλης και των τριγλυκεριδίων στο πλάσμα, τα επίπεδα της χοληστερόλης και τη συσσώρευση λιπιδίων στο ήπαρ καθώς και τη βιοσύνθεση λιπαρών οξέων και χοληστερόλης.

Συνοψίζοντας, η κατανόηση των μηχανισμών ρύθμισης της HDL είναι ιδιαίτερα σημαντική για την ανάπτυξη νέων στρατηγικών αύξησης των επιπέδων της HDL στο πλάσμα καθώς και της λειτουργικότητάς της, παρέχοντας προστασία από την ανάπτυξη αθηροσκλήρωσης.

Introduction

Cholesterol and atherosclerosis

Coronary artery disease (CAD), the major cause of morbidity and mortality in developed countries, is a complex multifactorial and polygenic disorder that is thought to be the result of interactions between an individual's genetic background and various environmental factors. Cigarette smoking, age, high body mass index, blood pressure, diabetes, dietary factors, lack of exercise and most importantly excess cholesterol consist the major risk factors contributing to the development of CAD and its underlying cause, atherosclerosis (1). Atherosclerosis, is characterized by the thickening of the arterial wall, due to the gradual accumulation of cholesterol, that results in the narrowing of the lumen of the artery and a consequent reduction in the amount of blood supplied to the organs, affecting mainly heart and brain. Upon atherosclerotic plaque rupture, a blood clot is formed causing myocardial infraction and stroke (2). Thus, cholesterol levels, the major risk factor for atherosclerotic disease and CAD, also implicated in the pathogenesis of dementias, diabetes and cancer must be tightly regulated both intracellularly and in plasma.

Cholesterol is an essential structural component of the cell membranes, accounting for 20-25% of the lipid molecules that helps to generate a semi-permeable barrier between cellular compartments and to regulate membrane fluidity. It affects cellular processes by interacting both with other membrane lipids as well as with specific proteins, modulating their functions and participating in several membrane trafficking and transmembrane signaling processes (3). Metabolites of cholesterol, such as bile acids and steroids (steroid hormones, vitamins) have important biological roles as signal transducers and solubilizers of other lipids. Excess cholesterol, however, is toxic to cells.

Lipoproteins

The transport of free cholesterol (FC) and cholesteryl esters (CE) as well as of other lipids (triglycerides (TG) and phospholipids (PL)) in the circulation, is achieved by their packaging into water-soluble complexes called lipoproteins. Lipoproteins are either spherical particles, consisting of a core of non-polar neutral lipid (mainly CE and TG) and coats of relatively polar materials such as PL, FC and proteins or discoidal particles consisting of mostly polar lipids and proteins in a bilayer conformation (4,5). The protein constituents of lipoproteins are called apolipoproteins. Representative members of the apolipoprotein family are: apoA-I, apoA-II, apoB, apoC-II, apoC-III, apoE and apoM. Apolipoproteins provide structural stability and functional specificity to the lipoprotein particle, playing an essential role in the control of plasma and tissue lipid homeostasis. They are involved in specific binding to cellular receptors, the regulation of lipolytic enzymes and the process of lipid exchange and transfer (4).

Based on their density, plasma lipoproteins are grouped into five major classes and various subclasses: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). HDL and LDL are rich in cholesterol, whereas TG constitute the major lipid in the remaining subclasses. ApoB-48, apoB-100 and apoA-I are the major protein components of chylomicrons, VLDL/ IDL/ LDL and HDL, respectively.

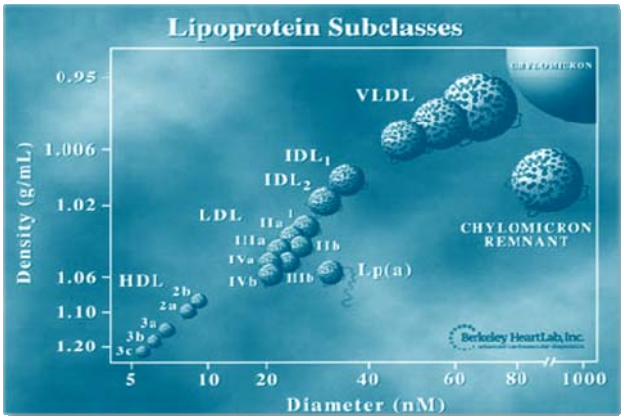

Figure 1. Classification of plasma lipoproteins on the basis of their density and size.

The lipoprotein pathways

Lipoproteins are synthesized and catabolized in three distinct but metabolically interrelated pathways: the chylomicron pathway, the VLDL/IDL/LDL pathway and the HDL pathway.

In the ***chylomicron pathway***, dietary lipids assemble along with apoB-48 in intestinal epithelial cells to form chylomicrons, a process that is facilitated by the microsomal triglyceride transfer protein (MTP). Chylomicrons are then secreted into the blood and hydrolyzed by the enzyme lipoprotein lipase (LPL) which is anchored on the surface of microvascular endothelial cells in muscle and adipose tissue so that fatty acids enter these tissues. The chylomicron remnants that are formed by triglyceride hydrolysis, are rich in cholesteryl esters (CE) and are rapidly taken up by the liver mainly through the LDL receptor (4).

In the ***VLDL/IDL/LDL pathway***, lipids assemble by the action of MTP with apoB-100 to form VLDL particles in hepatocytes. VLDL is then secreted into the plasma, hydrolyzed by the action of lipoprotein lipase to produce IDL, which in turn is further converted to LDL by the action of hepatic lipase (HL). IDLs and LDLs are recognized and catabolized by the LDL receptor in the liver (4).

In the ***HDL pathway***, biogenesis of HDL involves the interaction of apoA-I which is synthesized and secreted by the liver, with the cholesterol and phospholipid membrane transporter ATP Binding Cassette Transporter A1 (ABCA1). The premature HDL particles thus formed are subsequently remodeled by various plasma enzymes to form the mature spherical HDL particles which are catabolized by membrane receptors such as Scavenger Receptor Class B Type I (SR-BI) (4,6,7). This process also known as reverse cholesterol transport (RCT), is described in more detail below.

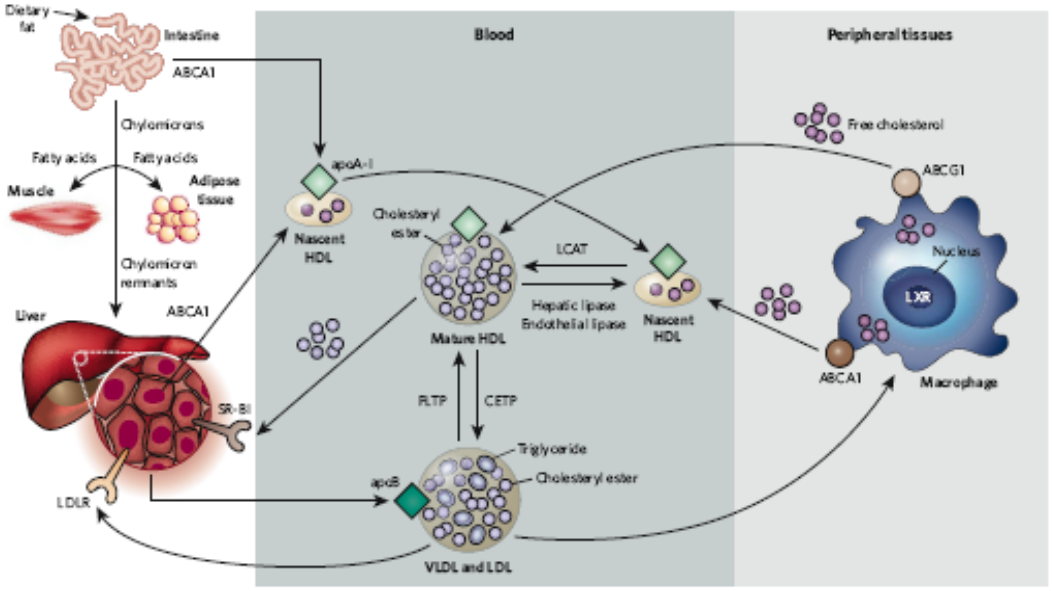

Figure 2. Lipoprotein metabolism. From reference (2). See text for details.

High Density Lipoprotein (HDL)

Structure of HDL

The HDL fraction in human plasma is heterogeneous in terms of size, shape, composition and surface charge (8). When viewed in the electron microscope, HDL can appear as either discoidal or spherical particles, with the latter being most abundant in normal plasma. Discoidal HDL particles contain two or three molecules of apoA-I and phospholipids with or without unesterified cholesterol while spherical HDL particles contain two or three molecules of apoA-I, phospholipids, unesterified cholesterol and a hydrophobic core consisting of cholesteryl esters and triglycerides (9).

When isolated on the basis of density by ultracentrifugation, human HDL separate into two major subfractions, which have been designated HDL_2 ($1.063<d<1.125 g/mL$,) and HDL_3 ($1.125<d<1.21 g/mL$). On the basis of their size, HDL is separated by nondenaturing polycaryamide gradient gel electrophoresis into five distinct

subpopulations with diameters ranging from 7.6nm to 10.6nm (3c, 3b, 3a, 2a and 2b) (10).

HDL in human plasma contains two main apolipoproteins, apoA-I and apoA-II, that account for 70% and 20%, respectively, of the total HDL protein. Spherical HDL particles have been classified into two main subpopulations on the basis of their apolipoprotein composition. The first subpopulation contains apoA-I but not apoA-II (A-I HDL), whereas the other contains both apoA-I and apoA-II (A-I/A-II HDL). In most human subjects, apoA-I is distributed approximately equally between A-I HDL and AI/A-II HDL. A-I/A-II HDL are mostly found in the HDL_3 density range, whereas A-I HDL are prominent components of both HDL_2 and HDL_3 (11,12).

The heterogeneity of HDL also extends to their surface charge. When HDL particles are separated on the basis of surface charge by agarose gel electrophoresis, they exhibit either alpha or pre-beta (β) migration. Alpha-migrating HDL are the spherical particles including both the HDL_2 and HDL_3 subfractions as well as most of the A-I HDL and A-I/A-II HDL subpopulations. Pre-β-migrating HDL represent either discoidal particles or monomolecular, lipid-free/poor apoA-I consisting of a single molecule of apoA-I with or without small amount of phospholipids (13).

Reverse Cholesterol Transport (RCT)

The most well known function of HDL relates to its role in reverse cholesterol transport (RCT). RCT is a complex process in which excess cholesterol from peripheral tissues is transferred to the liver for excretion into bile thus protecting cells from lipid toxicities and attenuating foam cell formation (Fig.2). Interaction of lipid-free apoA-I, that is synthesized and secreted by the liver, with ABCA1 lipid transporter results in the progressive lipidation of apoA-I and the formation of nascent HDL particles, called pre-β HDL. Nascent pre-β HDLs promote the efflux of cholesterol from peripheral tissues, including macrophages, through the actions of ABCA1. A portion of the unesterified cholesterol is then delivered to the liver whereas the remaining is esterified by the enzyme lecithin cholesterol acyl transferase (LCAT), that converts pre-β discoidal HDL

to small, spherical, mature α-HDL (HDL_3) and subsequently to larger HDL_2. Mature HDLs also promote cholesterol efflux from peripheral tissues through the action of the lipid transporter ABCG1. Plasma factors remodel HDL resulting in the formation of smaller α-HDL particles and the dissociation of small pre-β HDL particles, able to promote more cholesterol efflux. Cholesteryl esters within αHDL are finally delivered to the liver either directly via scavenger receptor class B type I (SR-BI) or indirectly as components of VLDL or LDL cholesterol (4-7,14).

HDL remodeling and pre-β HDL

HDL is subject to extensive remodeling by a range of plasma proteins. These include the enzymes LCAT and HL and the lipid transfer proteins cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). These enzymes alter the lipid content of HDL either by reducing the size of HDL or by promoting HDL particle fusion resulting in the generation of substantial amounts of pre-β HDL.

Pre-β HDLs are the precursor of mature HDLs. They are discoidal particles constituting only 5-10% of total HDL in plasma and exist as several subpopulations of discrete sizes (9). Pre-β HDLs contain apoA-I and phoshoplipids and as already mentioned, they can be derived from two sources: α) from newly synthesized hepatic apoA-I, acquiring free cholesterol and phospholipids through the interaction of apoA-I with ABCA1 and β) from α-HDL through the action of PLTP or the action of HL in concert with CETP. Pre-β HDL thus formed can then be relipidated either by incorporation into spherical α-HDL in a LCAT-dependent process that is associated with particle expansion (8,15), or by acquisition of lipids form other lipoproteins (16) and ABCA1-mediated cholesterol and phospholipid efflux from cells (9). Pre-β HDL therefore represent critical intermediates in the HDL pathway as they are the initial acceptors of excess cell cholesterol in the first step of RCT.

CETP is secreted by the liver and circulates in plasma principally bound to HDL. It promotes the exchange of cholesteryl esters for triglycerides between HDL and triglyceride-rich lipoproteins (VLDL/IDL) to form cholesteryl ester-depleted,

triglyceride-enriched HDL particles. The magnitude of transfer of cholesteryl esters out of HDL is greater than that of the transfer of triglycerides into the HDL. Subsequent hydrolysis of HDL triglycerides by hepatic lipase reduces the size of the HDL core. The consequent redundancy of surface constituents results in a dissociation of a proportion of the apoA-I from the HDL surface and thus the generation of a pool of lipid poor apoAI/ pre-β HDL (9,17).

PLTP is involved in the remodeling of HDL by transferring phospholipids between plasma lipoproteins, possibly by forming a ternary complex between donor and acceptor particles (18,19). This re-distribution of phospholipids between lipoprotein particles by PLTP results in the generation of small pre-β HDL and large α-HDL particles. Furthermore, during lipolysis of serum VLDL by lipoprotein lipase, surface remnants containing phospholipids and apolipoproteins are transferred by PLTP and contribute to pre-β HDL formation. Expression of human PLTP in transgenic mice increases the production of pre-β HDL and enhances hepatic uptake and clearance of cholesteryl esters (20,21).

Atherosclerosis

Atherosclerosis is the major cause of morbidity and mortality in the Western world, accounting for nearly 50% of all deaths. Both environmental and genetic factors affect the formation of the atherosclerotic plaque which is initiated by the accumulation and subsequent oxidation of low density lipoprotein in the arterial intima under inflammatory conditions (Fig.3).

Initiation and progression of atherosclerosis

Normal circulating LDL contains small quantities of lipid hydroperoxides derived from the lipoxygenase pathway (22). LDLs trapped in the subendothelial space receive additional lipid hydroperoxides produced by the lipoxygenase and the myeloperoxidase pathways operating in cells within the artery wall (22,23). When the level of oxidized

lipids in the trapped LDL exceed a critical threshold, the LDL phospholipids become oxidized and proinflammatory initiating and maintaining an active inflammatory process in the intima (22,23). Inflammation results in the production of chemokines such as monocyte chemoattractant protein 1 (MCP-1) and the expression of adhesion molecules by endothelial cells such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) that recruit monocytes into the subendothelial space. (24-26) Within the artery wall, LDLs promote the differentiation of monocytes into macrophages which express a range of scavenger receptors, SRAI, SRAII and CD36, that can bind and internalize oxidized (modified) LDLs in a process that converts them to foam cells (loaded with cholesterol) (27). The accumulation of foam cells leads to the formation of fatty streaks that are considered to be the initial lesions leading to the development of complex atherosclerotic plaques.

As the lesions progress, vascular smooth muscle cells, either recruited from the media into the intima or proliferating within the intima, contribute to this process by secreting large amounts of extracellular matrix components, such as collagen (28). The presence of these molecules increases the retention and aggregation of atherogenic lipoproteins. In addition to monocytes, other types of leukocytes, particularly T cells, are recruited to atherosclerotic lesions and help to perpetuate a state of chronic inflammation. As the plaque grows, compensatory remodeling takes place such that the size of the lumen is preserved while its overall diameter increases. Foam cells eventually die resulting in the release of cellular debris and crystalline cholesterol (2, 4, 28). In addition, smooth muscle cells form a fibrous cap beneath the endothelium, and this cap walls off the plaque from the blood. This process contributes to the formation of a necrotic core within the plaque and further promotes the recruitment of inflammatory cells. This non-obstructive plaque can rupture or the endothelium can erode, resulting in the exposure of thrombogenic material, including tissue factor, and the formation of a thrombus in the lumen. If the thrombus is large enough, it blocks the artery, which causes an acute coronary syndrome or myocardial infraction (heart attack). Ultimately, if the plaque does not rupture and the lesion continues to grow, the lesion can encroach on the lumen and result in clinically obstructive disease (29,30)

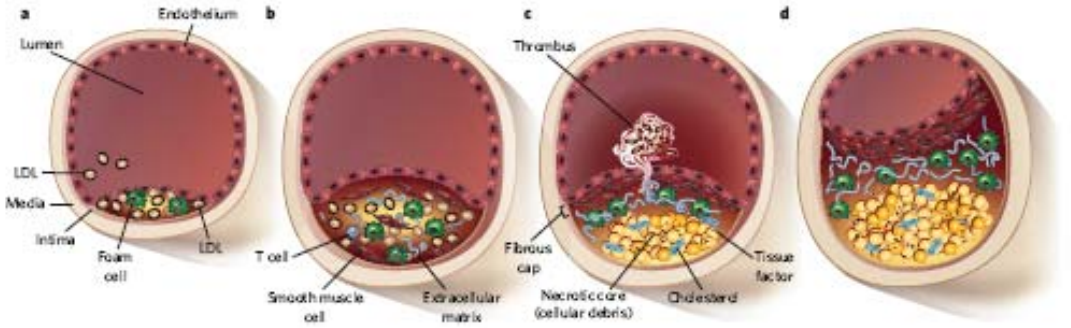

Figure 3. Initiation and progression of atherosclerosis. Atherosclerosis occurs at sites in the arterial tree where laminar flow is disrupted. A lesion begins as a fatty streak (a) and can develop into an intermediate lesion (b), and then into a lesion that is vulnerable to rupture (c) and, finally, into an advanced obstructive lesion (d). Taken from (2).

LDL as a risk factor for atherosclerosis and CVD

In agreement with their role in the initiation and progression of atherosclerosis, numerous epidemiological studies have identified high levels of LDL-C as a major contributor in atherogenesis and the development of cardiovascular disease (CVD) (1,31). The most effective and widely used drugs for LDL-C lowering in plasma are 3-hydroxyl-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, called statins. Statins inhibit cholesterol biosynthesis by reducing the activity of HMG-CoA reductase, the rate limiting enzyme in the de novo cholesterol biosynthetic pathway thus increasing the LDL receptor numbers on the cell surface and therefore the clearance of plasma cholesterol (32,33). However, although statins reduce CAD by approximately 30%, a substantial residual cardiovascular risk remains even with very aggressive reductions in levels of LDL-C (34-36), dictating the need for the development of new adjunctive therapies targeting other players in the lipoprotein pathway to prevent and treat CVD.

HDL as a risk factor for atherosclerosis and CVD

In contrast to LDL, epidemiological studies have shown that levels of HDL cholesterol in plasma are inversely related to atherosclerosis susceptibility in humans

(31,37,38). In the Framingham Heart Study, 43% to 44% of coronary events occurred in persons with HDL-C levels less than 40mg/dL (31). Individuals having HDL-C levels less than 35mg/dL had an 8-fold higher incidence of CVD compared with those having HDL-C levels of more than 65mg/dL (31,39). In addition, observational studies have shown that a decrease in plasma HDL cholesterol concentration by 1mg/ dL is associated with a 2-3% increased risk of CVD while an increase by 1mg/dL is associated with a 6 % lower risk of coronary death, independently of LDL-C level (40,41). Since low HDL-C levels are relatively common in the general population, with reported rates of HDL-C less than 35mg/dL of 16%-18% in men and 3% to 6% in women (31) and low level of HDL-C is strongly associated with obesity and insulin resistance states and is considered as a component of the metabolic syndrome (MetS), strategies for targeting HDL composition may contribute to the treatment of CVD. MetS is characterized by impaired glucose (insulin insensitivity, hyperinsulinemia, increased gluconeogeneis) and lipid metabolism (reduced suppression of lipopysis in adipose tissue, increased hepatic VLDL secretion-hypertriglyceridemia, low plasma HDL levels). Low HDL levels observed in obesity, insulin resistance states and MetS are believed to reflect increased core lipid exchange between HDL and triglyceride-rich lipoproteins, leading to modifications of HDL composition and size that result in increased catabolism of HDL particles.

HDL- based therapies

Currently available pharmacological agents that increase HDL-C levels include nicotinic acid, fibrates and statins while several HDL-based therapeutic strategies are currently being tested in clinical trials including CETP inhibitors, recombinant phospholipids/apoA-I complexes (rHDLs), apoA-I mimetic peptides, synthetic phospholipids and new PPARα agonists. Nicotinic acid (vitamin B_3) represents the most effective HDL-C increasing agent currently available (20% to 30% increase) (42-44) that acts mainly by increasing pre-β HDL and decreasing hepatic apoA-I catabolism (40). However, its therapeutic potential has been limited by its adverse effects, hepatotoxicity and glycemia being the most important. Fibrates reduce coronary events by 22% despite a

modest effect on HDL-C levels (6% increase) (45,46), which is probably due to some additional effects on apolipoprotein synthesis (increased hepatic apoA-I synthesis) and lipoprotein metabolism through their ability to activate the nuclear receptor peroxisome proliferator-activated receptor (PPAR) α. Similarly, statins only modestly increase HDL-C levels by 5% to 15% thus being inadequate as monotherapy for increasing HDL-C (47).

Torcetrapib, a direct CETP inhibitor, although significantly increased HDL-C levels increased all-cause mortality and other cardiovascular events in individuals at high risk for CVD as shown in the ILLUMINATE trial and as a result it's development was halted by Pfizer (48,49). The failure of torcetrapib could be attributed to a number of adverse effects such as increase in blood pressure while the production of dysfunctional HDL (production of large α-HDL particle, which are not the preferred ABCA1-mediated cholesterol efflux transporters) or production of proinflammatory HDL may also be a possible explanation (50). Recombinant phospholipids/apoA-I complexes (rHDLs), such as recombinant apoA-I Milano (mutant)/phospholipids complex and apoA-I mimetic peptides, such as L-4F and D-4F that are smaller peptides resembling the lipid binding domain of apoA-I exert their atheroprotective effects, at least in animal models, by generating HDL-like particles or modifying existing HDL (increased production of pre-β HDL) thus enhancing cholesterol efflux from foam cells rather than increasing HDL-C levels (51-53). In similar manner, synthetic phospholipids that mimic the integral components of HDL, have been shown in a small human study to increase pre-β HDL-C and apoA-I levels, to improve reverse cholesterol transport and to regress atherosclerosis (54,55). Finally, newer more selective and potent PPAR agonists, such as the PPARα agonist NS-220 and the PPARδ agonist GW501516 have been postulated to increase macrophage cholesterol efflux and the anti-inflammatory properties of HDL but the final results from clinical trials are still pending (56,57).

Atheroprotective properties of HDL

The atheroprotective properties of HDL involve the removal of cholesterol from macrophages of the arterial wall, anti-oxidation, anti-inflammation and anti-thrombotic functions as well as protection of endothelial cells from apoptosis (1,58-60).

Cholesterol elimination from macrophages is a key process that serves to prevent cholesterol retention in atherosclerotic lesions and represents the first crucial step in reverse cholesterol transport. Most of the cholesterol in foam cells is stored in lipid droplets and increasing macrophage cholesterol ester hydrolysis increases the flux of cholesterol from macrophages. ABC transporters, ABCA1 and ABCG1 are key regulators of cellular cholesterol export from macrophages, similar to other cell types.

HDL inhibits the pro-atherogenic oxidative modification of LDL mostly due to the activity of factors and enzymes that are co-transported with HDL, such as paraoxonase-1 (PON1), platelet-activating factor acetyl hydroplase (PAF-AH) and LCAT (61-63). PON1, PAF-AH and LCAT have different enzymatic activities but they all have the ability to destroy the lipid hydroperoxides that oxidize LDL phospholipids. Besides this, apoA-I, the main component of HDL and to a less extent apoA-II are capable of removing LDL lipid hydroperoxides (64). It has been reported that HDL cholesteryl ester hydroperoxides are rapidly and selectively removed by liver cells. Thus, one of the main antioxidant functions of HDL is mediated by a transport mechanism that binds and carries away oxidant molecules.

The anti-inflammatory function of HDL includes the inhibition of the cytokine-induced expression of endothelial cell adhesion molecules and chemotactic protein-1 (MCP-1) thus reducing the infiltration of monocytes into the artery wall. The inhibition of the expression of MCP-1 in response to oxidized LDL is a process linked to the antioxidant components of HDL, PON1 and PAF-AH (65,66), while the inhibition of adhesion molecule expression is associated with a reduction in the mRNA levels of these proteins due to inactivation of NF-kB, a critical regulator of their expression (67). In particular, HDLs inhibit endothelial cell sphingosine kinase, an enzyme that catalyzes a key step in the pathway by which TNF-α stimulates the expression of endothelial cell

adhesion molecules. This inhibition of sphingosine kinase has a downstream effect by inhibiting the nuclear translocation of NF-kB. Additionally, HDL inhibits adhesion molecule expression through its ability to inhibit reactive oxygen species generation and promote the synthesis of nitric oxide (NO) that contributes in the retention of NF-kB in an inactive state (68).

NO synthesis contributes to a number of additional vasoprotective and atheroprotective functions of HDL. NO is a potent vasodilator with multiple effects on endothelium, vascular smooth muscle cells and events at luminal surface (69). NO deficiency increases neutrophil adherence to the endothelium and enhances smooth muscle cell proliferation and platelet aggregation and adhesion thus accelerating the progression of atherosclerosis (70). It is generated by eNOS localized in caveolae and activated by agonists of diverse cell surface receptors, such as acetylcholine (Ach), and by physical stimuli such as hemodynamic shear stress (71). HDL promotes NO production by eNOS by a variety of mechanisms. It regulates eNOS subcellular distribution by maintaining the lipid environment in caveolae that is disrupted by oxidized LDL, it prevents eNOS uncoupling by LDL, which favors superoxide anions production over NO production and it increases eNOs activity and abundance through membrane-initiated signaling pathways (59).

The antithrombotic actions of HDL involve the promotion of blood flow, the attenuation of thrombin generation as well as the inhibition of platelet and endothelial activation. HDL increases blood flow by increasing NO and prostacyclin production. Prostacyclin acts synergistically with NO to induce vascular smooth muscle cell relaxation, inhibit platelet activation and diminish the release of growth factors that stimulate the local proliferation of smooth muscle cells (72). HDL promotes prostacyclin production both by providing the phospholipids required for its synthesis and by upregulating the expression of Cox-2, the rate limiting enzyme in its production (73,74). The HDL-related decrease in thrombin generation is mediated by enhanced activity of anticoagulant factors such as the protein C pathway and endothelial cell thrombomodulin (75) while the inhibition of platelet aggregation occurs through downregulation of platelet activating factor release and thromboxane A2 and upregulation of NO and

prostacyclin synthesis (59,76). The prevention of endothelial activation by HDL includes inhibition of tissue factor and cell surface adhesion molecule expression, promotion of NO production as well as blunting of endothelial cell apoptosis.

Blunting of endothelial cell apoptosis is achieved through the antagonism of HDL with a variety of proapoptotic mechanisms. It prevents the sustained increases in intracellular calcium induced by proapoptotic oxidized LDL (77), it inhibits the TNFα-induced activation of caspases 3 and 9 and it protects endothelial cells from growth factor deprivation-related apoptosis via inhibition of the mitochondrial pathway of apoptosis (78,79). Furthermore, it stimulates endothelial cell proliferation and migration by activation of multiple kinase cascades (80,81). These combined actions of HDL promote endothelial monolayer integrity that is very critical for normal homeostasis in the vascular wall and the prevention of atherogenesis.

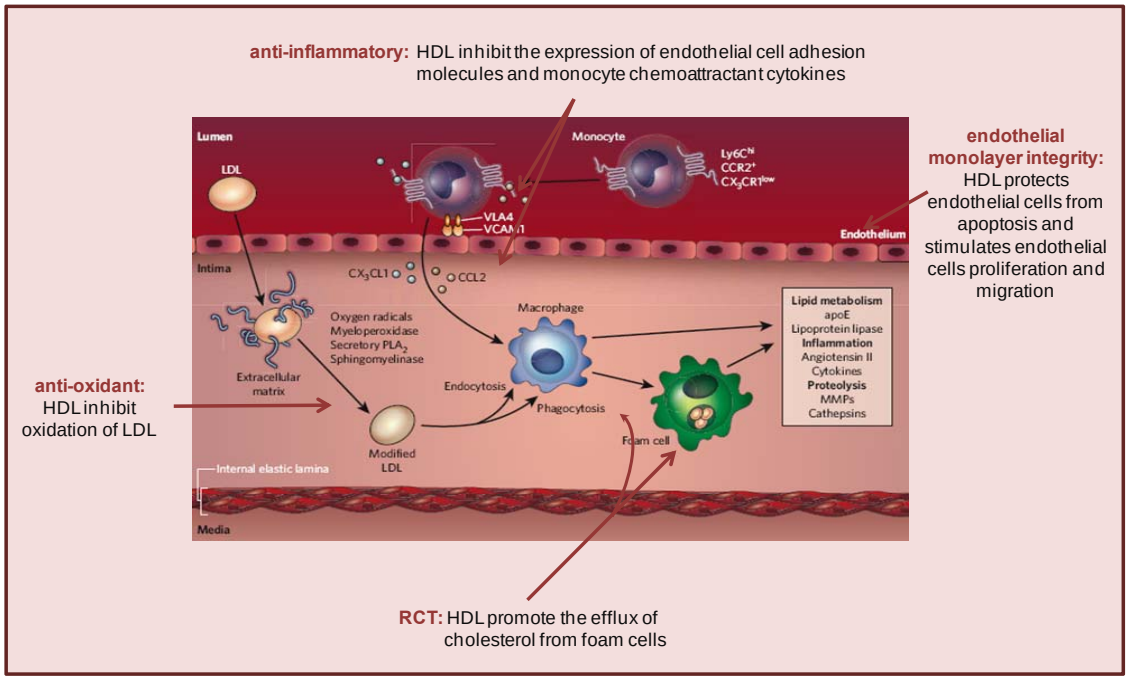

Figure 4. Atheroprotective properties of HDL. LDLs in the blood enter the intima, where they are retained through binding to the extracellular matrix. Modified LDLs then initiate and maintain an active inflammatory process that results in the generation of chemokines and the subsequent recruitment of monocytes in the intima. These monocytes then differentiate into macrophages, which take up modified LDL through endocytosis or phagocytosis and become foam cells. HDLs have the potential to impact at several points in this process. They promote the efflux of cholesterol from foam cells while through

their antioxidant, anti-inflammatory and vasoprotective effects, HDLs reduce the infiltration of monocytes into the artery wall. Modified from (2).

Apolipoprotein M

Apolipoprotein M was fist identified by Xu and Dahlbcak in 1999 (82). It is a lipoprotein-associated plasma protein with antiatherogenic properties that is synthesized predominantly in the liver and to a smaller degree in the kidney (82,83). Most human apoM in plasma is part of HDLs, (both HDL_2 and HDL_3 and mainly pre-β HDL) but minor amounts can also be found in other lipoprotein classes, including LDLs, VLDLs and chylomicrons (84-87). The apparent content of apoM in LDLs increases in LDL-R deficient hypercholesterolemic mice, in VLDLs from apoE deficient mice fed a high-fat high-cholesterol diet and in chylomicrons after ingestion of a fat-rich meal suggesting that apoM can be transferred between lipoprotein particles tending to associate with the most prominent plasma lipoprotein fraction (86). Five isoforms have been identified in both HDL and LDL in plasma that represent various degrees of glycosylation, sialylation or phosphorylation that possibly have different functions (87). The mean molar plasma concentration of apoM is approximately 0.9μM (23mg/L) which is 2-3% of that of apoA-I. Thus, in humans, apoM constitutes a minor apolipoprotein found in only 5% of total HDL and in 2% of total LDL(84).

apoM protein structure

Human apoM is a glycoprotein of 26kD, containing 188 amino acid residues that belongs to the lipocalin protein super-family (88-90). Lipocalins are small extracellular proteins with weak amino acid sequence similarity but high similarity at the tertiary structural level. They have diverse functions, some of which are antioxidants, have enzymatic activities or regulate cellular and immunological reactions but the majority of them are responsible for the storage and the transport of compounds that have low

solubility or are chemically sensitive such as vitamins, steroids and metabolic products (91-93).

Within the lipocalin protein superfamily, apoM has highest similarity with mouse major urinary protein (MUP), apolipoprotein D, and retinol binding protein (RBP) and these similarities were utilized to generate a computer-based three-dimensional model of apoM (88). According to the model, apoM contains an eight-stranded anti-parallel β-barrel and a α helix on the outside with the barrel forming a hydrophobic ligand-binding pocket for small lipophilic molecules (Fig. 5). Although the physiological ligand of apoM is unknown, recombinant human and mouse apoM (which is 79% identical to human apoM) have been shown to bind retinol and retinoic acid (89) as well as D-sphingosine and sphingosine-1-phosphate in vitro (94). Other structural features noticed during the evaluation of the 3D model were the six cysteines suggesting a disulfide-bond pairing of Cys23-Cys167, Cys95-Cys183 and Cys128-Cys157 and one site for N-linked glycosylation at Asn135 that is not preserved in mouse apoM (89).

A noteworthy property of the apoM protein shared only with haptoglobin-related protein (HRP) and paraoxonase-1 (PON-1) among the HDL-associated proteins is that it retains its N-terminal signal peptide, as it lacks a signal peptidase cleavage site (95,96). The retained signal peptide probably serves as a hydrophobic anchor, localizing apoM to the single phospholipid layer of lipoproteins. When a single-point mutation was introduced just after the signal peptide (Q22A) of apoM, creating a cleavage site, $apoM^{Q22A}$ was secreted without its signal peptide sequence and was unable to associate with lipoproteins while overexpression of this mutated $apoM^{Q22A}$ in the liver of mice resulted in its rapid removal from plasma by filtration in the kidney (95).

apoM functions

Although its biological function is not yet clear, apoM is suggested to be an important regulator of HDL metabolism bearing antiatherogenic properties while kidney-derived apoM is involved in the recycling of small lipophilic ligands via the multi-ligand receptor megalin.

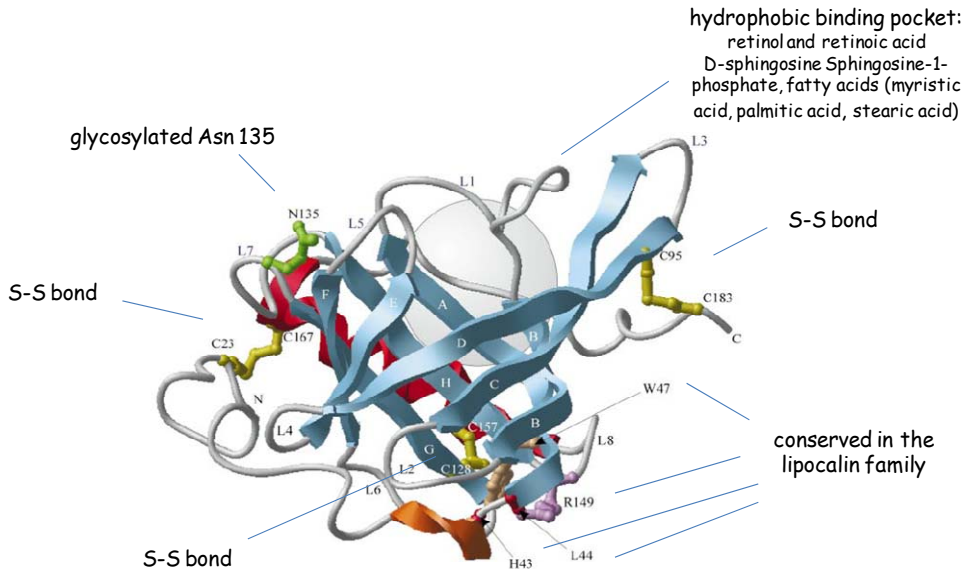

Figure 5. Predicted secondary structure of apoM protein. Modified from (88).

a) apoM functions in the liver

In the liver, apoM exerts its antiatherogenic properties mainly by influencing the formation of pre-β HDL and thus enhancing cholesterol efflux from foam cells (85,97-99), as well as protecting LDL against oxidation (84). The location of the apoM gene in the major histocompatibility complex class III (MHCIII) region on chromosome 6 in close proximity to genes related to the immune response, implies that apoM may also be related to the immune response system and thus affecting the anti-inflammatory, atheroprotective properties of HDL (99-101).

Studies in humans and in mice overexpressing or lacking apoM have shown a positive association between plasma apoM levels and total as well as HDL and LDL cholesterol concentrations (96,102,103). Lack of apoM expression in TCF-1α/HNF-1α (Transcription factor-1α/Hepatocyte Nuclear Factor-1α) knock out mice or in apoM small interfering RNA-injected mice leads to formation of larger size HDL 1 particles and disappearance of pre-β HDL particles in plasma suggesting that apoM may play a role in HDL remodeling, particularly with regard to the metabolism of pre-β HDL (97). Adenoviral apoM overexpression in LDL-receptor deficient mice (animal model for

premature atherosclerosis) or hepatic overexpression of apoM in apoM transgenic mice led to a reduction in atherosclerosis development (85,97). ApoM was also found to be positively associated with pre-β HDL formation in type 2 diabetes subjects (103). ApoM-containing HDL particles isolated from human plasma and apoM-transgenic mice have been shown to be more resistant to oxidation and more efficient in protecting against LDL oxidation as well as stimulating cholesterol efflux from macrophage foam cells (84,85).

Genetic association studies in Chinese populations have associated two single-nucleotide polymorphisms (SNPs) located in the apoM proximal promoter region (SNP T-778C and SNP T-855C) with the development of coronary artery disease (104,105). The T-778C polymorphism was also found to be associated with type 1 and 2 diabetes in Chinese supporting the possible involvement of apoM in the pathogenesis of diabetes as suggested by the reduced apoM expression levels found in animal models of diabetes and some patients with diabetes and metabolic syndrome (103,106-112).

The effect of apoM in pre-β HDL formation and thus cholesterol efflux and atherosclerosis seems to be due to a direct effect in the interconversion of α-HDL to pre-β HDL particles (85,97) as well as due to the facilitation of lipid addition by ABCA1 during intracellular nascent pre-β HDL particle formation (113) (Fig.6). The rates of interconversion of α-HDL to pre-β-HDL were increased in plasma from apoM transgenic mice and decreased in plasma from apoM-deficient mice although PLTP and HL activities towards synthetic substrates were not affected by apoM expression levels(85). Besides this, the LCAT-dependent interconversion of α to pre-α migrating HDL was delayed in plasma from apoM transgenic mice compared with controls. It is likely that apoM in HDL particles affects the ability of PLTP and HL to produce pre-β HDL from α-HDL while it reduces the ability of LCAT to esterify cholesterol possibly through sterical hindrance of the interaction between LCAT and apoA-I (85). Moreover, apoM expression into HEK293T cells expressing ABCA1 resulted in the formation of larger-sized nascent pre-β HDL particles. The formation of these larger-sized nascent pre-β HDL particles occurred at the expense of smaller pre-β1 HDL particles suggesting that apoM may facilitate the transfer of apoA-I, phospholipids and cholesterol during or after their

formation by ABCA1 or facilitate nascent HDL particle fusion as apoAI recycles through the endosomal compartment, resulting in appearance of larger pre-β HDL particles in the medium (113).

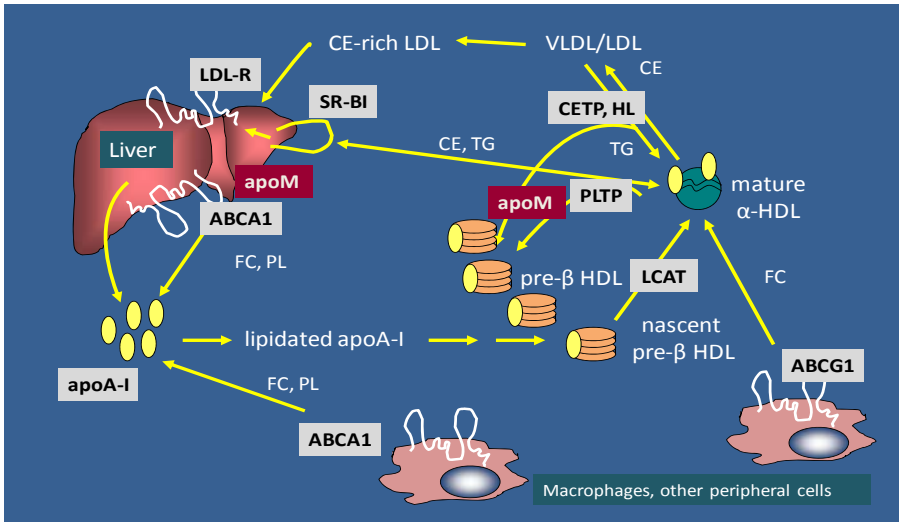

Figure 6. Apom function in RCT. See text for details.

b) apoM functions in the kidney

Kidney-derived apoM probably serves to bind through its ligand-binding pocket vital substances in the tubular space, such as lipophilic vitamins and other lipids, and through subsequent binding and reabsorption by the megalin receptor pathway prevent their loss in the urine (114). Megalin is a endocytosis-mediating membrane receptor that is highly expressed in the proximal tubule in the kidney (115,116) (117) and other absorptive epithelia e.g. brain lung, retina and inner ear (118,119). It binds a variety of substances, including albumin, basic drugs and apolipoproteins A-I, B, H and J (120) while it also binds apoM with high affinity. Mice lacking expression of megalin in the proximal tubule have low molecular weight proteinuria and secrete apoM in the urine, whereas apoM is normally undetectable in the urine. Similar to the liver, apoM is secreted with its intact signal peptide associated with particles that are larger than apoM

itself suggesting that apoM is secreted in complex with phospholipids to make it soluble in the urine (114).

apoM gene

The human apoM gene is located in the major histocompatibility complex class III region, on chromosome 6p21.31. This is a highly conserved chromosomal region rich in genes involved in innate immunity and inflammation, e.g. tumor necrosis factor (TNF) and lymphotoxins A and B, suggesting a potential involvement of apoM in inflammatory responses and a contribution to the anti-inflammatory functions of HDL (90,101).

The human apoM gene spans a genomic region of approximately 2.3kb and contains six exons and five introns. The mouse apoM gene is located on chromosome 17, it contains six exons and spans a 2.8kb genomic sequence. The homology between the human and the mouse apoM sequences is approximately 80% (121). Apart from mouse, this structure of the apoM gene is preserved among other mammalian species e.g. the orangutan (*Pongo pygmaeus*), the chimpanzee (*Pan troglodytes*), the cow (*Bos taurus*), the pig (*Sus scrofa*) and the dog (*Canine familiaris*) while it is also present in non-vertebrates, including zebra fish (Danio rerio), African clawed frog (Xenopus laevis) and puffer fish (Tetraodon nigroviridis). The widespread distribution of apoM in distantly related species implies an important biological role for the apoM protein (82,99,122).

Transcriptional regulation

ApoM is expressed mainly in hepatocytes and to a lesser extent (20% of that in liver) in the tubular epithelium of the kidney. Small amounts were also found in fetal liver and kidney while low-level expression (<5% of that in liver) was found in heart, brain, spleen, intestine and testis (82,86). The regulatory mechanisms that control human apoM gene transcription are not well understood. In the liver, apoM gene transcription has been shown to be regulated mainly by transcription factors that control hepatic lipid and glucose metabolism: Hepatocyte Nuclear Factor 1α (HNF-1α/TCF-1α) (106), Liver Receptor Homolog-1 (LRH-1) (123) and Forkhead box A2 (FOXA2) (106,123,124).

HNF-1α belongs to the helix loop/helix homeodomain transcription factor family, and binds to its target sequence 5'-ATTAACCATTAA-3' as a homodimer or heterodimer with its isoform vHNF-1/HNF-1β (125). It is primarily expressed in the liver, intestine, kidney and the exocrine pancreas where it plays important roles in development, cell differentiation and metabolism. HNF1α-/- mice exhibit hepatic, pancreatic and renal dysfunctions such as liver enlargement and extensive hypercholesterolemia and hyperphenylalaninemia, defective bile acid transport and increased bile acid and hepatic cholesterol synthesis, impaired HDL metabolism and glucose homeostasis and drastic reductions in the proximal reabsorption of several metabolites (126,127). In humans, mutations in the heterozygous state have been found to be responsible for a particular form of diabetes mellitus termed maturity-onset diabetes mellitus of the young type 3 (MODY 3) that is characterized by impaired insulin secretion due to defects in pancreatic β cells (128).

HNF-1α was shown to be a potent activator of apoM gene expression as HNF1α-/- mice do not express apoM in the liver and kidney and in turn, plasma apoM concentrations and pre-β HDL particles are virtually zero. Moreover, in these mice cholesterol is accumulated in large HDL particles (HDL1) and conversion of HDL to pre-β HDL is impaired while patients with MODY3 have reduced plasma apoM levels (97,106). HNF-1α regulates apoM gene expression through direct binding to a conserved DNA element located in the proximal apoM promoter region between nucleotides -55 to -41 in humans and -103 to -88 in mice (106).

LRH-1 is a member of the Ftz-F1 subfamily of the hormone nuclear receptor gene superfamily of transcription factors (129). It belongs to the orphan nuclear receptors, although its ligand-binding pocket can bind various phospholipids and it binds to its cognate target sequence 5'-(Py)CAAGG(Py)C(Pu)-3'as a monomer (130). It is highly expressed in liver, intestine, pancreas and ovary (130-132). In the liver, LRH-1 plays a key role in cholesterol homeostasis, through the control of the expression of genes that are implicated in bile acid biosynthesis and enterohepatic circulation (CYP7A1, CYP8B1, ABCG5/8) (131,133), reverse cholesterol transport (SR-BI, apoA-I) and HDL remodeling (CETP) (132,134-136) as well as in early development through the control of

the expression of genes that are involved in embryonic development (Oct4) and of the hepatic nuclear factors HNF-3β, HNF-4α and HNF-1α (137,138). Moreover, it is a negative regulator of the hepatic acute phase response antagonizing C/EBP transcriptional activity and inducing interleukin 1 receptor antagonist gene expression (139).

LRH-1 stimulates apoM gene transcription by binding to an LRH-1 response element located in the -29/-25 region of the proximal human apoM promoter (123). Small heterodimer partner (SHP), an atypical orphan nuclear receptor that lacks a DNA-binding domain, inhibits the transactivation function of LRH-1 by direct interaction with its AF-2 activation domain thus competing with the binding of coactivators. Overexpression of SHP or induction of its expression by bile acids (ligands for the nuclear receptor Farnesoid X Receptor, FXR), strongly increased SHP association to the apoM promoter, without modifying LRH-1 recruitment, resulting in reduced apoM gene expression levels (140). Similarly, apoM expression was reduced in the liver of mice fed with a diet supplemented with cholic acid or in the liver of SHP transgenic mice. Thus, bile acids suppress apoM expression *in vivo* by inhibiting LRH-1 transcriptional activity via recruitment of SHP to the apoM promoter.

Foxa2, also known as HNF-3β, is a forkhead transcription factor with a winged helix DNA binding domain that binds to its consensus DNA sequence 5'-T(G/A)TTT(A/G)(C/T)T-3' as a monomer (141,142). Due to its winged helix (or forkhead box) structure, Foxa2 is capable of chromatin remodeling and activation of transcription in the absence of chromatin remodeling complexes such as SWI/SNF, being thus a pioneer transcription factor that through opening of compacted chromatin it facilitates the recruitment of other transcription factors (143). It is mainly expressed in liver and pancreas and it is involved in pancreatic and liver development while in adulthood it regulates glucose homeostasis in the liver and pancreatic β cells (144,145). With its coactivator PGC-1β, it is a powerful activator of β-oxidation (146,147). Insulin inhibits Foxa2 through a mechanism that involves threonine phosphorylation mediated by PKB at amino acid position Thr-156 and nuclear exclusion (148).

Foxa2 has been identified as a transcriptional regulator of the apoM gene through its recruitment to a conserved binding site on the human apoM promoter located between nucleotides -398 and -388 (124). Haploinsufficient Foxa2+/- or hyperinsulinemic mice (with genetic mutations in leptin signaling, ob/ob or db/db, or high fat diet-induced obese mice that exhibit dyslipidemia, hyperglycemia, insulin resistance, and compensatory hyperinsulinemia) exhibited decreased apoM expression and plasma pre-β HDL levels due to inactivation of Foxa2 (124). Leptin is a multifunctional hormone encoded by the ob gene (149), synthesized mainly in adipocytes, and has been shown to have influences on hepatic lipid and lipoprotein metabolism through regulation of appetite and energy metabolism (150-152). Leptin signaling is mediated by the leptin receptor OB-R expressed in many organs including liver and kidney. Leptin treatment of ob/ob (leptin deficient) and db/db (leptin receptor deficient) mice that rapidly reduces plasma glucose and insulin levels, restored apoM expression through relocation of Foxa2 to the nucleus (124). In alloxan-induced diabetic mice, which have low leptin levels, plasma apoM concentrations as well as apoM expression in the liver and kidney was also reduced (110). On the other hand, *in vitro*, leptin treatment had no effect in primary hepatocytes (124) while it inhibited apoM gene expression in HepG2 cells (153). These contradictory effects of leptin on apoM expression *in vitro* and *in vivo* further indicate that the effects of leptin are not direct but are mediated by insulin and inhibition of Foxa2. A positive correlation between leptin levels and plasma apoM concentrations has also been observed in humans (154).

In agreement with the role of Foxa2 on apoM gene regulation and the mechanism of its inhibition by insulin, insulin, insulin-like growth factor I (IGF-I) and IGF-I potential peptide (IGF-IPP) significantly inhibited apoM expression in a dose- and time-dependent manner in HepG2 cells (110) and in primary human and murine hepatocytes (124). The inhibitory effect of insulin was mediated through the activation of phosphatidylinositol-3-kinase (PI3K) that in turn activates PKB resulting in inactivation of Foxa2 (110). Furthermore, glucose strongly inhibited apoM gene expression in HepG2 cells and the inhibitory effects of glucose and insulin were additive while in rats rendered hyperglycemic by short-term glucose infusion, resulting in enhanced insulin secretion,

serum apoM concentrations and hepatic apoM mRNA levels were significantly reduced (112).

Reduced apoM expression has been found upon infection and inflammation. Treatment of cells with lipopolysaccharide (LPS), Tumor Necrosis Factor α (TNF-α) or Interleukin 1 (IL-1) decrease apoM mRNA levels and the secretion of apoM while administration of LPS (model of gram negative infection), zymosan (model of fungal infection) or turpentine (model of sterile abcess) in mice, that mimic systemic inflammation and increase cytokine production resulted in a marked reduction in apoM expression levels in liver and kidney and decreased plasma apoM protein levels (155). Prolonged LPS administration has been shown to decrease HNF-1α gene expression in liver and kidney and that decrease may at least in part account for the decrease in apoM expression (156). Moreover, serum apoM levels are decreased in humans with acute bacterial infections or chronic HIV infection (155,157) suggesting that apoM may be considered as an acute phase response protein that decreases during infection and inflammation. The fact that the apoM gene is located in close proximity to genes related to immune response further support a role for apoM in infection and inflammation and its regulation by cytokines and inflammatory factors (100,101).

Platelet activating factor (PAF), however, a potent proinfammatory phospholipid, stimulates apoM expression and secretion in HepG2 cells (157). PAF is synthesized by various cells such as monocytes/macrophages, polymorphonuclear leukocytes, mast cells, endothelial cells and platelets and it initiates the rapid inflammatory response, through the induction of leukocyte-endothelium adhesion, the increase in endothelial and epithelial permeability and the activation of inflammatory cells to release cytokine mediators, which lead to local and/or systemic effects (158,159). The biological effects of PAF are mediated by its receptor PAF-R, a G-protein coupled receptor, and downstream induction of several signal transduction pathways (160) while it is hydrolyzed and thus inactivated by the enzyme PAF-acetylhydrolase (PAF-AH) (161). Human PAF-AH is associated with LDL and HDL containing apoE (162), suggesting that apoM might also be related to the HDL association and regulation by PAF-AH which would influence the inactivation of PAF thus providing an explanation for the opposing effect of the

proinflammatory PAF, relative to other poinflammatory mediators, on apoM expression levels. Lexipafant, a PAF-R antagonist (163) suppressed apoM mRNA levels and the secretion of apoM in a dose-dependent manner (157).

Other biological factors implicated in apoM gene regulation are the growth factors transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), epidermal growth factor (EGF) and hepatic growth factor (HGF) (164) as well as liver X receptor (LXR) agonists (165,166). TGF-α, TGF-β, EGF and HGF all downregulated apoM expression and secretion in HepG2 cells although to a different extent with TGF-β exhibiting the strongest effect (65% reduction), followed by HGF (50% reduction), EGF (30% reduction) and TGF-α (20 % reduction). TGF-β is the prototype member of a large, evolutionary conserved superfamily of pleiotropic cytokines that also includes activins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (167). It controls various physiological processes during embryogenesis and is an important homeostatic regulator in various cell types, such as epithelial and endothelial cells in adult organisms (167,168). TGF-β acts as a growth suppressor because of its cytostatic program (169) while during the late stages of cancer and metastasis, TGF-β acts as a tumor promoter due to its ability to enhance processes such as epithelial to mesenchymal transition (EMT), cell motility and invasion, immunosuppresion, angiogenesis and extracellular matrix production (169-172). TGF-β has also been implicated in lipid and lipoprotein metabolism, stimulating apoC-III promoter activity in hepatic cells (173,174), ABCA-1 expression and apoE secretion in macrophages and decreasing LCAT activity and mRNA levels or apoB secretion in hepatic cells (175).

The ligand-dependent nuclear receptor LXR is a key regulator of cholesterol and bile acid metabolism in hepatocytes (discussed later) that forms obligate heterodimers with RXR. LXR/RXR heterodimers can be activated by the endogenous oxysterols (oxidized cholesterol), that comprise a wide range of hydroxylated forms of cholesterol and intermediates of the cholesterol biosynthetic pathway, or by synthetic high affinity agonists such as T0901317 and GW39865. Treatment of HepG2 cells with the synthetic agonist T0901317 or administration of T0901317 in mice decreased heapatic apoM expression levels (165,166). Co-treatment of HepG2 cells with both T0901317 and the

ligand for RXR, 9-cis retinoic acid, further decreased apoM mRNA levels in an additive manner (165). However, T0901317 increased apoM expression levels in mouse intestinal cells (ileum and duodenum) or cultured human intestinal Caco-2 cells (166). This differential effect of T0901317 on apoM expression levels in liver and intestine probably reflects differences in the other factors expressed endogenously in these two cell types.

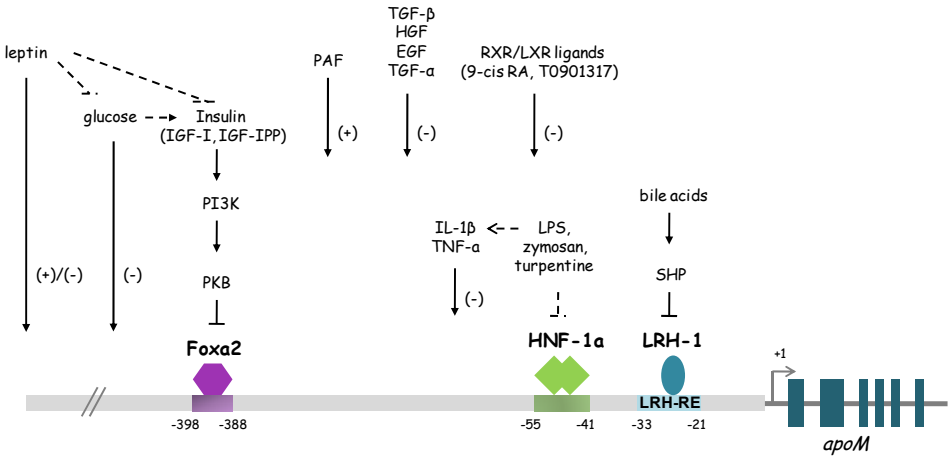

Figure 7. Summary of the current understanding of the transcriptional regulation of the human apoM gene.

Apolipoprotein E (apoE)

ApoE is a 37 kD ubiquitous protein synthesized by the liver and several other tissues and cell types, including kidney, adipose tissue, monocytes/macrophages, astrocytes and microglia (176-178). The majority of apoE is secreted by the liver as a lipoprotein-free protein or as part of the VLDL particles at times of increased lipoprotein synthesis and is readily redistributed among chylomicrons, chylomicron remnants, VLDL, IDL and HDL in human plasma (179,180), ApoE is a single glycosylated polypeptide of 299 amino acids that similar to other apolipoproteins, are organized in amphipathic α-helices that contribute to its ability to bind to lipids and form lipoproteins. (181,182).

Human apoE is a polymorphic protein. There are 3 isofoms of apoE, E2, E3 and E4, differing by a single unit of net charge. The three isoforms are expressed from multiple alleles at a single apoE genetic locus, giving rise to the three homozygous and three heterozygous apoE phenotypes (183,184). E3 (C112, R158) is considered the wild type allele product because it is expressed in highest frequency in all populations studied (184). E2, resulting from the substitution of cysteine by arginine at position -158 (C112, C158) is the rarest isoform and is associated with type III hyperlipoproteinemia (type III HLP), characterized by elevated plasma cholesterol and triglyceride levels, cholesterol-enriched VLDL and IDL particles, increased plasma apoE levels and premature coronary and periphereal atherosclerosis. (4,185). E4 (R112, R158), resulting from the substitution of arginine by cysteine at position-112 is the isoform with intermediate population frequency and is considered a risk factor for the development of Alzheimer's disease (181,186).

apoE functions

Studies in humans and mice have established a crucial role for apoE in the control of lipid metabolism in the liver and brain, affecting the development of atherosclerosis and Alzheimer's disease. In the liver, apoE has a functional role both in the clearance of the lipoprotein remnants from the circulation as well as in HDL metabolism promoting atheroprotection.

apoE functions in the liver

a) Role of apoE in triglyceride-rich lipoprotein metabolism

Lipoprotein-bound apoE is the ligand for the LDL receptor, as well as for other receptors, such as the LDLR-related protein 1 (LRP-1) (187-190) being essential for the clearance of the remnants of lipoprotein metabolism which are rich in cholesteryl esters. Mutations in apoE (mainly in the region between residues 136 and 152) that prevent binding of apoE-containing lipoproteins to the LDL receptor or apoE deficiency in

humans and mice is associated with increased plasma cholesterol levels, accumulation of remnants in the VLDL and IDL region and premature atherosclerosis (5,191-194). Overexpression, however, of full-length apoE in apoE deficient mice did not correct the high cholesterol levels and induced hypertriglyceridemia whereas overexpression of truncated apoE forms that lack the carboxy-terminal region corrected hyperlipidemia (195-198) due to increased affinity for the LDL receptor. Thus, the amino-terminal 1-185 domain of apoE is sufficient to direct receptor mediated lipoprotein clearance while the carboxy-terminal 261-299 domain of apoE induces hypertriglyceridemia resulting mainly from inefficient VLDL clearance and diminished lipoplysis by lipoprotein lipase. Both lipoprotein lipase and hepatic lipase have been shown to interact with apoE in the periphery (199-204) and the ability of apoE to modulate triglyceride hydrolysis may additionally contribute to the apoE-mediated uptake of remnant lipoproteins by the liver.

Despite its crucial role in lipoprotein remant processing and clearance, apoE is not necessary for their assembly and secretion (205,206) but it can affect the triglyceride content in newly formed hepatic VLDL, modifying the structure of the lipoprotein (191,194,207-209). In the absence of apoE, secreted VLDL particles are relatively deficient in triglycerides, while patients with type III HLP or transgenic mice expressing mutant apoE forms have higher triglyceride levels (191,194,207-209).

b) Role of apoE in HDL metabolism

In addition to its function in the clearance of triglyceride-rich lipoproteins, apoE is a critical component of the RCT pathway. ApoE promotes cholesterol efflux form macrophages and plays a role in HDL formation, maturation and hepatic uptake. ApoE participates in the biogenesis of apoE-containing HDL, in a process similar to that of apoA-I. Infection of apoA-I-/- mice with apoE4-expressing adenoviruses increased HDL and generated discoidal HDL particles that were converted to spherical upon co-infection of apoA-I-/- mice with an adenovirus expressing human LCAT. ABCA1-/- mice treated similarly failed to form HDL particles, suggesting that the biogenesis of apoE-containing HDL particles requires the functions of both ABCA1 and LCAT (210). Interestingly, adenovirus-mediated gene transfer in apoA-I-/- mice of apoE variants bearing

substitutions of either five residues (leu261, Trp264,Phe265, Leu268, val269) or three residues (Leu261, Trp264, Phe265) by Ala in either the apoE4 or apoE2 background were able to promote the formation of spherical apoE-containing HDL without inducing hypertriglyceridemia, as was documented for overexpression of wild type apoE in apoE-deficient mice, making them attractive therapeutic targets for the prevention of atherosclerosis (211).

Besides the formation of apoE-containing HDL, apoE affects the metabolism of apoA-I-containing HDL. HDL in apoE-/- mice is characterized by markedly abnormal lipid composition and apoprotein content with HDL particles carrying 45% of normal cholesterol, having reduced apoA-I and increased apoA-IV content (212) and a reduced capacity to accept cholesterol (213). In addition, the HDL cholesterol in apoE-/- mice is enriched in sphingomyelin, due to combined defects in its synthesis and clearance (208,214,215).

Studies in apoE deficient mice have indicated an important role for apoE in HDL maturation and HDL anti-inflammatory functions. ApoE-/- mice on a chow diet have impaired maturation of HDL due to, at least in part, a reduced (28%) LCAT activity in relation to wt mice and LDLR-/- mice(215). In response to a Western diet, the apoE-/- mice showed a further reduction in LCAT activity and reduced PAF-AH, while PON1 activity was reduced by 38%. Altered activities of these antiatherogenic enzymes are likely to contribute to the formation of dysfunctional HDL and accelerated atherogenesis in apoE-/- mice mice (215).

The key mechanism responsible for the antiatherogenic effect of apoE, however, seems to be the cholesterol efflux from mcrophage foam cells by macrophage-derived apoE. ApoE deficiency in macrophages promotes foam cell formation despite the presence of functional ABCA1 and abundant circulating apoA-I (216) and promotes atherogenesis in the absence of significant changes in plasma lipoproteins, in several murine models fed on atherogenic diets (217,218), LDLR-/- mice (219) and apoA-I-/- mice (220). The strong local effect of apoE in reducing cholesterol in arterial macrophages is evidenced by the fact that expression of apoE at levels too low to affect plasma lipoprotein levels significantly delays atherogenesis (221,222).

apoE functions in the brain

apoE is the only apolipoprotein present in the brain playing an essential role in lipid homeostasis in that organ. Similar to other tissues the interactions of apoE with ABCAI in the brain promote cholesterol efflux and the uptake of cholesteryl esters subsequent to the interaction of the apoE-containing HDL with the SR-BI receptor. Through its interaction with the LDL receptor, apoE coordinates the mobilization and redistribution of cholesterol and phospholipids with repair, growth and maintenance of myelin and neuronal membranes (223-225). Lipid homeostasis in the brain is central for the brain response to injury and neurodegeneration, amyloid precursor protein (APP) processing as well as the cholinergic system function, the most important neurotransmitter system in the brain that relies on lipid bioavailability to locally synthesize acetylcholine.

Following injury, macrophages synthesize and release apoE within the peripheral lesion in order to scavenge cholesterol from both cellular and myelin debris (223,226). Through its interaction with the LDL receptor, apoE regulates the transport of cholesterol and phospholipids during the early and middle phases of reinnervation as neurons undergo dendritic remodeling and synaptogenesis (224,226). The ability of neurons to undergo functional synaptic remodeling was markedly compomised in apoE deficient mice while reintroduction of human apoE gene in these mice drastically reduced the synaptic loss and improved cognitive performance in several behavioral tests (227).

APP processing and amyloid production is markedly affected by intracellular lipid homeostasis while apoE-containing HDL through receptor-mediated processes is involved in the clearance of extracellular beta amyloid, thus preventing amyloid deposition and plaque formation. Introduction of the apoE gene in amyloid precursor protein (APP)-overexpressing mice drastically reduced beta amyoid deposition in the brain of hybrid mice (228). In contrast, lipid-free apoE that can not bind to apoE-recognizing receptors may promote amyloid polymerization and plaque formation (229-231).

Cholinergic dysfunction, poor compensatory synaptogenesis and amyloid deposition are among the most common neuropathologic landmarks of Alzheimer's

disease (AD). Apolipoprotein E4 allele is strongly linked to both sporadic and familial late onset Alzheimer's disease (AD). In a typical control population, approximately 20% of the individuals carry at least one apoE4 allele. That percentage would rise to 65% in non-related patients with sporadic AD and to 80% in those with familial AD (232). As already discussed, apoE4 and apoE3 differ by a single amino acid at position 112 (apoE4 contains an arginine and apoE3 a cysteine). This Arg-112 results in compact protein structure due to domain interaction of Arg-61 with Glu-225 and instability to protein unfolding of the apoE4 molecule that leads to the formation of an ensemble of loosely folded structures referred to as a molten globule state (233). Although the exact mechanism of apoE4 association with AD is not yet defined, introduction of the interaction domain in mouse models resulted in synaptic, functional and cognitive deficits, independent of amyloid production (234-236) probably due to the elicitation of an edoplasmic stress (ER) response and an unfolded protein response (UPR) in astrocytes that renders cells dysfunctional and inefficient to support neuronal maintenance and repair. Under these conditions, stress neurons turn on their own apoE expression for repair (237) that in the case of E4 results in the generation of C-terminal-truncated fragments that are neurotoxic (238) leading finally to extensive degeneration and AD.

Transcriptional regulation

ApoE expression is regulated by hormonal and nutritional factors and is developmentally regulated (239). High levels of apoE expression are observed during organ development while its expression levels in astrocytes have been shown to increase during nerve regeneration or after treatment with 17β-estradiol (177,239,240).

The human apoE gene contains four exons and is located in the long arm of chromosome 19 in a cluster with the genes encoding apoCI, apoCIV, and apoCII. Studies in vitro and in transgenic mice carrying different apoE gene constructs established that the expression of the genes of the apoE/apoCI/apoCIV/apoCII cluster are controlled by two homologous hepatic control regions, designated HCR-1 and HCR-2, of approximately 600bp that are located 15 and 27kb 3' of the apoE gene, respectively (241) Either region is sufficient to direct gene expression *in vivo*, although HCR-1 appears to

have a dominant effect on apoE. (242-245). Two other homologous regulatory region, designated multienhancer-1 and multienhancer-2, that are located 3.3 and 15.9 kb downstream of the apoE gene can direct independently the expression of the apoE gene in macrophages and adipocytes (246). In kidney, in contrast to the liver, the proximal promoter region between nucleotides -336 and -44 is sufficient to direct apoE gene expression (242,247). Important factors in this proximal promoter region for apoE gene regulation appear to be SP1 bound on element III. SP1 bound on the proximal promoter is also essential for the transcription of the apoE gene in hepatic cells possibly working in synergy with factors bound to the HCR-1, including HNF-3, C/EBP and hormone nuclear receptors, such as HNF-4 (248,249).

ATP binding cassette transporter A1 (ABCA1)

ABCA1 is a member of the ATP binding cassette (ABC) family of transporters (250). Like all members of the A family, the ABCA1 protein is a full size ABC transporter containing two transmembrane domains, each composed of six α helices and two intracellular nucleotide-binding domains. It is a ubiquitous protein of 2261 amino acids encoded by the ABCA1 gene located on chromosome 9 in humans. ABCA1 gene contains 50 exons and spans a genomic region of more than 150kb. (251,252).

ABCA1 function

ABCA1 plays a critical role in the formation of nascent HDL by promoting efflux of cellular phospholipids and unesterified cholesterol to lipid-free or lipid-poor apoA-I particles, forming pre-β HDL, thereby exerting important antiatherogenic properties. (253-255). Loss of ABCA1 function leads to dyslipidemia known as Tangier disease, an autosomal recessive disorder characterized by very low plasma cholesterol levels, undetectable discoidal or spherical HDL particles and abnormal lipid deposition in various tissues (34,256-258). In agreement with this, fibroblasts of patients with Tangier

disease or from ABCA1-/- mice had severely reduced cellular cholesterol efflux (2,256,259). Moreover, selective inactivation of the ABCA1 gene in macrophages enhanced atherosclerosis independently of changes in plasma lipids and HDL levels (256) while overexpression of ABCA1 in liver and macrophages of transgenic mice increased plasma HDL levels and conferred atheroprotection upon feeding of mice a high-cholesterol diet (260).

Transcriptional regulation

ABCA1 is expressed abundantly in the liver, macrophages, brain, intestine, kidney, adrenal gland and heart and to a lesser extent in various other tissues (250,261). Its expression is mainly regulated at the level of transcription and is facilitated by the utilization of alternative promoters and transcription initiation sites present upstream of the first exon or inside the first intron of the gene (262-264). In accordance with its function in the efflux of cholesterol and phospholipids, ABCA1 gene transcription is tightly regulated by the intracellular cholesterol content through the nuclear receptor LXRα. LXRα, together with its heterodimeric partner RXRα, bind to a well characterized LXR response element (LXRE) of the DR4 type located at position -65 of the promoter upstream of exon1 (upstream promoter) (265). This LXRE can additionally bind RXR/RAR and RXR/TRβ heterodimers that activate or repress, respectively, ABCA1 gene transcription (266,267). The oxysterol/retinoid-induced transcription of the ABCA1 gene via RXR/LXR heterodimers has been shown to be modulated by the ubiquitous transcription factor SP1 that binds to two GC- boxes located on the proximal ABCA1 promoter (-90/-85 and -156/-151). The close proximity of SP1 and LXR responsive elements facilitates physical interactions between the two proteins resulting in synergistic activation of ABCA1 gene transcription (268). Besides RXR/LXR heterodimers and SP1, Hypoxia-Induced Factor 1 (HIF1) further stimulates ABCA1 gene expression through its recruitment to an E-box located at position -140/-135 of the upstream ABCA1 promoter. This E-box element also serves as a binding site for the Upstream Stimulatory Factors (USFs) 1 and 2 as well as for the sterol regulatory element binding protein 2 (SREBP-2) upon cholesterol depletion, which repress ABCA1 gene expression (269-

271). The zinc finger protein ZNF202 constitutes an additional repressor of ABCAI gene expression through its recruitment to a GT box located in the -234/-215 region of the upstream ABCA1 promoter (272,273). Finally, LPS and proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ downregulate ABCA1 mRNA and protein levels in macrophages and murine peritoneal macrophage-derived foam cells (274,275) whereas the anti-inflammatory cytokinTGF-β significantly increases ABCA1 expression and cholesterol efflux (276) supporting the concept that inflammatory mediators modulate reverse cholesterol transport and macrophage foam cell formation at least in part by directly acting on the expression levels of ABCA1.

Nuclear hormone receptors

Nuclear receptors (NRs) comprise a superfamily of conserved transcription factors that are activated by small lipophilic ligands such as steroids (estrogen, progesterone, glucocorticoids), retinoids (9-*cis* and *all-trans*-retinoic acid), thryroid hormone, peroxisome proliferators, and vitamin D as well as numerous orphan receptors with yet unidentified ligand. Nuclear receptors play essential roles in diverse biological processes including cell differentiation, development, homeostasis and reproduction (277,278) . Dysfunction of nuclear receptor signaling leads to proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity and diabetes.

The structure of nuclear receptors is highly conserved throughout evolution and consists of a N-terminal ligand-independent transactivation domain (A/B domain) called Activation Function 1 (AF-1), a conserved DNA-binding domain (DBD or C domain) composed of two zinc finger motifs , and a multifunctional ligand-binding domain (LBD or E domain) which harbors ligand-dependent activation function 2 (AF-2) and is connected to the DBD by a hinge region (D domain) (277,278) (Fig.8).

Nuclear receptors that are activated by steroids form homodimers and bind to Hormone Response Elements (HREs) on the promoters of their target genes in a ligand-dependent manner while nuclear receptors that are regulated by other ligands form

heterodimers with Retinoid X Receptor (RXR) and bind to HREs in the presence or absence of ligands, activating or repressing transcription, respectively. Upon ligand binding heterodimeric nuclear receptors undergo conformational changes that lead to the dissociation of correpressor complexes and the subsequent recruitment of coactivators resulting in a switch from repression to gene activation. (279).

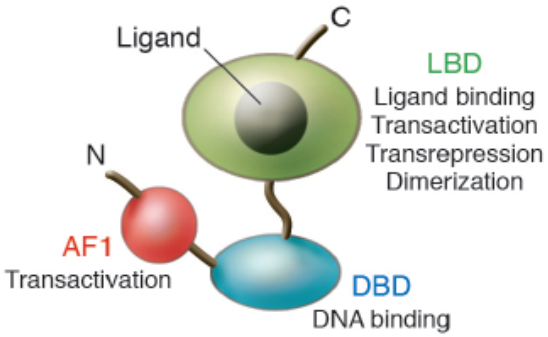

Figure 8. Domain structure of nuclear receptors. From (280)

HREs variously contain direct repeats (DRs), inverted repeats (IRs) or palindromic repeats (PRs) of the sequence 5'-AG(G/T)TCA-3'. These repeats are separated by one, two, three, four, or five nucleotides and are designated DR1, DR2, etc. (for direct repeats), IR1, IR2, etc. (for the inverted repeats), and PR1, PR2, etc. (for the palindromic repeats) (281). The exact sequence of the repeats and spacing as well as 5' extension of the HREs define the specificity of different nuclear receptor heterodimers. Besides their recruitment to HREs and direct gene regulation, nuclear receptors have also been reported to regulate the expression of genes that lack canonical HREs via physical and functional interactions with other promoter bound transcription factors such as Sp1, acting as superactivators, or AP1, acting as transrepressors (280,282).

Hepatocyte nuclear factor 4α (HNF-4α)

HNF-4α is liver-enriched transcription factor that plays a significant role in embryonal liver development and hepatocyte differentiation as well as in many liver-specific functions including lipid, glucose, drug and ammonia metabolism, bile acid synthesis and serum protein production (283, 284). High HNF-4α expression levels are also observed in intestine, kidney and pancreatic beta- islet cells (283,284). Disruption of its expression leads to embryonic lethality due to a visceral endoderm defect that prevents gastrulation (285,286) while conditional liver-specific inactivation of the HNF-4α gene in mice caused weight loss, increased mortality and lipid abnormalities (accumulation of lipid in the liver, greatly reduced plasma total and HDL cholesterol levels, and increased serum bile acids) due to impaired expression of genes involved in lipid metabolism, including apolipoproteins, cholesterol synthesis enzymes and bile acid transporters (287). Known target genes of HNF-4α include apoB, MTP, apoA-II, apoA-IV, apoC-II, apoC-III, PXR, HNF-1α, and HNF-4α itself. Furthermore, heterozygous mutations in the HNF-4α gene are associated with an early onset form of type II diabetes called maturity onset diabetes of the young 1(MODY1) characterized by impaired triglyceride metabolism and insulin secretion (288,289) whereas recent genome wide association studies (GWAs) identified a common variant in the coding region of HNF-4α gene (rs1899861) associated with low HDL concentrations in humans (290)

HNF-4α belongs to the orphan nuclear receptors and can activate gene transcription in the absence of exogenous ligands. However, recent crystallographic studies established that the ligand-binding domain of HNF-4α is occupied by fatty aids that lock the receptor in an active conformation (281, 291, 292). HNF-4α binds to DNA as a homodimer, preferentially to DR1 (291) and activates target gene transcription via the recruitment through its AF-1 and AF-2 domains of members of the p160 family of coactivators such as steroid receptor coactivator 1 (Src-1), glucocorticoid receptor interacting protein -1/transcriptional intermediary factor 2 (GRIP1/TIF2) as well as histone acetyltransferase CREB binding protein (CBP) and peroxisome proliferator activated receptor γ coactivator 1 (PGC-1).

The activity of HNF4α has been shown to be regulated in a number of ways including posttranscriptional mosdifications, such as phosphorylation by PKA (292),

MAPK and JAKs (293,294) or acetylation by CBP (295) that affect mainly its DNA binding activity and its interaction with coactivators, as well as through protein-protein interactions with other transcription factors such as Smads, NF-kB or SHP (296). NF-kB inhibits while Smads increase the transcriptional activity of HNF4α. The initial activation of the HNF4α gene depends on the synergistic action of HNF-1β with GATA-6. At later stages of hepatocyte differentiation when HNF-1α levels reach a critical threshold and HNF-1β expression gradually decreases, the HNF4α promoter activity depends on the synergism between HNF-1α and HNF-6. COUP-TFII transcription factors negatively regulate HNF4α gene expression and their repressive effect can be alleviated by retinoids bound on RXR/RAR via competition for the same binding site (297,298).

Retinoid receptors (RXRα, RARα)

Retinoids, vitamin A and its active derivatives, are non-steroidal hormones which play a critical role in the development and homeostasis of nearly every tissue through their regulatory effects on cell differentiation, proliferation and apoptosis (299) (300). The retinoid signal is transduced by two families of nuclear receptors, the retinoid X receptors (RXRs) and the retinoic acid receptors (RARs). RARs are activated by all-trans retinoic acid (RA) and its 9-cis isomer, while RXRs are only activated by 9-cis RA. Each family consists of three isotypes (α,β and γ) encoded by separate genes while for each isotype, there are at least two isofoms generated by differential promoter usage and alternative splicing that differ only in their N-terminal regions (278,301). The expression of RARα and RXRβ is ubiquitous and abundant (302,303), the levels of RARβ and γ are high in brain and skin, respectively (303) whereas the level of RXRα is especially high in the adult liver (302). RARs regulate the expression of target genes through the formation of obligate heterodimers with RXR and subsequent binding to RA response elements (RAREs) of DR1, DR2 or DR5 type whereas RXRs can also form homodimers binding preferentially to DR1 (304).

In agreement with the major role of retinoids in the regulation of many biological programs, gene-ablation experiments of RXRα/RARα generate embryonic development

defects, abrogating the diffrentiative and antiproliferative effects of RA, that result in midgestation embryonic lethality (305,306). Liver-specific disruption of the RXRα gene in mice caused lipid abnormalities including increased plasma triglyceride and cholesterol levels probably due to increased expression of apoA-I and apoC-III (their expression is increased by RXRα/RXRα and RXRα/RARα and decreased by RXRα/PPARα) and increased hepatic cholesterol levels due to at least in part increased CYP7A gene expression (caused by diminished inhibition of its expression by the RXR/FXR heterodimers). In mice fed a high cholesterol diet, hepatic cholesterol levels were even further increased due to compromised processing of cholesterol and inability of bile acid formation owning to the absence of the RXR/LXR activity (306,307).

Retinoids, however, have also been shown to regulate the expression levels of retinoid nuclear receptors themselves affecting both the transcription of their genes as well as their degradation by the proteasome, providing a mechanism to control the magnitude and the duration of retinoid-mediated transcription (310-312). In hepatic cells, RA differentially regulates retinoid receptor gene expression, inducing RARα and RARβ and downregulating RXRα. The inhibition of the RXRα gene expression by RA was shown to occur within 8h after addition of RA in Hep3B cells and was dose dependent (308). Furthermore, the activity of retinoid receptors is modulated by phosphorylation and interaction with other transcription factors (309). Phosphorylation by PKA (310), the cdk7 subunit of TFIIH (311) or upon activation of PKC (Delmotte, 1000) positively regulates their transcriptional activity while MAPK-mediated phosphorylation impairs the transcriptional activity of RXRα/RARα (312). Moreover, both RXRα and RARα strongly interact with the DBD of SP1through their DBD and LBD resulting in synergistic activation of target genes (313,314) and cooperate with STAT5 in the presence of cytokines (315). In contrast, retinoids antagonize the activation of a subset of AP-1 (316), or Smads-regulated genes in response to TGF-β (317) and inhibit the PI3K/Akt pathway (309).

Peroxisome proliferator activated receptor α (PPARα)

PPARα is a lipid-activated nuclear receptor that belongs to the nuclear receptor subfamily of PPARs consisting of three isoforms encoded by separate genes, α, γ and δ/β, The DNA-binding domain is approximately 85% homologous within the PPAR family, whereas the ligand-binding domain possess a 70% similarity among the family members. The tissue expression of PPAR family members is diverse. PPARγ is mainly expressed in the intestine and in adipose tissue (318-320) while PPARα is mainly expressed in tissues having a high metabolic rate such as liver, muscle, kidney and heart (320,321). In addition, it is expressed in steroidogenic tissues such as adrenals (322), in vascular cells including endothelial, smooth muscle cells, and macrophages/foam cells (323-327). PPARα is involved in the regulation of lipoproteins and fatty acids metabolism and has profound effects on vascular inflammation and the related atherosclerosis.

Activation of PPARα increases the catabolism of free fatty acids and leads to decreased serum triglyceride levels mainly through stimulation of free fatty acid liver uptake and subsequent activation of the β-oxidation pathway by several mechanisms, including enhancement of HDL-mediated reverse cholesterol transport through transcriptional upregulation of apolipoproteins A-I and A-II (328,329), stimulation of lipoprotein lipase (330) and repression of apoC-III expression (331), stimulation of liver fatty acid uptake through induction of fatty acid transport protein expression (332), stimulation of their transport into the mitochondria by increasing carnitine palmitoyltrasferase I gene expression (333-335) and finally stimulation of their catabolism by increasing the transcription of rate-limiting enzymes involved in β-oxidation (336). Mice deficient in PPARα exhibit metabolic changes including high plasma cholesterol levels and low β-oxidation (337-339).

Besides its important role in the metabolism of fatty acids, PPARα through its expression in vascular cells is implicated in atherogenic and inflammatory processes exerting atheroprotection. PPARα via negative regulation of NF-kB signaling pathway inhibits the expression of inflammatory genes such as IL-6 and COX-2 (340) and the

expression of monocyte-recruiting proteins such as VCAM-I (341) while it induces apoptosis in monocyte-derived macrophages (342). In accordance with this, PPARα-deficient mice show a prolonged response to inflammatory stimuli (343). These mice are also more sensitive to damage upon exposure to chemicals that induce oxidative stress. PPARα is believed to protect liver from oxidant-induced damage through inhibition of the NF-kB signaling pathway and upregulation of stress-modifier genes that maintain the health of the proteome including those that prevent protein aggregation, heat-stress inducible chaperones, and eliminate damaged proteins and, proteasome components (344).

PPARα is activated by peroxisome proliferators such as various fatty acids and their derivatives and synthetic PPARα ligands such as fibrates (345-348). Other pharamacological compounds such as nonsteroidal anti-inflammatory drugds (NSAIDs) have also been identified as PPARα ligands (349). It binds to specific PPAR consensus sequences (PPRE) consisting of DR1 in the promoters of target genes as an obligate heterodimer with RXRα (350-353). Binding of 9-cis retinoic acid to RXRα can activate PPARα/RXRα heterodimers as well (351). Coactivators that have been shown to interact with the PPARα/RXRα heterodimers include CBP, SRC-1 and PGC-1 (354,355) while besides NF-kB, STAT1 and AP-1 pathways are negatively affected by PPARα (356)

Liver X Receptors (LXR)

LXRs are nuclear receptors that play central roles in the transcriptional control of lipid metabolism. Two isoforms have been indentified, LXRα and LXRβ, that share 77% amino acid sequence identity in both DNA and ligand-binding domains. LXRα exists in three variants originating from alternative promoter usage and splicing, LXRα1, LXRα2 and LXRα3 (357). LXRα1 is the major variant in most tissues and although all three variants bind DNA, LXRα2 is less active in stimulating transcription than LXRα1 whereas LXRα3 is unable to bind ligands and fails to stimulate transcription. LXRα is highly expressed in the liver and at lower levels in the adrenal glands, intestine, adipose, macrophages, lung and kidney, whereas LXRβ is ubiquitously expressed (358). Both

isoforms are activated by intermediates or end products of sterol metabolic pathways, such as 22(R)-hydroxycholesterol, 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol and 27-hydroxycholesterol (359) as well as by synthetic high affinity agonists such as TO901317 and GW3965. LXRs bind to characteristic DNA sequences termed LXR response elements (LXRE) of DR-4 type as obligate heterodimers with RXR (360-362).

Due to their ability to bind to and be activated by endogenous oxysterols, LXRs function as whole body cholesterol sensors that regulate the expression of genes controlling cholesterol absorption, excretion, catabolism and cellular efflux as well as synthesis and esterification of fatty acids, thus playing a key role in the maintenance of cellular cholesterol and lipid homeostasis. ABCA1 (365-367) and SREBP1 (363,364), that have central roles in reverse cholesterol transport and fatty acid metabolism, respectively, are two of the main genes regulated by LXR. Other target genes of LXR include CYP7A1 (365), ABCG1 (366-368), ABCG5/8 (369,370), apoE (371), CETP, LPL, PLTP (372) and FAS (373). In addition to inducing genes involved in reverse cholesterol transport, LXRs reciprocally repress a set of inflammatory genes such as COX-2, IL-6, IL-1β, and MCP-1 through inhibition of the NF-kB pathway (374,375) thus attenuating inflammation in macrophages and atherogenesis. Treatment of atherosclerosis-prone apoE-/- and LDLR-/- mice with a synthetic LXR ligand induced the expression of ABCA1 and ABCG1 and repressed that of inflammatory genes leading to an approximately 50% decrease in lesion size (376) . In contrast, macrophage specific loss of LXRs achieved by transplantation of bone marrow from LXR-/- mice into either apoE-/- or LDLR-/- mice resulted in a marked increase in lesion size (377).

Glucose metabolism is also regulated by LXRs. Two different synthetic agonists of LXRs have been reported to improve glucose tolerance in diabetic mouse (378) and rat models (379). This effect was associated with repression of the hepatic gluconeogenic genes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase as well as with the induction of glucokinase and of the insulin-dependent glucose transporter 4 (GLUT4) in white adipose tissue (378,380).

An additional level of LXR regulation, beyond ligand availability is the levels of LXRα receptor expression. In macrophages, PPARα and PPARγ have been shown to

induce the expression of LXRα through a PPRE present in the 5'-flanking region of the LXRα gene (381). Four-month administration of fenofibrate resulted in the upregulation of LXRα in the peripheral blood monocytes of patients with type 2 diabetes (382) while high-fat diet or fasting, both associated with elevation of the plasma fatty acid level, up-regulated hepatic LXRα in the rat (381). Insulin has also been shown to increase LXRα expression in the liver both in vitro and in vivo suggesting that the stimulatory effect of insulin on hepatic lipogenesis may be partially mediated via LXRs (383) while a single dose of estradiol decreased LXRα mRNA levels in mice liver and white adipose tissue (384). Moreover, LPS and proinflammatory cytokines, such as TNF-α and L-1β reduce the expression of LXRα in the liver, kidney and adipose tissue (385) (386) (387) (388,389). In contrast, the anti-inflammatory cytokine IL-10 stimulates LXRα in cultured macrophages (390). Furthermore, LXRα expression is controlled by an auto-regulatory mechanism that may be responsible for the strong upregulation of LXRα induced by lipid loading of macrophages. Three functional LXREs are located in the human LXRα gene promoter that can bind both LXRα and LXRβ (391,392). However, this autoregulation does not seem to apply in the case of the LXRβ gene.

Thyroid hormone receptor β (TRβ)

Thyroid hormone receptor family comprises two members, TRα and TRβ encoded by separate genes on human chromosomes 17 and 3, respectively. TRs are activated by 3, 3', 5-triiodo-L-thyronine (T_3) and bind to thyroid response elements, termed TREs as homodimers or heterodimers with RXR. Heterodimerization with RXR has been shown to be enhanced by phosphorylation of TRs (393,394). TREs are usually DR4 but RXR/TR heterodimers have also been demonstrated to bind to DR1 and DR2 or even IR0 and PR albeit with different affinities (278,395-398). p300/CBP, p300/CBP associated factor (P/CAF) and Src-1 are among the coactivators that have been shown to interact and enhance TR transcriptional activity

TRs are ubiquitously expressed and control diverse biological functions including growth, development and homeostasis (278). Disruption of TR gene in mice leads to loss

of T_3 responsiveness (399). Studies in vitro have also indicated a role for TRs in lipid metabolism. Acute administration of T_3 increases the levels of apoC-III gene transcription while chronic administration of T_3 decreases significantly apoC-III mRNA levels. In agreement with this, RXRα/TRβ1 heterodimers have been shown to activate apoC-III gene transcription in the presence of 9-cis RA but repress its expression in the presence of T_3 (400). Similarly, RXRα/TRβ1 heterodimers repress apoA-I gene transcription in the presence of T_3 (401). Such repression in the presence of T_3 may be the result of unfavorable conformation of TRβ1 partner brought about by its binding to a non-preferred DR-1 or DR-2 and suggest that RXRα/TRβ1heterodimers can be either activators in the presence of 9-cis RA or confer slight repression in the presence of T_3. In contrast, RXRα/TRβ1 heterodimers have been shown to positively regulate apoC-II and apoA-II gene transcription. Interestingly, the promoter of apoA-II contains two HREs, one in the proximal region, AIIAB, and one in the distal, AIIJ, that although they both bind RXRα/TRβ1heterodimers with high affinity and specificity they respond selectively to thyroids and retinoids respectively. This differential response of the receptors to their ligands may be due to the functional interaction between RXRα/TRβ1 and the ubiquitous transcription factor USF bound to overlapping sites on regulatory element AIIAB that probably stabilizes the ligand binding domain or TRβ1 in the activated state (402).

Activator Protein-1 (AP-1)

Activator protein-1 (AP-1) family proteins are basic region-leucine zipper (bZIP) transcription factors that are implicated in the regulation of a variety of cellular processes including proliferation and survival, growth, differentiation, apoptosis, cell migration, and transformation. They consist of homo- or heterodimers which are formed by members of the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2) and ATF-2 (ATF-2, LRF1/ATF-3, B-ATF, JDP1 and JDP2) subfamilies (316,403). Some of the Maf proteins (v-Maf, c-Maf and Nrl) can also heterodimerize with c-Jun or c-Fos (404,405), whereas other Maf related proteins, including MafB, MafF, MafG and MafK,

heterodimerize exclusively with c-Fos (406,407). Fos proteins do not favor homodimerization and can only form heterodimers with members of the other families.

Homo- and heterodimerizarion between AP-1 proteins occurs via hydrophobic interactions between the leucine zipper regions located in the C-terminal half of the proteins (regions with an α helix structure having a leucine every seventh amino acid) as well as other hydrophobic residues present between the leucines (403,408). The N-terminal half, immediately upstream of the leucine-zipper, contains two clusters of basic amino acids responsible for binding to AP-1 palindromic consensus sequences 5´-TGAG/CTCA-3´, termed TREs [phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements] based on their ability to mediate transcriptional induction in response to the phorbol ester tumor promoter TPA (409). ATF/Jun heterodimers prefer cAMP response elements (CRE) [5´-TGACGTCA-3´] for binding to DNA.

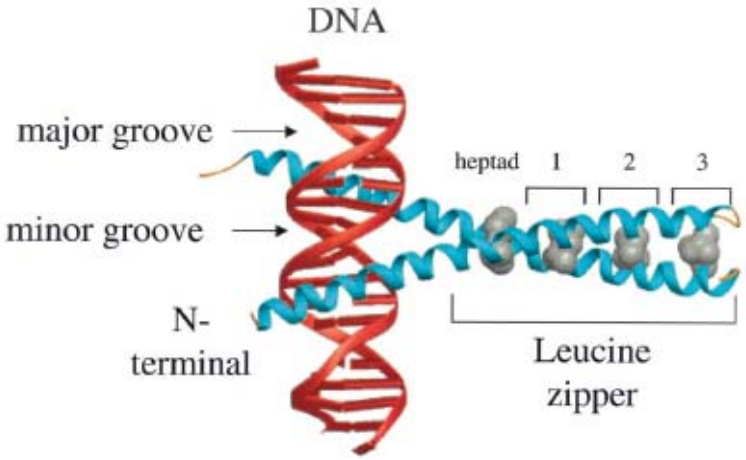

Figure 9. bZIP dimer (blue helices) bound to DNA. The N-terminal DNA recognition helix lies in the major groove of the DNA. A leucine present every two turns of the C-terminal helix is shown in gray. From (403).

Regulation of AP-1 activity is critical for the decision of cell fate and occurs at various levels, including dimer-composition, transcriptional and post-translational events, and interaction with accessory proteins (410). A plethora of physiological stimuli and

environmental insults differentially regulates the expression of AP-1 proteins and their phosphorylation status, modulating the abundance of dimerization partners and dimer composition so that the final decision as to whether a given AP-1 factor positively or negatively regulates a specific target gene will depend on the concurrent events. Rather than being a specialized executor of a unique response to exogenous stimuli, the AP-1 complex plays a general role in the transduction of signals from the membrane to the nucleus.

Jun

The Jun subfamily of AP-1 family of transcription factors is comprised of three proteins, c-Jun, JunB and JunD. Jun proteins share a highly conserved DNA-binding basic region in their C-terminal half and three short regions in their N-terminal half that are important for transcriptional activation. These regions consist of clusters of negatively charged amino acids and correspond to the only sequences which are highly conserved among the N-terminal halves of the three Jun proteins (408). An additional region, a proline-glutanime rich sequence, close to the basic domain is required for transactivation by c-Jun.

Despite their homologies in their DNA binding domains, the transactivation capacities of the Jun proteins vary considerably, with c-Jun exhibiting the highest activation potential. Heterodimerization with c-Fos further increases c-Jun's transcriptional capacity through the formation of more stable dimmers while heterodimerization with JunB attenuates it (411). JunB and JunD homodimers are poor transactivators of promoters containing single TREs but can efficiently activate transcription in the presence of c-Fos. Due to their limited transactivation potential, JunB and JunD homodimers can act as inhibitors of c-Jun activity, possibly by competitive binding to the TRE. CNC, ATF2, ATF3 and c-Maf have also been reported to heterodimerize with Jun proteins (403).

Regulation of AP-1 activity

Physiological and pathophysiological stimuli: Serum, growth factors (EGF, TGF-α, TGF-β, PDGF), proinflammatory cytokines (TNF-α, IL-1) T cell activators, neurotransmitters, UV radiation and oncoproteins such as v-Src or Ha-Ras are all potent inducers of AP-1 activity acting both at the level of transcription of the c-Jun and Fos genes and by posttranslational modification of preexisting AP-1, thus activating c-Jun promoter by positive autoregulation (412). The growth factor responses are mediated by the activation of the ERK (MAPK) cascade, which through phosphorylation of ternary complex factors (TCFs) causes induction of Fos genes, whose products then heterodimerize with Jun proteins to form more stable AP-1 dimers (412). Through AP-1 sites in the c-Jun promoter, these newly formed Jun/Fos heterodimers can lead to increased Jun transcription (413). ERK activation may also contribute to c-Jun induction through MEF2 proteins, another group of transcription factors that bind to the c-Jun promoter (414). The response to proinflammatory cytokines and UV radiation are mostly dependent on two other MAPK cascades, JNK and p38 (415) . In addition to phosphorylation of TCFs and Fos gene induction, p38s contribute to c-Jun gene induction through phosphorylation of MEF2c (416), while JNKs contribute to c-Jun gene induction through phosphorylation and enhancement of the transcriptional activity of c-Jun itself and ATF-2(415). The binding of c-Jun/ATF2 heterodimers to divergent AP-1 sites within the c-Jun promoter results in transcriptional activation. In all cases the primary phosphorylation event results in potentiation of transactivation without exerting a considerable effect on DNA binding activity(415). In contrast, JunB and JunD are less responsive to JNK activation.

Phosphorylation: c-Jun is a nuclear phosphoprotein, phosphorylated on at least five and up to seven serine and threonine residues. Phosphorylation at a cluster of sites located next to its basic region inhibits DNA binding by c-Jun homodimers but not by c-Jun/c-Fos heterodimers (417). On the other hand, phosphorylation of c-Jun at Ser-73 and Ser-63, located within its transactivation domain, potentiates its ability to activate

transcription either as a homodimer (418,419) or as a heterodimer with c-Fos (420). The inhibitory phosphorylation at the region located next to the C-terminal DNA binding domain is mediated by ERK1, ERK2 (421,422) and GSK-3 while JKNs are responsible for the phosphorylation of the N-terminal activation domain of c-Jun (423,424). This phosphorylation may potentiate c-Jun transcriptional activity through recruitment of CREB binding protein (CBP) that connects the phosphorylated activation domains of c-Jun to the basal transcriptional machinery. The induction of c-Jun gene transcription by the PMA-PKC signaling pathway correlates with a net dephosphorylation of preexisting c-Jun protein at GSK-3 target sites that accounts for the increased DNA-binding activity of AP-1.

Interaction with other proteins: In addition to the modulation of its activity by phosphorylation/dephosphoryation, the AP-1 complex is also subject to regulation via interactions with other proteins which do not belong to the Jun or Fos families. Three proteins that have been shown to interact with the AP-1 complex and modify its activity are the activated glucocorticoid receptor (GR), the adenovirus-2 E1A protein and the retinoic acid receptor α (RARα). The glucocorticoid receptor interacts with the DNA-binding domain of c-Jun resulting in significant inhibition of c-Jun DNA binding activity (425). In agreement with this, glucocorticoids and the synthetic glucocorticoid receptor agonist dexamethasone negatively regulate AP-1 activity. The therapeutical effects of glucocorticoids in inflammatory diseases such as rheumatoid arthritis are in part due to their repressive effect on AP-1 inducible collagenase gene expression that contributes to tissue destruction during inflammation (425-427). In a mutual way, the formation of this AP-1/glucocortioid receptor complex inhibits binding of the glucocorticoid receptor to its cognate response element (GRE) (425,428). Similar to GR, RARs inhibit binding of c-Jun to the AP-1 site, thus repressing AP-1 activity and c-Jun inhibits RAR binding to DNA while the viral protein E1A does not inhibit DNA binding of AP-1, but the transactivation function of AP-1 possibly through competition for CBP (429).

Function

Jun proteins play important role in the control of cell proliferation, neoplastic transformation and apoptosis mainly through their ability to regulate the expression and function of cell cycle regulators such as cyclin D1, p53, p21 $^{cip1/waf1}$, p16 and p19^{Arf} (412). Amongst the Jun proteins, c-Jun is unique in its ability to positively regulate cell proliferation through the repression of tumor suppressor gene expression and function, such as p53 and p16, and induction of cyclin D1 transcription in cooperation with c-Fos or FosB. These actions are antagonized by JunB which upregulates tumor suppressor genes and represses cyclin D1 (430) while the role of JunD in the control of cell proliferation is more complex, being growth promoting under some conditions (negatively regulating p19^{Arf} and protecting cells from p53-dependent senescence and apoptosis) and inhibitory under other conditions (431). Mice with tissue-specific loss of JunB develop a myeloproliferative disorder (432) and sever osteopenia (433) (434) while c-Jun induction is critical for hepatocyte cell survival during liver regeneration (435). Liver-specific c-Jun deficiency in mice caused a significant reduction in hepatocyte DNA replication and an aberrant increase in hepatocyte apoptosis and lipid accumulation.

In addition to its growth promoting function, c-Jun is also involved in the activation of programmed cell death, being upregulated under extreme conditions of genotoxic stresses such as exposure to rather high levels of UV radiation or alkylating agents. While some of the proapoptotic activity of c-Jun is due to transcriptional activation of apoptotic mediator genes, such as FasL, a substantial contribution to this biological activity, seems to derive from the transcriptional repression of p21 $^{cip1/waf1}$, which promotes p53-dependent apoptosis.

Studies in mice have also indicated an important role of c-Jun and JunB proteins in development. Disruption of the c-Jun gene caused embryonic lethality at midgestation and an arrest of liver development (436,437). Inactivation of the JunB gene results in earlier embryonic lethality with defects in placentagenesis and vascularization (438) while inactivation of the JunD gene had no effect on development, but caused an age-dependent sterility phenotype associated with disrupted spermatogenesis.

The PKC family of serine/threonine protein kinases comprises a large number of isoenzymes that play a fundamental role in numerous biological facets such as differentiation, proliferation and survival, apoptosis, immune reaction and neuronal transmitter release. Individual isoenzymes have also been implicated in the pathogenesis of clinical disorders such as atherosclerosis, fibrosis, cardiac hypertrophy, pulmonary disease and colon carcinogenesis (439-441). In the case of atherosclerosis, it was shown that inhibition of PKC blocks the production of superoxide anion by activated monocytes and the subsequent oxidation of LDL (442). Both α and β PKCs were shown to be up-regulated upon stimulation of monocytes to translocate to the particulate fraction (443). These findings suggest that specific PKC isozymes may increase the pro-oxidant environment of the atherosclerotic plaque and may lead to plaque instability. Each of the steps in the process of atherosclerosis in which PKC has been found to play a role are indicated in Fig.10.

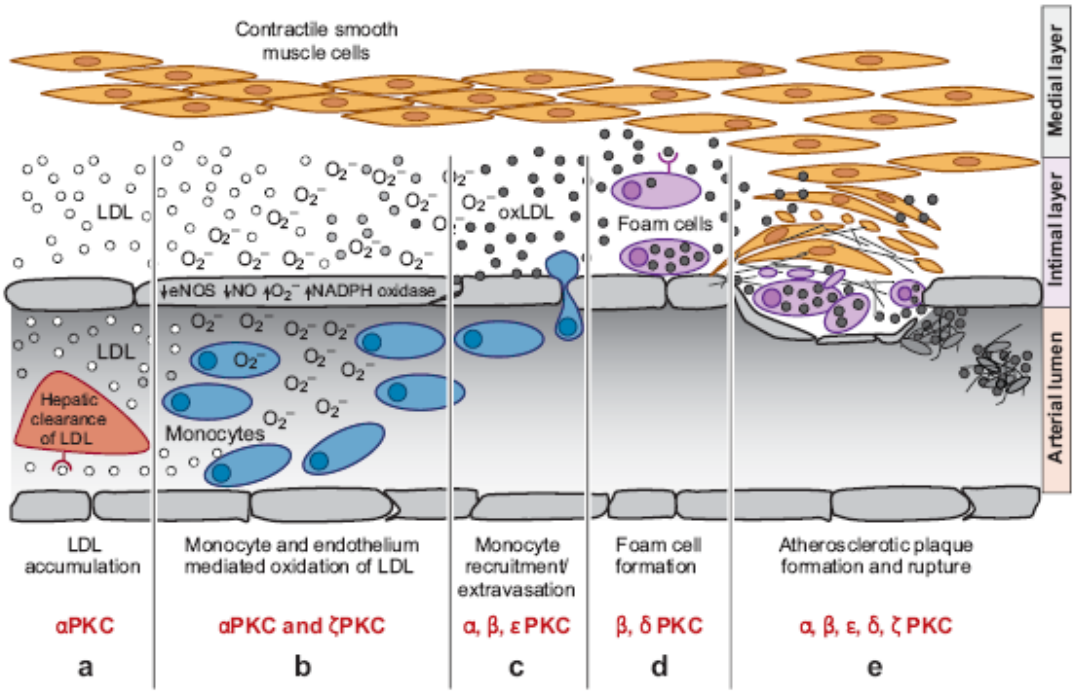

Figure 10. PKC is intimately involved in many stages of atherosclerotic disease progression. (*a*) Hepatic clearance of LDL is regulated, in part, by αPKC. (*b*) α and ζPKC-mediated increases in reactive oxygen species generated from monocytes, endothelium, and smooth muscle oxidizes LDL. (*c*) Oxidized LDL in the subendothelial space acts as a chemotractant for monocytes. Recruitment, adherence, and extravasation of monocytes are regulated in part by α, β, and εPKC. (*d*) β and δPKC-mediated engulfment of oxLDL by macrophages leads to foam cell formation. (*e*) Recruitment and apoptosis of smooth muscle cells and apoptosis of foam cells, as well as accumulation of fibrous tissue, leads to further atherosclerotic plaque progression and subsequent rupture, resulting in thrombi formation into the arterial lumen. From (439).

PKC signaling pathway

The PKC signaling pathway is activated by a broad spectrum of extracellular stimuli that promote lipid hydrolysis. Diacylglycerol that is generated upon lipid hydrolysis acts an essential cofactor for PKC activation. Phorbol myristate acetate, (PMA), a tumor promoter that affects a number of cellular functions including gene expression, protein and DNA synthesis, mimics diacylglycerol in the cellular membrane and potently activates PKC within minutes (444). Signals that elevate intracellular Ca^{2+} , IL-1, TNF, serum and growth factors, have also been shown to lead to PKC activation. PKC in turn activates JNK, p38, ERK and $p90^{RSK}$ and stimulates the activity of the AP-1 transcriptional complex (445,446). Other PKC-responsive elements include the serum response element (SRE) of the c-fos gene, the NF-kB site and the AP-2 recognition site. Besides this, PKC signaling affects the activity of multiple nuclear receptors including the androgen receptor (AR), glucocorticoid receptor (GR), vitamin D receptor (VDR), peroxisome proliferator activated receptor (PPAR), retinoid acid receptor (RAR) and liver X receptor (LXR), increasing the activity of AR and PPAR and repressing the activity of VDR, RAR and LXR (447-451).

PKC structure and regulation

The multiple isoforms of the PKC family are grouped into 3 subclasses based on the requirements for activation which depends on the composition of the regulatory moiety. Similar to PKA and PKB kinases, PKC kinases have a conserved kinase core

whose function is regulated allosterically by a corresponding regulatory moiety. The regulatory domain serves two key functions (i) it targets the kinases to the appropriate cellular location and (ii) it regulates kinase activity by serving as an autoinhibitory module (pseudosubstrate sequence that sterically blocks the active site). Autoinhibition is relieved upon binding of the cofactor diacylglycerol to PKC that recruits PKC to the membrane and through conformational changes unmasks the active site. The two basic modules of the regulatory moiety are the C1 and C2 domains. The C1 domain is the diacylglycerol sensor (also binds phorbol esters) and the C2 domain is the Ca^{2+} sensor that serves as a membrane-targeting module that binds anionic phospholipids in a Ca^{2+} dependent manner. Based on the presence or absence of the C1 and C2 domains, PKC isoenzymes are grouped into: conventional PKCs (α, βI, βII and γ), that contain functional C1 and C2 domains and respond to diacylglycerol and Ca^{2+} signals-, novel PKCs (δ, ε, θ, and η/L), that contain functional C1 domain and respond only to diacylglycerol-, and atypical PKCs (ζ,ι/λ) that contain a non-ligand C1 domain and no C2 domain and as a consequence they respond to neither diacylglycerol nor Ca^{2+} (440).

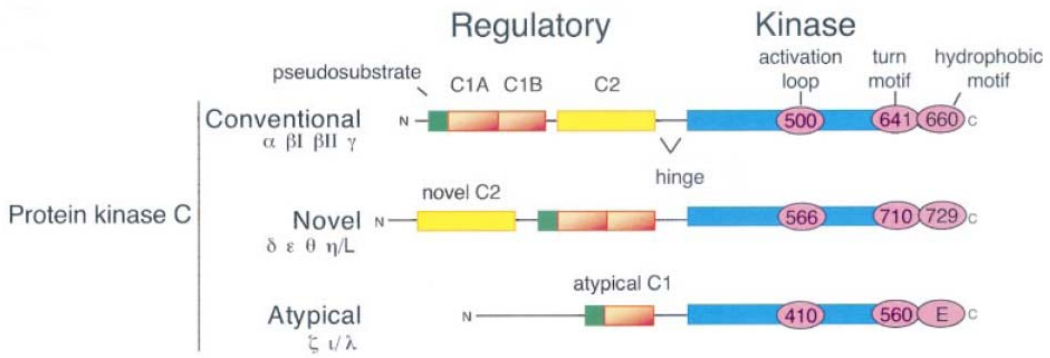

Figure 11. Domain composition of PKC protein kinase family members. From (440).

Apart form diacylglycerol and Ca^{2+}, the structure, subcelluar localization and function of PKCs is regulated by phosphorylation triggerd by 3-phosphoinositide-dependent kinase -1 (PDK-1). PDK-1 is often activated by PI3K that in turn can be activated by phorbol esters. This phosphorylation is a priming event of newly synthesized

PKCs that renders them catalytically competent without directly activating them and is a prerequisite step to allow substrate phosphorylation by PKC. Newly synthesized PKCs associate with a membrane compartment in the cell being in an 'open' conformation that allows docking of PDK-1 and subsequent phosphorylation at a segment near the entrance to the active site, termed the activation loop. PDK-1 is then released exposing sites for autophosphorylation, the hydrophobic motif and the turn motif. Upon phosphoryltaion PKC adopts its 'close' conformation, loses its primary membrane anchor and localizes to the cytosol in its mature but inactive form. Generation of diacylglycerol and Ca^{2+} will then activate PKC, targeting it to the membrane and thus providing the energy for the release of the pseudosubstrate from the substrate-binding cavity, allowing downstream signaling. In its active conformation, PKC is rapidly dephosphorylated. The molecular chaperone Hsp70 binds the dephosphorylated turn motif and stabilizes PKC, allowing it to become rephosphorylated and to re-enter the pool of signaling-competent PKC. In the absence of Hsp70 binding, or as a result of chronic activation, dephosphorylated PKC accumulates in a detergent-insoluble cell fraction, where it is eventually degraded (440).

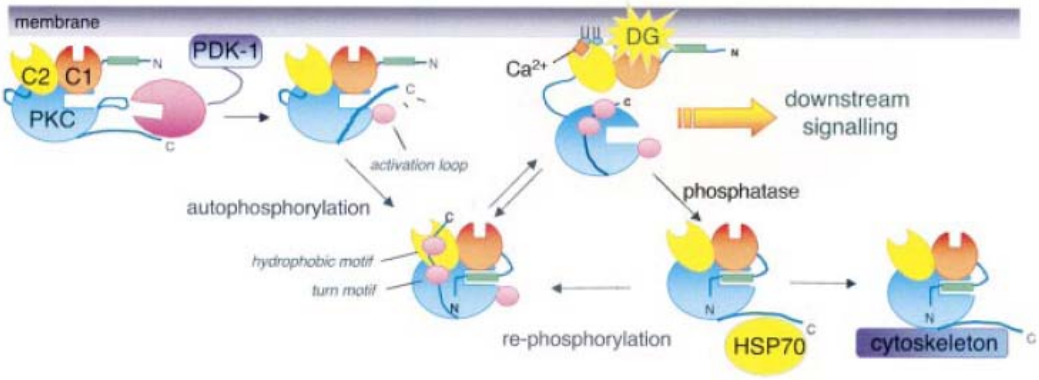

Figure 12. Model showing the life-cycle of PKC, from its biosynthesis to its eventual down-regulation. From (440).

MicroRNAs (miRNAs) comprise a large family of small ~21-nucleotide-long noncoding RNAs that have emerged as key posttranscriptional regulators of gene expression in animals, plants and protozoa. In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes (452) and participate in the regulation of important cellular processes such as cell proliferation and differentiation, apoptosis, development and metabolism (347-354). Deregulation of their expression can lead to pathological conditions. Indeed, aberrant expression of numerous miRNAs has been associated with the development of several diseases including cancer (453), diabetes, obesity, infectious, inflammatory, autoimmune, cardiovascular and Alzheimer's disease (454-456). The importance of miRNAs in normal development and physiology, as well as in disease development, is highlightened by their unique expression profile in each tissue and in different types or stages of disease suggesting their use as biomarkers for disease diagnostics and representing a new therapeutic target.

miRNA biogenesis (transcription and processing)

In humans, approximately 720 miRNA genes are scattered throughout the genome. 70% of these genes are located in regions avoid of protein-coding genes and 30% within introns and exons of protein-coding transcripts. They can be expressed as single genes (monocitronic) or as clusters of miRNAs within one locus (polycystronic) being transcribed from their own promoters, promoters of nearby genes or promoters of host genes by RNA polymerase II. Once transcribed the imperfect complementary sequence of the miRNAs leads to the formation of a complex secondary structure within the primary miRNA transcripts (pri-miRNA).

Mature miRNAs are produced from pri-miRNAs by sequential processing in the nucleus and cytoplasm (457,458). Pri-miRNAs are processed by Rnase III Drosha with its partner DGCR8 into the precursor miRNAs (pre-miRNAs), 69- to 70-nucleotide long

imperferct hairpin structures (459-462) which are then exported into the cytoplasm by the nuclear export factor Exportin 5 and its cofactor Ran-GTP (463,464). In the cytoplasm the pre-miRNAs are further processed by another RNase III Dicer into 22-nucleotide duplexes, one strand of which is the mature miRNA (465) The mature miRNA is incorporated into the RISC complex and negatively regulates protein synthesis by base-pairing to target mRNAs.

In animals, most studied miRNAs form imperfect hybrids with sequences in the 3'-untranslated region (3'UTR) of the mRNAs, with the miRNA 5'-proximal "seed" region (positions 2-8) providing most of the pairing specificity (466,467). Usually, multiple sites, either for the same or different miRNAs are required for effective repression and when sites are close to each other they tend to act cooperatively (468,469). However, targeting of 5'UTR or coding regions of mRNAs by miRNAs has also been documented (470,471).

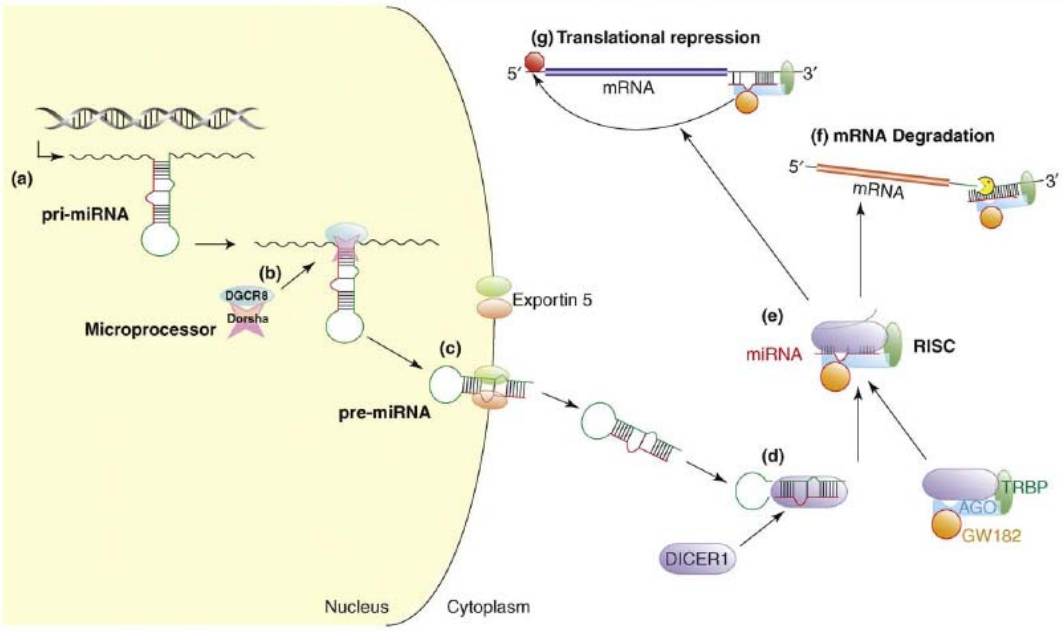

Figure 13. Biogenesis of miRNAs. See text for details. From (472).

Biological action of miRNAs

Generally, miRNAs inhibit protein synthesis either by promoting target mRNA cleavage when miRNA target complementarity is high or by translational repression and/or deadenylation and subsequent degradation of mRNA targets in cases of low miRNA-target complementarity (469, 474, 475). More recently, however, activation of mRNA translation by some miRNAs was reported (354-358).

miRNAs function as part of ribonucleoprotein complexes, miRISCs (miRNA-induced silencing complexes). Core components of miRISC include the Argonaute (AGO) and GW182 [glycine-tryptophan (GW) repeat-containing protein of 182 kDa] family of proteins (473,474). There are four AGO proteins in mammals, AGO1 through AGO4 but only AGO2 contains an enzymatically competent RNaseH-like PIWI domain able to endonucleolytically cleave perefectly complementary RNA targets (475). AGO proteins function to directly anchor miRNAs and bridge the miRNA to the silencing effectors, the GW182 proteins. GW182 proteins interact with AGO proteins via their GW repeats to effect miRNA-mediated repression.

miRNAs repress translation either at initiation or postiniation steps. Inhibition at the level of translation initiation occurs through interfering with eukaryotic initiation factor elF4F-cap recognition and 40S ribosomal subunit recruitment or through antagonizing 60S subunit joining and thus preventing 80S ribosomal complex formation (476-479). The inhibition of cap-dependent translation by miRSC although still questionable has been suggested to be due to competitive binding of AGO2 protein to the cap structure (480) or interaction of miRISC with a component of the cap-binding complex, eIF4F. In Drosophila, dAGO2 was found to compete with eIF4G for binding to eIF4E (481). The inhibition of 80S complex assembly was proposed to be the result of miRISC association with eIF6 which prevents the 60S joining to 40S (482-484) although subsequent studies failed to verify the validity of this assumption. In addition, GW182 protein contributes to the translational initiation repression by interfering with the closed-loop formation mediated by the elF4G-PABP [poly(A)-binding protein] interaction through competitive binding to PABP (485,486). The mechanism of inhibition of translation at postinitiation steps is not well understood but it might include inhibition or

ribosome elongation, induction of ribosome drop-off or facilitation of nascent polypeptides proteolysis (487-490).

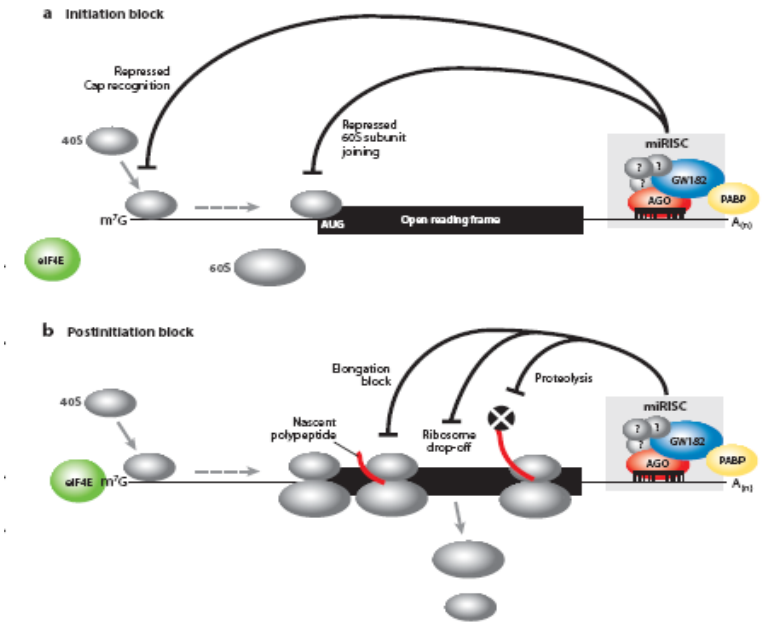

Figure 14. Schematic diagram of miRNA-mediated translational repression. See text for details. From (491).

miRNA-mediated mRNA deadenylation and decay requires the AGO and GW182 component of miRISC (492). GW182 through its direct interaction with the PABP recuits the CCR4-NOT1 (carbon catabolite repression-4-negative on TATA-less) deadenylase complex to promote deadenylation of miRNA-targeted mRNAs (486, 493). Following deadenylation, the 5'-terminal cap is removed by the decapping DCP1-DCP2 complex (492). The mechanism of miRNA-mediated translational activation is not known, but in most cases an association of the miRNAs with the 5'UTR of the target mRNA was reported that did not seem to follow the classical miRNA-mRNA interaction rules for seed region base-pairing (493). Besides this, miR-122-binding to the 5' UTR of HCV RNA was shown to stimulate HCV RNA translation possibly by modifying RNA structure and thus increasing ribosome loading (494,495).

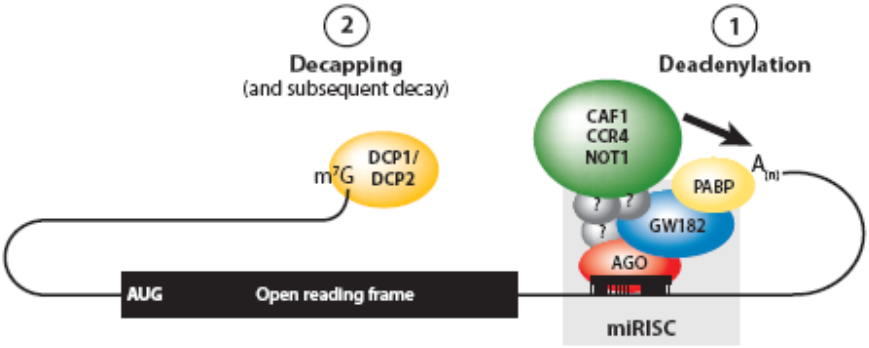

Figure 15. Schematic diagram of miRNA-mediated mRNA decay. See text for details. From (492).

miRNA-mediated repression seems to be modulated by 3'UTR-binding proteins , such as HuR, as well as by their accumulation in discrete cytoplasmic foci, such as P bodies or multivesicular bodies (MVBs) and interaction with the P body components. HuR, is an AU rich element (ARE)-binding protein which modulates miRNA-mediated suppression possibly by modifying mRNA folding and accessibility of RISC to miRNA-binding sites (496,497). P bodies and MVBs, specialized late endosomal compartments with a characteristic multivesicular morphology, are enriched in AGOs, GW182s and miRNAs as well as in proteins involved in mRNA deadenylation, decapping, and degradation Knocking down of some P body components compromises miRNA-induced repression while blocking of MVB formation inhibits silencing by miRNAs (492,498-500) .

Role in metabolism

miRNAs have been implicated in the regulation of gene expression in several metabolically active tissues including the endocrine pancreas, liver and adipose tissue such that alteration in miRNA expression can result in impaired glucose and lipid homeostasis. Disruption of Dicer 1 in mice resulted in severe defects in the formation of the pancreatic exocrine and endocrine systems, in the differentiation of human

multipotent stromal cells to adipocytes and profound hepatocyte apoptosis and steatosis (472). Among the miRNAs implicated in the regulation of metabolism, miR-375 has been shown to play a central role in pancreatic islet cell viability and function, negatively regulating glucose-stimulated insulin secretion (501), miR-1/206 and the miR-133a/133b families appear to be most important for muscle development and growth (502) while miR-122 has been demonstrated to be important for normal lipid metabolism. Other known examples include miR-9, miR-124α2 and miR-96 that target pancreatic and brain mRNAs that are involved in exocytosis (503,504), miR-29b that control genes that affect amino acid metabolism and miR-143 that affect genes that are essential in adipocyte differentiation (505).

In the liver, besides miR-122, which is the predominant miRNA in this tissue (discussed in more detail later), recent studies have suggested a role for miR-370, miR-126, miR-21 and miR-33α and -b in lipid metabolism. miR-370 has been proposed to affect lipid metabolism both directly through targeting the 3'UTR of the carnitine palmitoyl transferase 1α (Cpt1a) involved in fatty acid β oxidation, and indirectly through upregulation of miR-122 and subsequent activation of lipogenic gene expression (SREBP-1c, FAS, ACC, DGAT-2) (506). The effect of miR-370 on the upregulation of the lipogenic genes in HepG2 cells is greatly reduced by treatment with antisense miR-122. Thus upregulation of miR-370 and/or miR-122 may have a causative role in the accumulation of hepatic triglycerides by promoting lipogenesis and inhibiting β oxidation. miR-126 and miR-21 may have a role in the development of atherosclerosis as they have been shown to inhibit the expression of VCAM-1and affect vessel remodeling thus regulating neointimal lesion formation (507).

miR-33α and b differ in only 2 of 19 nucleotides that constitute the mature microRNA and have very recently been shown to target ABCA1 (508-510). Both isoforms are located in the intronic region of genes encoding for the SREBP (sterol regulatory element binding protein) transcription factors. In particular, miR-33α is present in intron 16 of the SREBP-2 gene on chromosome 22 while miR-33b is located in intron 17 of the SREBP-1 gene on chromosome 17. The mature forms of miR-33α/b appear to be coexpressed with the SREBP host genes upon cholesterol depletion and

since SREBPs promote cholesterol uptake and synthesis they probably cooperate with SREBPs to boost intracellular cholesterol levels. The coordinated regulation of SREBPs and miR-33α/b could also provide an explanation for the decreased plasma HDL cholesterol levels observed in patients with metabolic syndrome. Lentiviral delivery of miR-33 to mice repress ABCA1 expression in the liver and reduces plasma HDL levels while silencing of miR-33 in vivo increases hepatic expression of ABCA1 and plasma HDL levels.

miR-122

miR-122 is the predominant miRNA in the liver, playing an important role in the regulation of cholesterol and triglyceride metabolism (511,512). It has been estimated that there are 135,000 copies of this miR per human hepatocyte (513) which accounts for the 70% of the miRNA population, while it is undetectable in other tissues such as lung, heart, kidney, and spleen (513,514). It is derived from a liver-specific noncoding polyadenylated RNA of 160nt transcribed from the *hcr* gene located on chromosome 18q21.31. Both the primary sequence and the predicted secondary structure of the *hcr* mRNA sequences flanking miR-122 are conserved in 12 different animal species, ranging from mammals to birds and fish (513).

The mRNA coding for cationic amino acid transporter (CAT-1 or SLC7A1) was the first mRNA identified to be a direct target of miR-122 (513). Since then a number of studies have demonstrated an important role for miR-122 in the control of lipid metabolism in the liver (472) Antagomir-122 induced degradation of miR-122 in the liver of mice led to a significant reduction in plasma cholesterol levels and decreased the expression of several genes important for cholesterol biosynthesis whereas adenoviral overexpression of miR-122 increased cholesterol biosynthesis, suggesting that the effects of miR-122 on cholesterol biosynthesis are indirect (512). A second complementary study found that miR-122 knockdown in mice decreased circulating cholesterol and triglyceride levels, hepatic cholesterol and fatty acid synthesis and increased liver fatty acid oxidation (511). Furthermore, miR-122 inhibition reduced both hepatic cholesterol accumulation and liver steatosis during the development of diet-induced obesity in mice

(511). Furthermore, fat feeding resulting in hepatic lipid accumulation was found to be associated with increased miR-122 expression in the liver of mice and rats (506). In contrast, in patients with Nonalcoholic Fatty Liver Disease (NAFLD) (515) miR-122 gene expression was found to be downregulated and this was correlated with increased expression of lipogenic genes. In this latter case, it seems likely that miR-122 downregulation is a compensatory mechanism for the increase lipid levels in the liver, rather than a causative agent in the development of NAFLD (516). Inhibition of miR-122 in primates with antisense molecules reduced plasma cholesterol levels without any apparent liver toxicity (517), suggesting miRNA inhibition as a feasible therapeutic approach in humans.

The increase in fatty acid oxidation rate and the decrease in fatty acid and cholesterol synthesis after miR-122 inhibition was probably due to the associated downregulation observed in the mRNA levels of acyl-CoA carboxylase-1 and -2 (ACC-1, ACC-2), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), phosphomevalonate kinase (PMVK) and 3-hydroxy-3-methylglutaryl-CoA-reductase (HMG-CoA reductase) (511). In agreement with this, a recent study has shown an increase in the expression levels of the lipogenic genes FAS, ACC-1, diacylglycerol acyltransferase -2 (DGAT-2) as well as of the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) upon miR-122 overexpression in HepG2 cells (506). The increase in FAS and ACC-1 was attributed to SREBP-1c and DGAT-2 activation as both FAS and ACC-1 responded later to the miR-122 treatment in comparison to SREBP-1c and DGAT-2 and both FAS and ACC-1 are known to be regulated directly (through an SRE) or indirectly by SREBP-1c and DGAT-2, respectively. In addition, the increased β oxidation rate could result from the increased levels of phosphorylated AMP-activated protein kinase (AMPK) observed upon miR-122 inhibition in mice (511), that in turn might be due to increased levels of PPARβ/δ (518). PPARβ/δ was shown to enhance AMPK stimulation (519) and PPARβ/δ protein levels were found to be upregullated upon miR-122 inactivation.

miR-122 affects PPARβ/δ expression levels via three mechanisms: by directly targeting the 3'UTR of PPARβ/δ leading to reduced protein levels, by affecting ligand

(fatty acids) availability and by inhibiting the expression of Smarcd1/BAf60α, through direct targeting of Smarcd1/BAf60α 3'UTR. Smarcd1/BAf60α is a core subunit of the SWI/SNF chromatin remodeling complex that interacts with PPARs and the coactivator PGC-1α enhancing PPARs transcriptional activity. Smarcd1/BAf60α upregulation upon miR-122 inhibition could also contribute to increased β oxidation independently of PPARβ/δ as its overespression in hepatocytes was shown to positively affect the transcriptional activation of genes involved in fatty acid oxidation. Finally, miR-122 was recently shown to downregulate the protein levels of Nocturnin, through direct binding to its 3'UTR, Nocturnin is a circadian clock regulated deadenylase that is thought to be involved in lipid metabolism as mice lacking nocturnin are resistant to diet-induced obesity and hepatic steatosis (520) .

Besides its role in lipid metabolism, miR-122 has been shown to be implicated in hepatocarcinogenesis and the replication of hepatitis C virus (HCV). HCC accounts for 80-90% of primary liver cancers and is the third cause of death from cancer worldwide. Studies in human and mice have reported a significant and specific downregulation of miR-122 in hepatocellular carcinoma (HCC) that contributes to the development and progression of HCC, at least in part, by targeting the expression of cyclin G1 (521,522). By modulating cyclin G1, miR-122 was found to enhance p53 protein stability and transcriptional activity (cyclin G1 negatively regulates p53 protein by dephoshorylating Mdm2), sensitize cells to doxorubicin-induced apoptosis and reduce invasion capability of HCC-derived cell lines. Moreover, lower miR-122 levels in HCC patients were associated with a shorter TTR (time to recurrence) whereas higher cyclin G1 expression was related to a lower survival, suggesting that miR-122 might represent an effective molecular target for HCC (523). However, an upregulation of miR-122 expression was reported in patients with HCV-related HCC and HBV-related HCC. HCV (hepatitis C virus) is an enveloped RNA virus which is capable of causing both acute and chronic hepatitis in humans by infecting liver cells. miR-122 has been reported to facilitate the replication of HCV through binding to the viral 5' non-coding region (495). In accordance with this, HCV RNA could replicate in Huh 7 cells, which express miR-122,

but not in HepG2 cells, which do not express miR-122 while silencing of miR-122 in hepatocytes resulted in a marked loss of replicating RNAs from HCV (495).

Let-7

The lethal-7 (let-7) miRNA is a founding member of the miRNA family. It was originally discovered in the nematode *Caenorhabditis elegans* to control the transition form late-larval to adult cell fates (524,525). Homologs of the let-7 gene were subsequently identified in human and fly genomes representing the first miRNA found in humans (525). Now it is well known that let-7 is highly conserved across animal species both in sequence and in function. In line with their role in C.elegans in promoting differentiation, in mammals let-7 miRNAs are developmentally regulated, being expressed at high levels in differentiated adult tissues while inappropriate expression of let-7 results in oncogenic loss of differentiation (526).

Let-7 family consists of 12 members in humans that generally act as tumor suppressors. Their closely related genes are located in regions that are frequently deleted in humans while their expression levels have been found to be reduced in several cancer types, including lung, breast, ovarian, HCC, and acute lymphoid leukemia (ALL) (527). The reduced expression of let-7 RNAs in lung cancer patients and lung epithelial cells after irradiation is associated with poor prognosis (527) and increased resistance to radiation (528), respectively, that is attributed to the role of let-7 in the control of oncogenes and genes involved in the DNA damage response pathway. The human oncogenes Ras and HMGA2, which are commonly overexpressed in human tumors, are directly regulated and suppressed by let-7 through their 3' UTR (529, 531). However, let-7 was shown to induce translation upregulation of HMGA2 under growth arrest conditions through recruitment of AGO and fragile X mental retardation related protein 1 (FXR1) to the ARE present in its 3'UTR (529).

Apart from their role as tumor suppressors, let-7 RNAs are involved in cell proliferation. Let-7 was shown to directly regulate CDK6, CDC25a and cyclin D, thus inhibiting the transition from G1 to the S phase of the cell cycle, as well as other cell-

cycle related genes such as cyclin A2, CDC34 and genes coding for DNA synthesis and DNA replication functions through an indirect mechanism. Furthermore, the implication of the let-7α isoform in cell survival was proposed as it was found to modulate the interleukin-6-dependent STAT-3 survival signaling in human malignant cholangiocytes, by targeting the tumorsupressor gene NF2, (530) and regulate apoptosis by directly targeting the expression of caspase-3. Overexpression of let-7α increased the resistance of squamous carcinoma and hepatocellular carcinoma cells to drug-induced apoptosis while knock-down of let-7α reduced the survival of malignant human cholangiocytes upon treatment with drugs (530,531).

More recently, the tumor suppression function of let-7 was suggested to be additionally exerted through its involvement in the inflammatory process associated with carcinogenesis. Expression of the cytokine IL-6, a major mediator of the inflammatory response, was shown to be repressed by let-7 both directly through an interaction with its 3'UTR but also indirectly through downregulation of RAS and subsequent reduction of NF-kB activity, resulting in reduced STAT-3 activation and attenuation of cellular transformation in an immortalized breast cancer cell line. In a feedback loop, IL-6 and NF-kB were found to inhibit let-7 posttranscriptionally through the trancriptional activation of lin-28B. Furthermore, a role for let-7 in inflammation is supported by its contribution to endotoxin tolerance. Let-7e was found to be transcriptionally activated by Akt1 in LPS-stimulated macrophages and through targeting TLR4 expression inhibit LPS-signaling and production of proinflammatory cytokines (532). Inhibition of TLR4 expression by another let-7 isoform, let-7i, has also been demonstrated in human cholangiocytes (533). Finally, let-7 are highly expressed in endothelial cells being involved in angiogenesis. Both let-7b and let-7f were found to promote angiogenesis by targeting the angiogenesis inhibitor tissue inhibitor of metalloproteinase (TIMP) and thrombospondin-1 respectively (534).

Purpose of study

Coronary heart disease (CHD) is the leading cause of morbidity and mortality in Western countries accounting for nearly fifty percent of all deaths. Numerous epidemiological and clinical intervention trials have established that HDL is a strong, independent risk factor for the development of atherosclerosis and CHD and that there is an inverse correlation between plasma HDL cholesterol levels and incidence of acute cardiovascular events. On the other hand, it has been well documented in recent years that the concentration of plasma HDL is not by itself a good biomarker for CHD risk prediction but the functionality of HDL particles is also a critical parameter that should be taken into consideration. HDL structure is strongy related to its multiple atheroprotective functions which include the removal of excess cholesterol from peripheral cells and protective functions on the vascular endothelium. The atheroprotective functions of HDL have been shown to be disturbed in patients with chronic inflammatory diseases such as systemic lupus erythematosis, rheumatoid arthritis and chronic kidney disease.

The purpose of the present study was:

a) To understand the mechanism of regulation of the gene encoding apolipoprotein M (apoM), a recently described apolipoprotein that plays an essential role in HDL maturation in plasma. The emphasis was on the role of hormone nuclear receptors and members of the pro-inflammatory Jun family on apoM gene regulation in hepatic cells in hepatic cells under basal or inflammatory conditions.

b) To understand the mechanisms that are responsible for the downregulation of the gene endocing the ATP Binding Cassettee Transporter A1 (ABCA1) by inflammatory factors. The focus was on the role of Protein Kinase C and Jun factors in ABCA1 gene regulation in hepatic cells.

c) To understand the role of miRNAs in the regulation of the expression of genes that are involved in lipoprotein metabolism. The focus was on the role of miR-122 in the expression of several lipoprotein-related genes in hepatic cells and on the role of let-7b in the regulation of apoE gene expression in macrophages.

Understanding the mechanisms of HDL physiology and its deregulation in pathological conditions is very importnant for developing novel methods to increase HDL levels in plasma, to correct its functional abnormalities and to effectively treat patients with CHD and other chronic inflammatory diseases.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin and trypsin/EDTA for cell culture were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Charcoal-stripped serum was prepared after treatment of FBS with charcoal and dextran. Restriction enzymes and T4 DNA ligase were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). Go Taq DNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega Corp. (Madison, WI). 9-cis retinoic acid (9-cis RA), fenofibrates, 22(R)-hydroxycholesterol, T3, PMA, LPS, poly (dI/dC), ONPG (*o*-nitrophenyl β-D-galactopyranoside), PMSF, aprotinin, and benzamidine were purchased from Sigma-Aldrich (St.Louis, MI). Go6976 and bisindolylmaleimide I inhibitors (Calbiochem) were kindly provided by Dr. Krasagakis (Medical School, University of Crete, Greece). T0901317 was purchased from Cayman Chemicals (Ann Arbor, MI). QuickChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Protein G sepharose were purchased from GE healthcare (Waukesha, WI). Trizol reagent for RNA extraction, SuperScript RNAse H-reverse transcriptase, random hexamers, OPTIMEM, Lipofeactamine 2000 and Dynabeads M-280 streptavidin were purchased from Invitrogen/Life Technologies (Carlsbad, CA). The Super Signal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). Biotinylated oligonucleotides were synthesized at VBC Biotech (Vienna, Austria). All other oligonucleotides were synthesized at the microchemical facility of the Institute of Molecular Biology and Biotechnology (Heraklion, Greece). MicroRNA mimics, let-7b and miR-122, inhibitor as-let-7b and negative control, scr-miR, were purchased from Dharmacon and Ambion (TX, USA). MicroRNA mimics let-7a, let-7c, let-7d and let-7f were kindly provided by Dr. D.Iliopoulos (Harvard Medical School).

Antibodies

Anti-HNF-4α (C-19), anti-RXRα (D-20), anti-RARα (C-20), anti-LXR (H-144), anti-TRβ1 (J51), anti-PPARα (H-98), anti-c-Jun (H-79), anti-JunB (C-11) and anti-HNF-1α (C-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti cmyc(9E-10), anti-Flag M2 (F 3165) and the anti-goat peroxidase-conjugated secondary antibody were purchased from Sigma-Aldrich (St.Louis, MO, USA). Anti-LRH-1 (ab18293) antibody was purchased from Abcam (Cambridge, MA). Anti-apoM (NB100-57088) and ABCA1 (NB400-105) were purchased from Novus Biologicals (Littleton, CO). Anti-apoE was purchased from Biodesign International (Meridian Life Science, Inc.). Anti-actin and anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Chemicon International Inc. (Temecula, CA). Anti-PKCα (H-7) and anti-p-PKCα (Ser 657) from Santa Cruz Biotechnology (Santa Cruz, CA) were kindly provided by Dr. Krasagakis (Medical School, University of Crete, Greece).

Cloning

DNA isolation and purification

a) from agarose gel

PCR products and digestion reactions, run on agarose gels, are purified with the use of Wizard SV Gel and PCR Clean-Up System according to the manufacturer's instructions (Promega). In cases a single enzyme is used for vector digestion, 1μl shrimp alkaline phosphatase (SAP) is added to the digestion reaction followed by incubation at 37^{o}C for 30min to 1h to catalyze the dephosphorylation of 5' phosphates from DNA and prevent recircularization.

b) ethanol precipitation

1) add 2 $_{1/2}$ volumes of absolute ethanol and 1/10 volume of CH_3COONa 3M

2) incubate o/n at -20 °C or for 20min at -80 °C
3) centrifuge at 13.000rpm for 15 minutes
4) remove supernatant
5) add 500µl 75% (v/v) ethanol
6) centrifuge at 13.000rpm for 5min
7) remove supernatant
8) resuspend pellet DNA in ddH_20

Ligation reaction

Combine plasmid DNA and DNA to be inserted in a total volume of 15µl containing 1x T4 DNA ligation buffer (New England BioLabs) supplemented with ATP and 1µl T4 DNA ligase. Adjust volume with ddH_20 and incubate at 4 °C o/n.

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Transformation

1) add 100 DH10β competent cells to the ligation reaction
2) leave 30 min on ice
3) transfer at 42 °C for 45sec
4) cool on ice for 2min
5) add 900µl growth medium (LB)
6) incubate at 37 °C for 45min-1h
7) centrifuge at 2.000rpm for 5min
8) remove supernatant and resuspend pellet
9) spread cells on an LB agar plate
10) incubate 37 °C o/n

Purification of plasmid DNA (miniprep)

For the preparation of up to 10-20 µg of plasmid DNA
1) pick a single colony from a selective plate and inoculate 2-3 ml LB medium containing the appropriate selective antibiotic. Incubate at 37°C for 16 h with vigorous

shaking

2) harvest cells by centrifugation at 13.000 rpm for 1 minute

a) bacterial lysis by lysozyme

1) add 600µl lysis buffer and 20-30µl lysozyme (10mg/ml)
2) resuspend pellet by vortex
3) incubate for 10min at RT
4) boil for 90sec
5) keep on ice for 15min
6) centrifuge at 13.000rpm for 15min
7) discard pellet
8) add 600µl cold isopropanol and mix
9) keep at -20 ^{o}C for 20-30min
10) centrifuge at 13.000rpm for 15min
11) discard supernatant
12) add 500µl 75% (v/v) ethanol
13) centrifuge at 13.000rpm for 5min
14) discard supernatant and airdry pellet
15) resuspend pellet in 50µl H20 or TE supplemented with 100µg/ml RNase A

b) alkaline lysis

3) resuspend the bacterial pellet in 150 µl P1 buffer supplemented with RNase A (100 µg/ml)
4) add 150 µl buffer P2, mix gently by inverting 4-6 times
5) add 150µl of chilled buffer P3, mix immediately by inverting 4-6 times
6) centrifuge at 13.000 rpm for 15 minutes at 4^{o}C. remove supernatant containing plasmid DNA
7) carefully transfer supernatant to a new eppendorf tube
8) precipitate DNA by adding 300 µl RT isopropanol. Mix and centrifuge at 13.000 rpm for 15 minutes at 4^{o}C. Carefully decant the supernatant

9) wash DNA pellet with 500 µl RT 70% EtOH, and centrifuge at 13.000 rpm for 10 minutes
10) airdry pellet
11) redissolve DNA in 30 µl H_2O

Purification of plasmid DNA (midiprep/ maxiprep)

For the preparation of up to 500 µg of plasmid DNA, QIAGEN-tips-100 or -500 were used according to the protocol provided by QIAGEN (QIAGEN Plasmid Midi and Maxi Protocol).

1) pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 3 ml LB medium containing the appropriate selective antibiotic. Incubate at 37°C for 6 h with vigorous shaking
2) dilute the starter culture and inoculate 200 ml selective LB medium. Grow at 37°C for 16 h with vigorous shaking
3) harvest the bacterial cells by centrifugation at 3.500 rpm for 30 minutes at 4°C for 100ml culture:
4) resuspend the bacterial pellet in 4 ml buffer P1 supplemented with RNase A (100 µg/ml)
5) add 4 ml buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at RT for 5 minutes
6) add 4 ml of chilled buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 15 minutes
7) centrifuge at 3.500 rpm for 30 minutes at 4°C. remove supernatant containing plasmid DNA
8) equilibrate a QIAGEN-tip 100 by applying 4 ml buffer QBT, and allow the column to empty by gravity flow
9) apply the supernatant from step 7 to the QIAGEN-tip and allow it to enter the resin by gravity flow
10) wash the QIAGEN-tip with 2 x 10 ml buffer QC
11) elute DNA with 5ml buffer QF

12) precipitate DNA by adding 3.5 ml RT isopropanol to the eluted DNA. Mix and centrifuge immediately at 11.000 rpm for 30 minutes at 4°C. Carefully decant the supernatant
13) repeat steps 8 – 12 for the rest 100 ml culture using the same QIAGEN-tip and tubes to precipate DNA
14) wash DNA pellet with 2ml RT 70% EtOH, and centrifuge at 11.000 rpm for 10 minutes
15) airdry pellet and redissolve the DNA in 200 µl TE buffer
16) determine DNA concentration from the absorbance at 260nm

Plasmid constructions

Promoter constructs

The human *apoM* promoter constructs (-950/+42)h*apoM*-luc, (-642/+42)h*apoM*-luc, (-402/+42)h*apoM*-luc, (-241/+42)h*apoM*-luc, (-105/+42)h*apoM*-luc, and (-49/+42)h*apoM*-luc were generated by PCR amplification of the corresponding fragments using human genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector (Promega Corp.). The human *apoM* promoter construct (-20/+8)h*apoM*-luc was generated by ligation of a double-stranded oligonucleotide corresponding to the -20/+8 region of the human *apoM* promoter into the KpnI-HindIII sites of the pGL3basic vector. The human apoM promoter constructs (-950/+42)mut h*apoM*-luc, which bear mutations in the HRE, the HNF-1α/Ap-1 site or both the HRE and the HNF-1α/Ap-1 site were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers shown in Table 3 according to the manufacturer's instructions (described below). The human *apoM* promoter constructs (-642/+42)mut h*apoM*-luc, (-402/+42)mut h*apoM*-luc, (-241/+42)mut h*apoM*-luc and (-105/+42)mut h*apoM*-luc bearing mutation in the HRE were generated by PCR amplification of the corresponding fragments using (-950/+42)mut h*apoM*-luc as template and subsequent cloning into the KpnI-HindIII sites

of the pGL3basic vector (Promega Corp.). The mouse *apoM* promoter construct (-761/+7)m*apoM*-luc was generated by PCR amplification of the corresponding fragment using mouse genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector. The sequence of all primers is shown in Table 1.

The human apoA-II and ABCA1 promoter constructs, (-911/+29)h*apoA-II*-luc and (-668/+33)h*ABCA1*-luc, were available in the lab. The human ABCA1 promoter construct (-668/+33)mut h*ABCA1*-luc, which bears mutations in the putative Ap-1 site was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers shown in Table 3, according to the manufacturer's instructions.

The human apoE promoter construct hapoE-3'UTR-luc was generated by PCR amplification of the corresponding fragment using human genomic DNA as template and the primers shown in Table 1, and subsequent cloning into the XbaI site of the pGL3promoter vector (Promega Corp.). The human apoE promoter construct hapoE-3'UTRmut-luc, which bears mutations in the seed sequence was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) and the primers shown in Table 3, according to the manufacturer's instructions.

Table 1.
Oligonucleotides used for cloning promoter fragments

name	sequence
hapoM-950F	5' -CGGGGTACCGGTGGTGTTTTGTTTGTTTGGAGAC- 3'
hapoM-642F	5'-CGGGGTACCTTGGACACATTCTGCCTGACACCAA- 3'
hapoM-402F	5'-CGGGGTACCCGACTTCTTCAAGATCAGGGGCTATG- 3'
hapoM-241F	5'-CGGGGTACCAACAAATAACCAGCTCAGATACAGG- 3'
hapoM-105F	5'-CGGGGTACCGAAGTCCCAAACACTAAGTAATCCA- 3'
hapoM-49F	5'-CGGGGTACCTCATTAGCAGGTGAAAGGGTCAAGG- 3'
hapoM-20F	5'-CAACGCAAGGGAGCTGAAAGCAGAGTGGAGAATTC A- 3'
hapoM+42R	5' -CCCAAGCTTGCCTTAACTGCTCTCTCCCCTACTG- 3'
hapoM+8R	5'-AGCTT GAATTCTCCACTCTGCTTTCAGCTCCCTTGCGTTGGTAC- 3'
mapoM-761F	5' -CGGGGTACCCTGAATAAACGTGAGCCCATTTGTC- 3'
mapoM-7R	5' -CCCAAGCTTACCTCCGCCTTAACTGTTCTCTGAT- 3'
hapoE3'UTRF	5' -TGCTCTAGAGGATCCGACAATCACTGAACGCCGAAG-3'
hapoE3'UTRR	5' -TGCTCTAGAGCGTGAAACTTGGTGAATCTTT-3'

*Kpn*I (GGTACC), *Hind*III (AAGCTT), *Eco*RI (GAATTC) and *Xba*I (TCTAGA) sites are underlined

Sh-RNA-producing vectors

The sh-HNF-4, sh-HN1α, sh-cJun, sh-JunB and sh-control-producing vectors were generated by ligation of a double-stranded oligonucleotide that contained the siRNA-expressing sequence targeting HNF-4, HNF-1α, c-Jun, JunB or a scrambled sequence (si-control), respectively, into the BglII-HindIII sites of the pSuper.GFP/neo vector (Oligoengine,Seattle). The sequence of all oligonucleotides is shown in Table 2.

Table 2.
Oligonucleotides used for sh-RNA

name	sequence
HNF-4 shRNA sense	5′-GATCCCC*GGCAGTGCGTGGTGGACAA*TTCAAGAGA*TTGTCCACCACGCACT GCC*TTTTTA- 3′
HNF-4 shRNA antisense	5′ -AGCTTAAAAA*GGCAGTGCGTGGTGGACAA*TCTCTTGAA*TTGTCCACCACGC ACTGCC*GGG- 3′
HNF-1α shRNA sense	5′-GATCCCC*TGACAGCACTGCACAGCTT*TTCAAGAGA*AAGCTGTGCAGTGCTGTCA* TTTTTA-3′
HNF -1α shRNA antisense	5′ -AGCTTAAAAA*TGACAGCACTGCACAGCTT*TCTCTTGAA*AAGCTGTGCAGTGCTG TCA*GGG-3′
c-Jun shRNA sense	5′-GATCCCC*GAGCGGACCTTATGGCTAC*TTCAAGAGA*GTAGCCATAAGGTCCGCTC* TTTTTA- 3′
c-Jun shRNA antisense	5′ -AGCTTAAAAA*GAGCGGACCTTATGGCTAC*TCTCTTGAA*GTAGCCATAAGGTCCG CTC*GGG-3′
JunB shRNA sense	5′-GATCCCC*GCATCAAAGTGGAGCGCAA*TTCAAGAGA*TTGCGCTCCACTTTGATGC* TTTTTA- 3′
JunB shRNA antisense	5′ -AGCTTAAAAA*GCATCAAAGTGGAGCGCAA*TCTCTTGAA*TTGCGCTCCACTTTGA TGC*GGG-3′
scrambled shRNA sense	5′ -GATCCCC*TTCTCCGAACGTGTCACGT*TTCAAGAGA*ACGTGACACGTTCGGA GAA*TTTTTA- 3′
scrambled shRNA antisense	5′ -AGCTTAAAAA*TTCTCCGAACGTGTCACGT*TCTCTTGAA*ACGTGACACGTTCG GAGAA*GGG- 3′

si-RNA sequence targeting HNF-4α, HNF-1α, c-Jun, JunB or si-control (scrambled) sequence is italic and underlined

Expression vectors

The expression vectors pcDNA3-myc-LRH-1 and pCMV-SHP were kindly provided by Dr. Ioannis Talianidis (Biomedical Sciences Research Center Alexander

Fleming, Vari, Greece). The pPKCα-EGFP expression plasmid was from Clontech (Mountain View, CA). The expression vectors pMT2-HNF-4, pMT2-RXRα, pMT2-RARα, pMT2-PPARα, pCMX-LXRα, pMT2-TRβ1, pCMX-FXR, pMT2-Ear-3, pcDNAI/Amp-HNF-4-DN, pCMV-SPORT6-HNF-1α, RSV-cJun, pcDNA3-JunB, RSV-JunD, pAdTrack-CMV-HNF4 and pAdTrack-CMV-RXR were available in the lab.

Site-directed mutagenesis

The mutated promoter constructs were generated by site-directed mutagenesis using the QuickChange II Site-Directed Mutagenesis Kit from Stratagene according to the manufacturer's instructions.

1) PCR reaction:

   5 µl 10x buffer for Pfu Ultra HF DNA polymerase
   10 ng ds DNA template
   125 ng primer #1
   125 ng primer #2
   2,5 µl dNTPs 10mM
   31,5 µl H_20
   add last 1 µl Pfu Ultra HF DNA polymerase (2.5 U/µl)

   cycling parameters:
   95°C 30 sec
   95°C 30 sec
   55°C 1 min } 18 cycles
   68°C 6 min

2) add 1 µl of the DpnI restriction enzyme (20U/ml). Gently and thoroughly mix. Spin down in a microcentrifuge for 1 minute
3) incubate 37°C for 1 hour
4) dilute reaction 1:3 with ddH_20 and transform 1 µl into electrocompetent DH10β cells (ElectroMAX DH10B cells, Invitrogen).

Thaw electroMAX DH10B cells on ice. Add 20 µl of cells to the chilled eppendorf tube containing DNA. Pipette the cell/DNA mixture into a chilled cuvette. Gently tap the cuvette to ensure tat the cell/DNA mixture makes contact all the way across the bottom of the cuvette chamber. Avoid formation of bubbles. Electroporate samples (2 kV). To the cells in the cuvette, add 100-150 of S.O.C medium and transfer the solution to a 15ml tube. Shake at 37^{o}C for 1 hour.

5) spread cell on LB-ampicillin agar plates
6) incubate the transformation plates at 37^{o}C for 16 hours
7) select colonies to grow for plasmid DNA purification
8) verify the presence of the desired mutation in the selected clones by sequencing

Table 3.
Oligonucleotides used for site-directed mutagenesis

name	sequence
hapoM-44Fmut	5'-AGCAGGTGAAAGGGT***T***AAG***T***GTCGA***C***CGCAAGGGAGCTGAA-3'
hapoM-4Rmut	5'-TTCAGCTCCCTTGCG***G***TCGAC***A***CTT***A***ACCCTTTCACCTGCT-3'
hapoM-65Fmut	5'-ACAAGGTTGAAGTTAC***GGG***TTAGCAGGTGAAAGGG-3'
hapoM-31Rmut	5'-CCCTTTCACCTGCTAA***CCC***GTAACTTCAACCTTGT -3'
hABCA1-137Fmut	5' -GTGCTTTCTGCTGAGT***A***AC***C***G***C***ACTACATAAACAGAGG-3'
hABCA1-100Rmut	5' -CCTCTGTTTATGTAGT***G***C***G***GT***T***ACTCAGCAGAAAGCAC-3'
hapoE3'UTRFmut	5' -CACCCCGTGCCTCCTG***A***C***G***CCGCGCAGCCTGCAGC-3'
hapoE3'UTRRmut	5'-GCTGCAGGCTGCGCGG***C***G***T***CAGGAGGCACGGGGTG- 3'

Nucleotide substitutions in the primers are in bold and italic

Cell culture and transient transfections

Cell culture

Human hepatoma HepG2 cells, human embryonic kidney cells (HEK293T), human colorectal adenocarcinoma cells Caco-2, 911 cells and mouse macropahages RAW 264.7 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (P/S) at 37 ^{o}C in a 5 % CO_2 atmosphere. For human monocytes

THP-1 cells, RPMI medium 1640 was used instead of DMEM. For the treatment of cells with 9-cis retinoic acid, fibrates, 22(R)-hydroxycholesterol, T0901317 and T3, 10 % FBS was replaced by 5 % charcoal-stripped serum (CSS). For the treatment of cells with TNF-α, TGF-β, LPS or PMA, cells were starved for 3 hours (DMEM 0.5 % FBS, 2 % P/S).

Charcoal Stripped Serum, CSS

1) In 50 ml FBS add: 0.125 g charcoal and 0.0125 g dextran
2) mix and incubate at 56 ^{o}C for 30 minutes
3) centrifuge at 3.000 rpm for 15 minutes
4) transfer supernatant to a new tube and add again: 0.125 g charcoal and 0.0125 g dextran
5) mix and incubate at 37 ^{o}C for 30 minutes
6) centrifuge at 3.000 rpm for 15 minutes
7) transfer supernatant to a new tube
8) filter-sterilize
9) store at -20 ^{o}C

Transient transfections

Transient transfections were performed using the calcium phosphate [$Ca_3(PO_4)_2$] co-precipitation method. In brief, one day before transfection, plate cells in the appropriate amount of growth medium such that they will be 60% confluent at the time of transfection. For each transfection sample, prepare complexes as follows:

1) dilute $CaCl_2$ (2M) and DNA in ddH_20
2) add equal volume 2x Hepes Buffered Saline (HBS) while vortexing
3) incubate for 10min at RT
4) add transfection complexes to cells. Mix gently by rocking the plate back and forth
5) incubate the cells at 37 ^{o}C in a CO_2 incubator for 8-18h
6) replace medium with fresh medium
7) incubate the cells at 37 ^{o}C in a CO_2 incubator for 24h

8) harvest cells

Transient transfections with the sh-RNA producing vectors or miRNAs were performed with the lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. For miRNA overexpression, reverse transfection with siPORT ™ NeoFX ™ Trasnfection agent (Ambion) was alternatively performed according to the manufacturer's instructions.

Reporter assays

For reporter assays, cells were plated in 6-well plates and were subsequently transfected with the calcium phosphate method. Luciferase assays were performed using the luciferase assay kit from Promega Corp. according to the manufacturer' instructions. Normalization for transfection efficiency was performed by β-galactosidase assays

Cell lysis:

1) wash with 1 ml ice-cold PBS
2) add 200 µl 1 x lysis buffer (Promega)
3) incubate for 5 minutes at RT in a shaking platform
4) scrape and collect cells
5) vortex and freeze at -80 ^{o}C for 10-30 minutes
6) thaw at 37 ^{o}C for 2 minutes, vortex
7) centrifuge for 1 minute at 13.000 rpm at RT
8) take supernatant (cell extract)

Measurement of luciferase activity:

Mix 60 µl of cell extract with 60 µl luciferase substrate (Promega) and measure relative light units (RLU) in the luminometer

Measurement of β-galactosidase activity:

1) add to 5-20 µl of cell extract 594 µl of the following mix:
   456 µl sodium phosphate buffer (P buffer)
   132 µl ONPG (8 mg/ml)
   6 µl Mg^{2+} buffer (salt)
2) incubate at 37 ^{o}C until the samples are colored yellow
3) stop reaction with the addition of 200 µl $NaCO_3$ 1M
4) measure OD at 410 nm

Sodium phosphate buffer (P puffer):
Adjust the pH of Na_2HPO4 0.1M to 7.3 with NaH_2PO_4 0.1M

ONPG:
Resuspend the appropriate amount of O-nitrophenyl-galactopyranoside (ONPG) in P buffer to a final concentration 8 mg/ml

Mg^{2+} buffer	
KCl	1M
$MgCl_2$	0.1M
β-mercaptoethanol	5M

RNA isolation and RT-PCR assay

RNA isolation

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions. For cultured cells grown in 60 mm dishes, containing $1x10^6$ cells per dish:

1) wash with 1 ml ice-cold PBS
2) add 1 ml Trizol until homogenized and collect cells in 2 ml eppendorf tubes
3) incubate for 5 minutes at RT
4) add 200µl $CHCl_3$, vortex for 10 seconds and incubate for 3 minutes at RT
5) centrifuge at 12.000rpm for 15 minutes at 4^oC
6) transfer the upper layer to 1.5ml eppendorf tube (500-600 µl)
7) add 500µl isopropanol and incubate for 10 minutes at RT
8) centrifuge at 12.000rpm for 15 minutes at 4^oC
9) wash with 1ml 75% EtOH
10) centrifuge at 12.000rpm for 15 minutes at 4^oC
11) airdry pellet
12) resuspend pellet in 50µl water for injection
13) store at -80^oC

RNA concentration is calculated from the absorbance at 260nm

Protein isolation by Trizol reagent

1) keep the bottom layer from step 5 (above).
2) add 300µl absolute ethanol. Mix
3) 2-3 minutes RT
4) centrifuge at 13.000rpm for 15minutes at 4^oC
5) transfer supernatant to 2ml eppenforf tubes
6) add 1.5ml (up to 2ml) isopropanol
7) $-20\ ^oC$ o/n
8) centrifuge at 13000rpm for 10 minutes at $4\ ^oC$
9) aspiration
10) wash three times with 2ml 0,3M guanidine hydrochloride
    (each wash includes: 20 minutes at RT

centrifuge at 9.000rpm for 5 minutes at 4 °C

aspiration)

11) add up to 2ml 95% ethanol (v/v)
12) 20 minutes at RT
13) centrifuge at 9.000rpm for 5 minutes at 4 °C
14) aspiration
15) airdry pelet for 5 minutes.
16) depeding on the pelet, add 100-150µl 1% SDS
17) 50 °C to dissolve
18) store at -20 °C

Reverse transcription PCR (RT-PCR)

Reverse transcription PCR was done with the use of the enzyme SuperScript Rnase H-reverse transcriptase from Invitrogen according to the manufacturer's instructions.

1) mix: 5 µl RNA (from 0.2 µg/µl)
   3µl random hexamers (from 100 ng/µl stock)
   5 µl dNTPs (from 2mM stock)
   20.5 µl H_2O
2) incubate at 65°C for 5 minutes
3) place the samples on ice and add 10 µl 5x first strand buffer
   5 µl DTT 0.1M
4) incubate at 25 °C for 2 minutes
5) add 1µl SuperScript Rnase H-reverse transcriptase (200U/µl) and 0.5 µl RNase OUT (40U/µl)
7) incubate at 25 °C for 10 minutes
8) incubate at 42 °C for 50 minutes
9) incubate at 70 °C for 15 minutes
10) store cDNA at -20°C

Table 4.
primers used in RT-PCR analysis

name	sequence
hapoA-I F	5’ -AGTTTGAAGGCTCCGCCTTGGGAAA - 3’
hapoA-I R	5’ -CACTTCTTCTGGAAGTCGTCCAGGTA - 3’
hapoC-III F	5’ -AGGAGTCCCAGGTGGCCCAGCAG- 3’
hapoC-III R	5’ -CACGGCTGAAGTTGGTCTGACCTCA- 3'
hapoA-IV F	5’ -ATGTTCCTGAAGGCCGTGGTCCT - 3’
hapoA-IV R	5’ -GGGTGAGTTCAGATTTCTGGAGATGTT - 3’
hapoA-II F	5’ -CGTGACTGACTATGGCAAGGAC- 3’
hapoA-II R	5’ -AGCAAAGAGTGGGTAGGGACAG- 3'
hapoB F	5’ -ATGAGCTCAAGCTGGCCATTCCAGA - 3’
hapoB R	5’ -CTTTGATGAGAGCAAGTGGGCTGATG- 3'
hapoE F	5’ -CCAGCGGAGGTGAAGGAC- 3’
hapoE R	5’ -CGCTTCTGCAGGTCATCG- 3'
mapoE F	5’ - ACAGATCAGCTCGAGTGGCAAA- 3’
mapoE R	5’ - ATCTTGCGCAGGTGTGTGGAGA- 3'
hapoM F	5’ -TATCCTTAACTCCATCTACCAGTGC- 3’
hapoM R	5’ -AGTTACAGGTCAGTTATTGGACAGC- 3’
hABCA1 F	5’ -GAAGCCACAAAAACATTGCTGCAT- 3’
hABCA1 R	5’ -CCTCATACCAGTTGAGAGACTTGAT - 3’
hSR-BI F	5’ -CTCATCAAGCAGCAGGTCCTTA- 3’
hSR-BI-R	5’ -GCATGACGATGTAGTCGCTCT - 3’
hSREBP-1α F	5’ -ATGGACGAGCCACCCTTCAGCGAGGCG - 3’
hSREBP-1c F	5’ -CATGGATTGCACTTTCGAAAGACATGCTTCAGC - 3’
hSREBP-1 R	5’ -CAATGTGGCAGGAGGTGGAGACAAG - 3’
hSREBP-2 F	5’ -ATGGACGACAGCGGCGAGCTGGG - 3’
hSREBP-2 R	5’ -AGGGAGAGAAGGAAGGTAATGTGAC - 3’
hSCD-1 F	5’ -GAGAAGGGGAGTACGCTAGACTTGT - 3’
hSCD-1R	5’ -CTTGTCATAAGGACGATATCCGAAG - 3’
HNF-4α F	5’ -CGAGCAGATCCAGTTCATCA- 3’
HNF-4α R	5’ -CCAGCGGCTTGCTAGATAAC- 3’
RXRα F	5’ -GTTGAACTCGCCTCTTTTGC- 3’
RXRα R	5’ -GACACTTTCTTCCCCACCAA- 3’
HNF-1α F	5’ -TCATCGAGACCTTCATCTCCAC- 3’
HNF-1α R	5’ -ATGAACAGGCTTTGCTCCTAGC- 3’
hSHP F	5’ -GGAGTCCTTCTGGAGCCTGGAGCTT - 3’
hSHP R	5’ -TCTTCACTCAGCCACACCCCACATT - 3’
hPGC-1α F	5’ -AAGCAAAGGGAGAGGCAGAGGC - 3’
hPGC-1α R	5’ -GGTGGAAGCAGGGTCAAAGTCATA - 3’
hCAT-1 F	5’ -CCCACCCCCATAGCTCC - 3’
hCAT-1 R	5’ -TCCTGAAGTAGACTCAGTGGAACG - 3’

GAPDH F	5’ -ACCACAGTCCATGCCATCAC- 3’
GAPDH R	5’ -TCCACCACCCTGTTGCTGTA- 3’

Purification and analysis of protein extracts

For isolation of protein extracts, cells were plated in p-60 or p-100 plates. Following treatment, cells were collected in Co-IP lysis buffer and analysed by immunoblot as follows.

Purification of protein extracts

1) wash with 5 ml ice-cold PBS
2) scrape and collect cells in 1 ml ice-cold PBS
3) centrifuge at 5.000 rpm for 5 minutes at 4^{o}C
4) depending on the pellet resuspend in 100-500 µl Co-IP lysis buffer
5) rotate for 30 minutes at 4^{o}C
6) centrifuge at 13.000 rpm for 5 minutes at 4^{o}C
7) take supernatant and store at -80^{o}C

Co-IP lysis buffer	
Tris-Cl pH 7.5	20mM
NaCl	150mM
glycerol	10%
Triton X-100	1%
PMSF	1mM
benzamydine	0.5mM

Calculation of protein concentration (Lawry)

Protein concentration in cell extracts is calculated from the absorbance at 750 nm using the Biorad *DC* protein Assay kit

1) prepare reaction:
   190 µl H_20
   10 µl protein extract
   100 µl reagent A´ (A´= 1 ml reagent A + 20 µl reagent S)
   800 µl reagent B
2) mix and incubate for 15 minutes at RT
3) measure OD at 750 nm
4) calculation of protein concentration:
   x = (y-a)/b, x = proteins µg/µl measured
   a = 0.02348
   b = 0.00745
   y = OD_{750nm}

Immunoblot analysis

After protein concentration measurement, equal amounts are loaded on SDS-polyarylamide gels followed by electrotransfer onto nitrocellulose membranes.

Sample preparation:

Boil samples at 100^{o}C for 5 - 10 minutes in 4 x sample buffer

SDS-PAGE:

Based on the protein size, samples are run on 8.5, 10.5 or 12.5 % polyacrylamide gels consisting of the separating and stacking gel. For the electrophoresis 1 x TGS buffer is used.

Separating gel			
	8.5%	**10.5%**	**12.5%**
ddH_20	4.6 ml	3.9 ml	3.2 ml
30 % acrylamide	2.8 ml	3.5 ml	4.2 ml
separating buffer	2.5 ml	2.5 ml	2.5 ml
10 % APS	160 µl	160 µl	160 µl
TEMED	8 µl	8 µl	8 µl
Volume	10 ml	10 ml	10 ml

Stacking gel	
ddH_20	3.6 ml
30 % acrylamide	900µl
stacking buffer	1.5 ml
10 % APS	60 µl
TEMED	6 µl
Volume	6 ml

10x TGS	
Tris	30.3 g
Glycine	144.2 g
SDS	10 g
Volume	1lt

	Separating buffer		**Stacking buffer**	
	18.165 g	1.5M	6.05 g	0.5 M
SDS	0.4g	0.4% w/v	0.4 g	0.4 % w/v
Volume	100 ml		100 ml	
pH (with HCl)	8.8		6.8	

Western Blot:

Following SDS-PAGE, proteins are transferred onto nitrocellulose membranes using transfer buffer. Proteins are then visualized by probing the membrane with appropriate monoclonal or polyclonal antibodies and subsequent incubation with horseradish-peroxidase-conjugated secondary antibodies, as follows:

1) wash membrane for 5 minutes with TBS-T 0.05 %
2) incubate for 1 hour at RT in 5 % w/v non-fat milk in TBS-T 0.05 %
3) incubate with the primary antibody o/n at 4 ^{o}C (1:500 in 5 % w/v non-fat milk in TBS-T 0.05 %, 0.02 % NaN_3)
4) wash 3 times for 10 minutes with TBS-T 0.05 %
5) incubate with the secondary antibody for 1 hour at RT (a-mouse, a-goat, a-rabbit IgG HRP 1:10000 in 5 % w/v non-fat milk in TBS-T 0.05 %)
6) wash 3 times for 10 minutes with TBS-T 0.05 %
7) wash once for 5 minutes with TBS
8) detect signals by enhanced chemiluminescence
9) to normalize the variations for proteins amounts, membranes are stripped (incubate with stripping buffer for 30 minutes at 50 ^{o}C, wash 2 times for 10 minutes with TBS-T at RT, blocking for 1 hour at RT in 5 % w/v non-fat milk in TBS-T 0.05 %) and re-probed with an antibody to β-actin (1: 5000 in TBS-T 0.05%, 0.02% NaN_3)

Transfer buffer		**10 x TBS**	
10 x TGS	100 ml	**NaCl**	180 g
Methanol	200 ml	**Tris**	121.14 g
ddH_20	700 ml	**pH 7.3**	(with HCl)
Volume	1 lt	**Volume**	1 lt

Stripping buffer	
SDS	2 %
Tris-HCl pH 6.8	62.5 mM
β-mercaptoethanol	100 mM

DNA affinity precipitation assay (DNAP)

Nuclear extract isolation

For cultured cells grown in 100 mm dishes:

1) wash with ice-cold PBS
2) scrape cells in 1ml ice-cold PBS
3) centrifuge at 4.000 rpm for 10min at 4^{o}C
4) measure pellet and resuspend quickly in 5x (cell volume) Hypotonic Buffer
5) centrifuge at 4.000rpm for 5min at 4^{o}C
6) resuspend in 3x (cell volume) Hypotonic Buffer
7) incubate for 10 minutes on ice
8) homogenization with insulin syringe (27G) (20 times)
9) pellet the nuclei at 6.000rpm for 15 minutes at 4^{o}C
10) measure volume and resuspend in ½ (pellet vol) low salt buffer, vortex
11) add ½ (pellet vol) high salt buffer, drop by drop during vortex
12) rotate for 30 minutes at 4^{o}C
13) centrifuge at 13.000 rpm for 40min at 4^{o}C
14) take supernatant and store at -80^{o}C

Hypotonic buffer		Low salt buffer		High salt buffer	
Hepes pH 7.9	10mM	**Hepes pH 7.9**	20 mM	**Hepes pH 7.9**	20 mM
$MgCl_2$	1.5 mM	**Glycerol**	25 %	**Glycerol**	25 %
KCl	10 mM	**$MgCl_2$**	1.5 mM	**$MgCl_2$**	1.5 mM
EDTA	0.2 mM	**KCl**	0.02 M	**KCl**	1.2 M
PMSF	1 mM	**EDTA**	0.2 mM	**EDTA**	0.2 mM
DTT	2 mM	**PMSF**	1 mM	**PMSF**	1 mM
benzamydine	0.5 mM	**DTT**	2 mM	**DTT**	2 mM

Beads preparation:

1) wash 5µl (50µg) Dynabeads M-280 Streptavidin with 500 µl 1 x B&W buffer
2) add 7 µl 2 x B&W buffer and 7 µl biotinylated oligo (0.58µM)
   (for control, add to the beads 14µl of 1 x B&W buffer)
3) incubate for 15 minutes at RT. Shake occasionally. In the meantime, prepare nuclear extract.
4) wash 2 times with 500µl 1x B&W buffer
5) wash one time with D buffer

Nuclear extract preparation (on ice):

6) add: 6 µl of carrier mix [1µl dI-dC (3µg/µl) + 5µl 10x binding buffer]
   30µg of nuclear protein extract
   D Buffer until 50µl
7) incubate on ice for 15 minutes. Shake occasionally.

Protein – DNA binding interactions

8) add the prepared nuclear to the beads and incubate on ice for 30 minutes. Shake occasionally.

9) wash three times with 500µl D buffer

10) add 10µl loading buffer (+input) and boil for 10 minutes.

2 x B&W		D buffer		10x binding buffer	
Tris-HCl pH 7.5	10mM	**Hepes pH 7.9**	20 mM	**Hepes pH 7.9**	100 mM
EDTA	1 mM	**Glycerol**	10 %	**KCl**	0.5 M
NaCl	2 mM	**KCl**	40 mM	**$MgCl_2$**	20 mM
		DTT	0.5 mM	**spermidine**	40 mM
				BSA	1 mg/ml
				Zn-acetate	0.2 mM
				NP-40	0.5%
				DTT	2 mM

Preparation of ds biotinylated oligonucleotides

1) reaction:
   5 µg biotinylated oligonucleotide
   5 µg unbiotinylted complementary oligonucleotide
   2 µl NEB2 10 x buffer
   ddH_20 up to 20 µl
2) incubate at 92 ^oC for 2 minutes
3) allow to cool gradually
4) adjust volume with H_20 to a final concentration of 5 µM
5) store at -20 ^oC

In cases that a biotinylated PCR product was used instead of biotinylated oligonucleotides, PCR products were amplified with the use of a biotinylated primer

and isolated by gel extraction. The amount used in the reaction was calculated based on the fact that for a 600bp fragment, 2 μg of the PCR product is required.

Table 5.
oligonucleotides used in DNAP assays

name	sequence
hapoM-241F-Bio	5' -Bio-CGGGGTACCAACAAATAACCAGCTCAGATACAGG- 3'
hapoM-105F-Bio	5' -Bio-CGGGGTACCGAAGTCCCAAACACTAAGTAATCCA- 3'
hapoM-40F-Bio	5'-Bio-GGTGAAAGGGTCAAGGGTCGAACGCAA- 3'
hapoM-40F-Bio-mut	5' -Bio-GGTGAAAGGGT*T*AAG*T*GTCGA*C*CGCAA- 3'
hapoM-61F-Bio	5'-Bio-GGTTGAAGTTACTCATTAGCAGGT- 3'
hapoM-61F-Bio-mut	5' -Bio-GGTTGAAGTTACGGGTTAGCAGGT- 3'
mapoM-109F-Bio	5' -Bio-GGTTGAAGTTACTTATTAGCAGGT- 3'
hABCA1-535F-Bio	5' -Bio-AGGCCTTTGAAAGGAAACAAAAGACAAGACAAA - 3'
hABCA1-118F-Bio	5' -Bio-TGAACTACATAAACAGAGGCCGGGA - 3'
hRhoB-76F-Bio	5' -Bio-GCCGGCTGGTTTCCCATTGGACGGCTATATTAAG- 3'
hapoM-81R	5' -TGGATTACTTAGTGTTTGGGACTTC- 3'
hapoM+42R	5' -CCCAAGCTTGCCTTAACTGCTCTCTCCCCTACTG- 3'
hABCA1+205R	5' -CCGCTCGAGGTCTCTTTCTCCTACCCCTTGACA - 3'
hapoA-II-573F	5' - CGTCTCATTACACATTAACTCAAA - 3'

Chromatin immunoprecipitation assay (ChIP)

For cultured cells grown in 100 mm dishes, containing $10x10^6$ cells per dish:

1) wash with 7 ml DMEM/10% FBS
2) replace medium with 9 ml DMEM/10% FBS, add 1 ml formaldehyde (from 10% stock) and mix immediately. Incubate at 37^{o}C for 10 minutes
3) add 1 ml glycine (from 1.375M stock) and mix immediately
4) place the plate on the top of ice and wash 3 times with 10 ml ice-cold PBS/0.5 mM PMSF
5) scrape cells in 7 ml ice-cold PBS/0.5% NP-40/0.5 mM PMSF. Centrifuge at 1.000 rpm for 5 minutes at 4^{o}C
6) resuspend pellet in 5 ml Swelling buffer. Incubate on ice for 10 minutes

7) dounce 20-30 times up-down (check nuclei in microscope by mixing 5µl with equal volume of 0.4%Trypan Blue)
8) centrifuge at 2000 rpm for 5 minutes at 4°C
9) resuspend pellet (nuclei) in 2 ml Sonication buffer
10) sonicate 12 times for 30 sec at 50% setting. Keep sample in ice and allow sample to cool in ice for 5 minutes between each sonication
11) centrifuge at 14.000 rpm for 15 minutes at 4°C
12) take the supernatant and centrifuge again at 14.000rpm for 15 minutes
13) take the supernatant. Keep 50µl for sonication check. Store the rest at -80°C.

Sonication check:

to the 50 µl aliquot:
14) add 150 µl H_2O and 10.5 µl NaCl (from 4M stock)
15) incubate at 65°C o/n
16) add 2µl RNAse A (from 10 mg/ml stock)
17) incubate at 37°C for 1 hr
18) add 2 µl EDTA (from 0.5M stock)
19) add equal volume phenol/chloroform/isoamylalcohol. Mix.
20) centrifuge at 13000 rpm for 5 minutes
21) take upper phase and add equal volume chloroform. Mix
22) centrifuge at 13000 rpm for 5 minutes
23) take upper phase and add 4 µl glycogen (from 5 µg/µl stock), 1/10 V Na-acetate (from 3M stock) and 2.5 V 100% EtOH
24) transfer to -80°C for 30 minutes
25) centrifuge at 13000 rpm for 10 minutes
26) wash with 300 µl 75% EtOH
27) centrifuge at 13000 rpm for 10 minutes
28) airdry pellet
29) resuspend pellet in 20 µl H_20
30) run on agarose gel (fragment size should be less than 1000nt)

Beads equilibration-blocking:

For each sample use 160 µl Protein-G Sepharose (40µl for IP, 40µl for control and 80µl for preclearing).

31) centrifuge the appropriate amount of Protein-G Sepharose at 6000 rpm for 3 minutes (or at 2000 rpm for 5 minutes) at 4 ^{o}C
32) wash 3 times with 1.5 ml sonication buffer
    - rotate for 10 minutes at 4^{o}C
    - centrifuge at 6000rpm for 3 minutes at 4^{o}C
33) add 494 µl sonication buffer, 5µl BSA (from 100 mg/ml stock), and 1 µl sonicated λ DNA (from 0.5 µg/µl stock)
34) rotate for 2 hours at 4^{o}C
35) transfer 180 µl Protein-G Sepharose in 2 eppendorf tubes and store at 4^{o}C (will be used for IP and control)

Preclearing:

36) spin the rest Protein-G Sepharose (340 µl) at 6000rpm for 3minutes at 4^{o}C
38) thaw chromatin stored at -80 ^{o}C from step 13. Measure OD at 260nm and 280nm. Divide each OD_{260} with the smaller OD_{260} (factor)
39) add to the Protein-G Sepharose 1500 µl from the sample with the smaller OD_{260} and 1500/factor (µl) from the rest. Add up to 1500 µl sonication buffer
40) add 15µl BSA (from 100 mg/ml stock) and 3 µl sonicated λ DNA (from 0.5µg/µl stock)
41) rotate for 2 hours at 4^{o}C
42) centrifuge at 2000 rpm for 5minutes (or at 6000rpm for 3minutes) at 4^{o}C
43) take the supernatant. Save a 65 µl (1/10th of amount used per IP) aliquot at -20^{o}C (input DNA)

IP:

44) divide the sample into 650 µl aliquots in eppendorf tubes

45) add 5µg ab (IP) or nothing (control)

46) rotate for 2 hours at 4°C

47) centrifuge Protein-G Sepharose stored at 4°C from step 35 at 6000 rpm for 3 minutes

48) add IP and control samples to the Protein-G Sepharose

49) rotate o/n at 4 °C

Washes-Elution:

50) centrifuge at 6000 rpm for 3 minutes at 4°C

51) wash 2 times with 1ml Wash buffer A

- rotate for 10 minutes at 4°C
- centrifuge at 6000rpm for 3 minutes at 4°C

52) wash 2 times with 1ml Wash buffer B

53) wash 2 times with 1ml Wash buffer C

54) wash 2 times with 1ml TE buffer

55) add 150 µl Elution buffer, vortex and incubate at 65°C for 10 minutes

56) Vortex and centrifuge at 14000 rpm for 1minute

57) transfer supernatant (150 µl) to a new tube and elute beads again

58) combine eluates (300ul final volume, adjust with elution buffer if necessary)

59) add 100 µl H_2O and 21µl NaCl (from 4M stock)

60) thaw the input sample (65 µl) and supplement with elution buffer (300µl final volume). Add 100µl H_2O and 21µl NaCl 4M (from 4M stock)

Decrosslinking-Purification:

61) incubate at 65°C o/n (or for 5 hours)

62) add 1µl RNAse A (from 10 mg/ml stock) and incubate at 37°C for 1 hour

63) add 2µl EDTA (from 0.5M stock) and 2 µl Proteinase K (from 10 mg/ml stock)

64) incubate at 42°C for 2 hours

65) add 200µl H_2O, 1/10 V Na-acetate (from 3M stock) and equal volume

phenol/chloroform/isoamylalcohol 25:24:1

66) vortex and centrifuge at 10000 rpm for 5 minutes

67) take upper phase and add equal volume chloroform

68) vortex and centrifuge at 10000 rpm for 5 minutes

69) take upper phase and add 1µl glycogen (from 20 mg/ml stock) and 2.5 V 100% EtOH

70) vortex and leave to precipitate at -20 ^{o}C o/n

71) centrifuge at 13000 rpm for 30 minutes at 4 ^{o}C

72) wash with 500µl 75% EtOH

73) centrifuge at 13000rpm for 10 minutes at 4 ^{o}C

74) airdry pellet

75) resuspend input sample in 100 µl and IP samples in 50µl 10 mM Tris (pH 7.5)

Swelling buffer		**Sonication buffer**	
Hepes pH 7.9	25 mM	**Hepes pH 7.9**	50 mM
$MgCl_2$	1.5 mM	**NaCl**	140mM
KCl	10 mM	**EDTA**	1 mM
NP-40	0.5 %	**Triton X-100**	1 %
DTT	1 mM	**Na-deoxycholate**	0.1 %
PMSF	0.5 mM	**SDS**	0.1 %
aprotinin	2 µg/ml	**PMSF**	0.5 mM

Wash buffer A		Wash buffer B		Wash buffer C	
Hepes pH 7.9	50mM	**Hepes pH 7.9**	50 mM	**Tris-Cl pH 8.0**	20 mM
NaCl	140 mM	**NaCl**	500 mM	**EDTA**	1 mM
EDTA	1 mM	**EDTA**	1 mM	**LiCl**	250 mM
Triton X-100	1 %	**Triton X-100**	1 %	**Na-deoxycholate**	0.5 %
Na-deoxycholate	0.1 %	**Na-deoxycholate**	0.1 %	**NP-40**	0.5 %
SDS	0.1 %	**SDS**	0.1 %	**PMSF**	0.5 mM

TE		Elution buffer	
Tris-Cl pH 8.0	10 mM	**Tris-Cl pH 8.0**	50 mM
EDTA	1 mM	**EDTA**	1 mM
PMSF	0.5 mM	**SDS**	1 %
aprotinin	2 μg/ml	**$NaHCO_3$**	50 mM

Adenovirus generation and infection

The recombinant adenoviruses ad-HNF-4α and ad-RXRα were produced and amplified using the AdEasy adenoviral system

Construction of recombinant DNA:

1) clone cDNA in the pAdTrack-CMV vector
2) digest with *Pme*I to linearize
3) isolate DNA with gel extraction
4) transformation into E.coli BJ5183-AD1which are already transformed with the adenoviral backbone plasmid pAd-Easy

5) select recombinants for kanamycin resistance
6) screen for positive clones by digestion with *Pac*I (the lower band after *Pac*I digest is either 3 or 4.5 kb depending on the recombination)
7) transform one of the positive clones into E.coli DH10β to amplify
8) select for kanamysin resistance
9) select one colony to grow for purification of plasmid DNA in large scale

Amplification of viruses in 911 cells

1) digest approximately 15 μg of the recombinant DNA with *Pac*I
2) purify DNA with ethanol precipitation
3) infect 1.5 x 10^6 911 cells with approximately 5 μg of the digested DNA using lipofectamine
4) the next day replace medium with fresh DMEM supplemented with 2 % Heat Inactivated Horse Serum (HS)
5) 48h later assess viral production by monitoring GFP expression by fluorescence microscopy
6) incubate cells for 7-10 days until cell lysis occurs
7) use harvested viruses to infect 3 x 10^6 911 cells (T175 flask)
8) after lysis, use harvested viruses to infect 12 x 10^6 911 cells (4 T175 flasks)
9) collect cells 1-2 days later, before lysis occurs
10) centrifuge at 1.000 rpm for 10 minutes at 4^{o}C
11) discard supernatant and resuspend pellet in 1ml medium
12) freeze at -80^{o}C
13) thaw at 37^{o}C
14) repeat freeze-thaw cycle 2 times
15) centrifuge at 3.500 rpm for 10 minutes at 4^{o}C
16) store viral lysate at -80^{o}C

Estimation of viral titers

Viral titers were estimated by a fluorescence forming assay

1) add to 35 mm plates 1 ml collagen (25 mg/ml)
2) incubate at RT for 30 minutes
3) remove collagen and let plates to dry
4) make serial dilutions of the viral lysate and add them to the plates covered with collagen
5) incubate at 37^{o}C for 2 hours
6) aspirate and replace with fresh medium
7) incubate at 37^{o}C for 48 hours
8) aspirate medium
9) wash with PBS
10) let plates dry for 10-20 minutes
11) add 1 ml 3 % PFA
12) incubate for 5 minutes at RT
13) discard PFA and wash with PBS
14) add 1 ml 1 x PBS and monitor GFP expression by fluorescence microscopy
15) calculation of viral titer: number of green cells x dilution x 200 (plate depth)

Infection of HepG2 cells

HepG2 cells were infected at a multiplicity of infection (MOI) 10 by adding virus stocks directly to the cell culture medium.

Results and Discussion

PART I: Regulation of human apolipoprotein M gene expression by orphan and ligand-dependent nuclear receptors

Hepatocyte Nuclear Factor 4 (HNF-4) is a positive regulator of human apoM gene expression in hepatic cells

HNF4-α is an orphan member of the hormone nuclear receptor gene superfamily (278,535) and a key transcription factor in the liver, regulating numerous target genes involved in lipoprotein metabolism, including apolipoproteins, cholesterol synthesis enzymes and bile acid transporters (283). Mice lacking HNF4-α expression in the liver have altered lipid metabolism exhibiting lipid accumulation in the liver and greatly reduced plasma total and HDL cholesterol levels (287). Recent genome-wide association studies (GWAs) identified a common variant in the coding region of HNF-4α gene (rs1899861) associated with low HDL concentrations in humans (290). A cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells showed a 4.5 fold increase in apoM expression levels (536).

To investigate the role of HNF-4 in apoM gene regulation in the liver, RNA interference by short hairpin RNAs (shRNAs) was employed. For this purpose, an HNF-4 sh-RNA (sh-HNF-4) producing vector was constructed along with a scrambled sh-RNA (sh-control) producing vector and used in transient transfection assays in the human hepatoblastoma-derived cell line HepG2. Transient expression of increasing amounts of sh-HNF-4 dose-dependently reduced HNF-4 mRNA levels up to 70% (Fig 1A) and HNF-4 protein levels by approximately 50% compared to cells expressing sh-control (Fig. 1B). Decreased HNF-4 expression in sh-HNF-4 transfected cells was associated with a 50% reduction in apoM mRNA levels (Fig. 1A) similar to the reduction observed in the mRNA levels of the apoC-III gene, a well established target gene of HNF-4 (400,537,538). Both the intracellular and the secreted levels of apoM protein were also decreased in sh-HNF-4 treated cells compared to sh-control treated cells (Fig. 1B).

In agreement with these findings, overexpression in HepG2 cells of a dominant negative mutant of HNF-4 (HNF-4 DN) that lacks the Activation Function 2 (AF-2) (539) reduced significantly both apoM mRNA levels to 40% of control (Fig. 1C) and protein levels (Fig. 1D). The mRNA levels of the apoC-III gene that was used as a positive control showed a comparable reduction in the presence of the HNF-4α DN mutant (Fig. 1C).

The effect of HNF-4 on apoM gene expression was next examined using adenovirus-mediated gene transfer. For this purpose, a recombinant adenovirus expressing wild type HNF-4 (Ad-HNF-4) was generated and used to infect HepG2 cells. As a control, a recombinant adenovirus expressing the green Fluorescent Protein (Ad-GFP) was used. Ad-HNF-4 infection in HepG2 cells led to a 2.4 fold increase in HNF-4 mRNA levels (Figure 2A) and protein levels (Fig. 2B) compared to control Ad-GFP infected cells. Importantly, HNF-4 overexpression was associated with a 1.9 fold increase in apoM mRNA levels (Fig. 2A) and a significant increase in both intracellular and the secreted apoM protein levels (Fig. 2B). Furthermore, overexpression of HNF-4 and its co-activator, PGC-1α, in HEK293T cells that do not express endogenously neither apoM nor HNF-4, induced apoM and apoC-III gene expression while GAPDH mRNA levels remained nearly constant (Figure 2C). HNF-4 or PGC-1α alone, however, were not sufficient to induce apolipoprotein gene expression

In summary, the combined findings of Fig. 1 and 2 indicate that HNF-4 is an important regulator of apoM gene expression in hepatic cells.

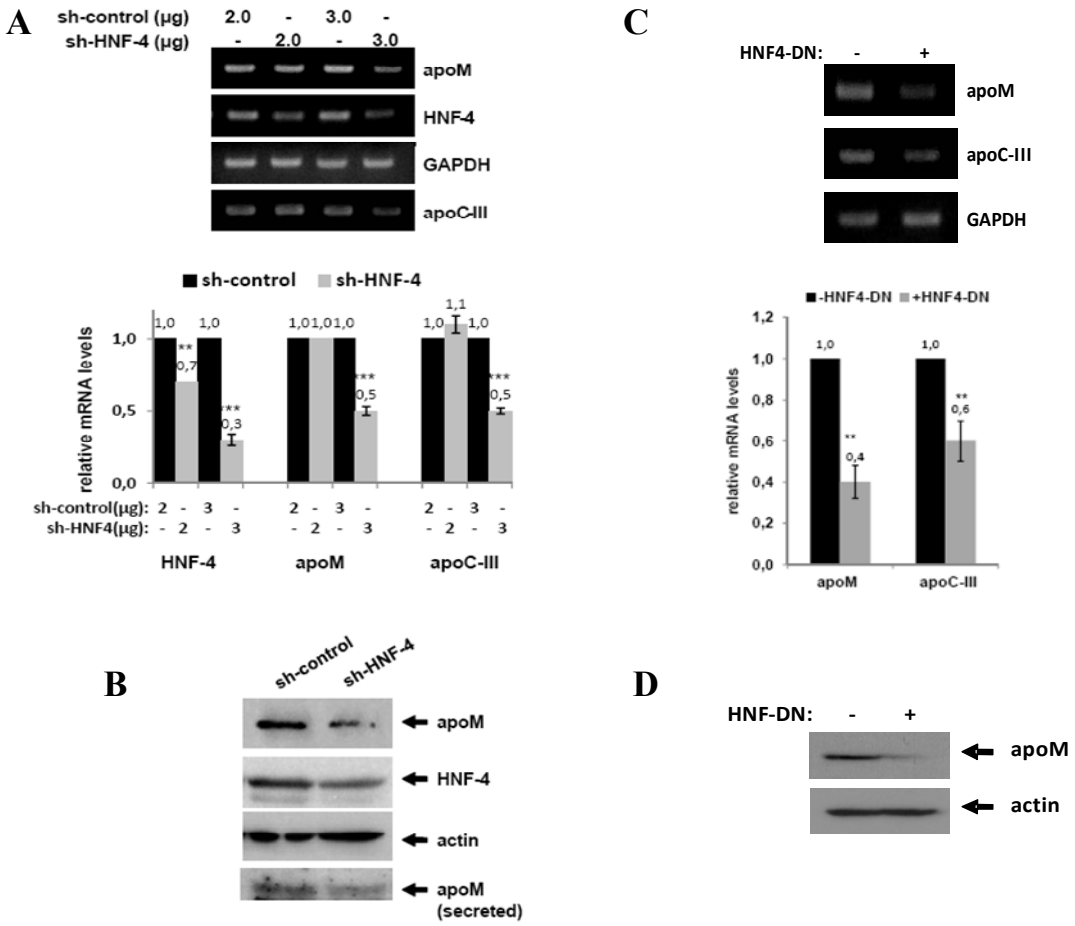


Figure 1. Hepatocyte nuclear factor 4α (HNF-4α) is a positive regulator of human apoM gene expression. (A) Human hepatoma HepG2 cells were transiently transfected with increasing amounts (2.0 and 3.0μg) of sh-HNF-4α or sh-control producing vectors. 48h later, total RNA was extracted and apoM, HNF-4α and apoC-III mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM, HNF-4α and apoC-III genes were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were transiently transfected with 3.0μg of sh-HNF-4α or sh-control producing vector and the intracellular protein levels of apoM, HNF-4α and actin (loading control) as well as of the secreted apoM were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (C). HepG2 cells were transiently transfected with an expression vector for HNF-4α DN (10μg) and 48h later, total RNA was extracted and apoM and apoC-III mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoM and apoC-III genes are shown as a histograph. Each value represents the average from three independent experiments. (D) HepG2 cells were transiently transfected with 10μg of the HNF-4α DN expressing vector and the protein levels of apoM and actin (loading control)

were determined by immunoblotting using the corresponding antibodies. Key: **p<0.01, ***p<0.001

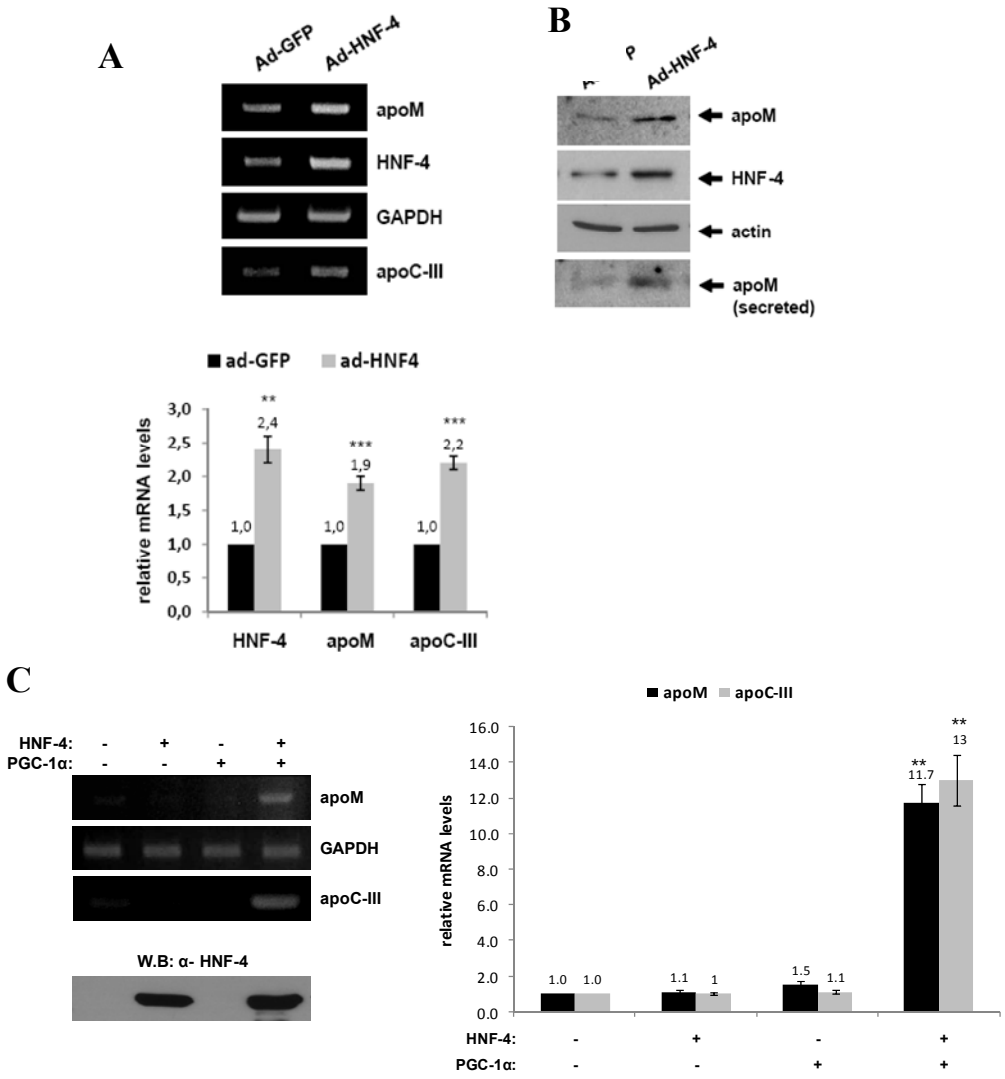


Figure 2. Hepatocyte nuclear factor 4α (HNF-4α) is a positive regulator of human apoM gene expression. (A) HepG2 cells were infected with a control adenovirus expressing GFP (Ad-GFP) or with a recombinant adenovirus expressing HNF-4α (Ad-HNF-4) at a multiplicity of infection (M.O.I.) 10. 24h later, total RNA was extracted and the mRNA levels of apoM, HNF-4α and apoC-III genes were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM, HNF-4α and apoC-III genes were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were infected for 24h with Ad-GFP or Ad-HNF-4 at an M.O.I. of 10 and the intracellular protein levels of apoM, HNF-4α and actin (loading

control) as well as of the secreted apoM were determined by immunoblotting. The experiment was performed three times and representative images are presented. (C) HEK293T cells were transiently transfected with an expression vector for HNF-4α (4μg), PGC-1α (4μg) or both (4μg each) as indicated. 48h later, total RNA and protein was extracted and apoM, apoC-III or HNF-4 expression levels were analyzed by RT-PCR and immunoblotting, respectively. The normalized (relative to GAPDH) mRNA levels of the apoM and apoC-III genes are shown as a histograph. Each value represents the average from three independent experiments. Key: **p<0.01, ***p<0.001

Transactivation of the human apoM promoter by HNF-4 requires the proximal -49/-20 region

To investigate further the mechanism of apoM gene regulation by HNF-4, a 1kb genomic fragment bearing the human apoM promoter from nucleotide -950 to +42 was amplified by PCR using human genomic DNA as a template and cloned upstream of the luciferase reporter gene (Fig. 3A). Transactivation assays in HepG2 cells showed that HNF-4 transactivated the human -950/+42 apoM promoter in a dose dependent manner (Fig. 3B).

In order to identify the minimal regulatory region of the apoM promoter that is required for the HNF-4-mediated transactivation, a series of luciferase reporter plasmids containing consecutive 5΄ deletions of the apoM promoter were constructed (-642/+42, -402/+42, -241/+42, -105/+42, -49/+42 and -20/+8) as shown in Fig. 3A. The transcriptional activity of these truncated apoM promoters in the presence of HNF-4 was analyzed by transient transfection assays in human embryonic kidney HEK293T cells which do not express endogenously HNF-4 or other hepatocyte-specific nuclear factors. As shown in Fig. 3C, HNF-4 overexpression in HEK293T cells was associated with a 10 fold increase in the -950/+42 apoM promoter activity. Using the deletion mutants of the apoM promoter, it was shown that the proximal apoM promoter between nucleotides -49 to +42 was the shortest apoM promoter fragment that was responsive to HNF-4 overexpression (5.7 fold), because further deletion to nucleotide -20 completely abolished the HNF-4-mediated transactivation (Fig. 3C). In agreement with these findings, overexpression of the dominant negative form of HNF-4 in HepG2 cells was

associated with a reduction in the activity of all apoM promoter fragments tested (to 20-35% relative to the control) except of the -20/+8 apoM promoter, the activity of which remained unaffected (Fig. 3D).

Taken together, the findings of Fig. 3 indicated that the proximal apoM promoter region between nucleotides -49 and -20 is required for transactivation by the orphan nuclear receptor HNF-4 in hepatic cells.

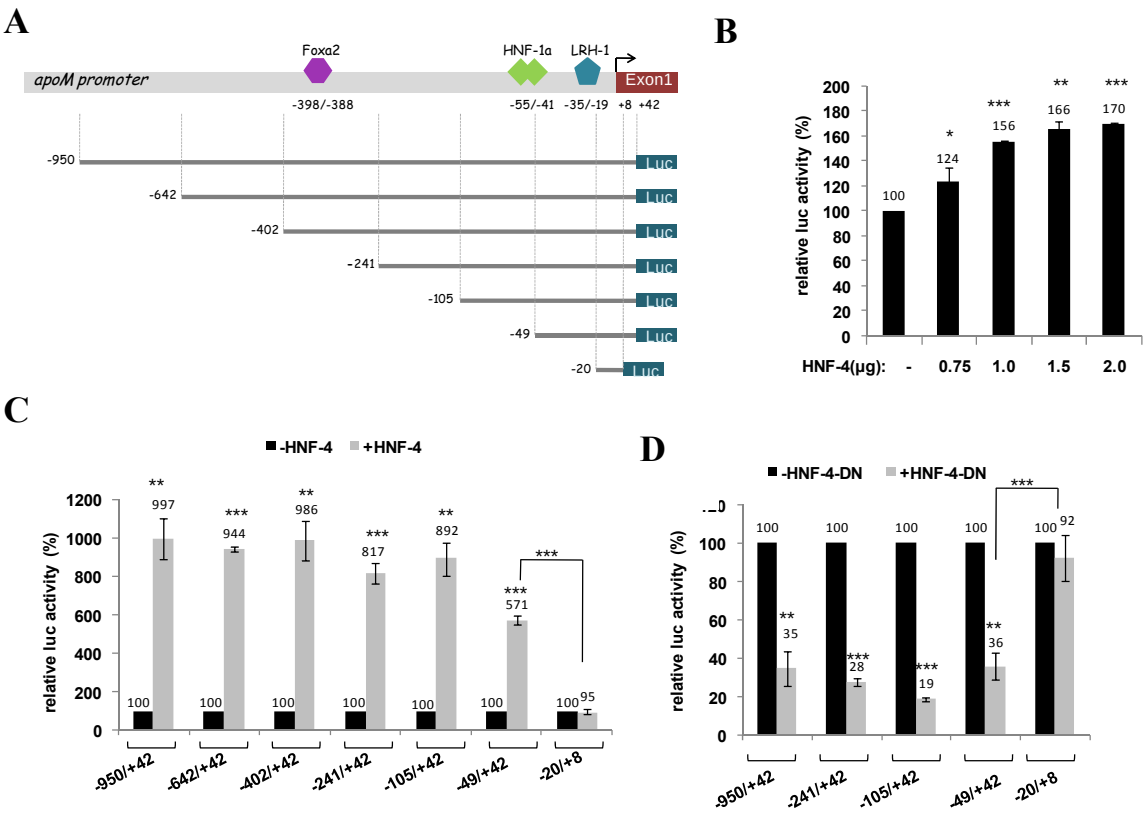


Figure 3. Transactivation of the human apoM promoter by HNF-4α requires the proximal -49/-20 region. (A) Schematic representation of the 5′deletion derivatives of the (-950/+42)hapoM promoter fragment that were cloned upstream of the luciferase reporter gene and used in the transactivation experiments of panels C and D. The position of previously described regulatory elements and factors is also shown. (B) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1μg) along with increasing concentrations (0.75, 1.0, 1.5 and 2.0μg) of an HNF-4α expression vector. An expression vector for β-galactosidase (1.0μg) was included in each sample for normalization purposes. (C) HEK293T cells were transiently transfected with the

luciferase reporter plasmids indicated at the bottom of the graph (1.0μg) along with an HNF-4α expression vector (1.0μg) and a β-galactosidase expression vector (1.0μg). (D) HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the bottom of the graph (1.0μg) along with the HNF-4α-DN (dominant negative) expression vector (1.0μg) and a β-galactosidase expression vector (1.0μg). In panels B-D, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

HNF-4 regulates apoM gene expression via a hormone response element located in the -33 to -21 region of the apoM promoter

Binding of HNF-4 to the human apoM promoter *in vivo* was established using chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig. 4B, an antibody against endogenous HNF4 could efficiently immunoprecipitate the proximal -241/+42 region of the apoM promoter but not the distal -950/-616 region of the apoM promoter. In control reactions, HNF-4 was found to be associated with the proximal promoter of the human apolipoprotein C-III gene (region -256/-45) but not with the unrelated proximal promoter of the small GTPase RhoB gene (region -313/-185) (540) confirming the specificity of the chromatin immunoprecipitation experiment.

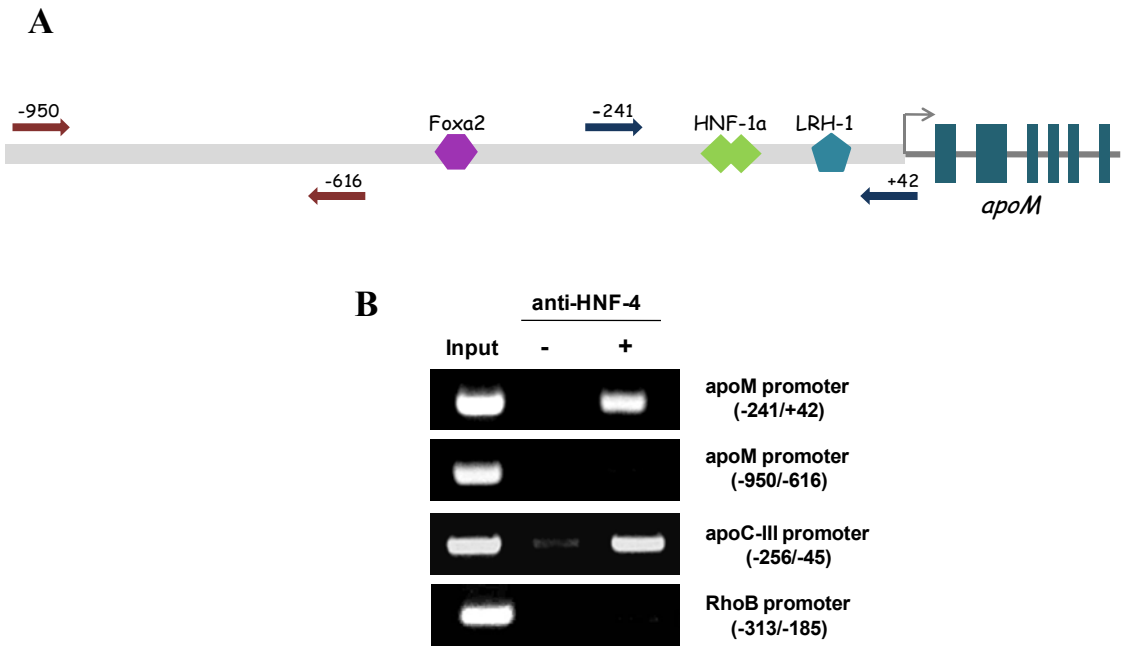


Figure 4. Chromatin immunoprecipitation assays establishing the recruitment if HNF-4α to the proximal region of the human apoM promoter in HepG2 cells. (A) Schematic representation of the human apoM promoter region, showing by arrows the location of the oligonucleotide primer sets (distal region: -950/-616, proximal region: -241/+42) that were utilized in the chromatin immunoprecipitation assays. (B) HepG2 cells were subjected to chromatin immunoprecipitation in the absence (second lane) or presence (third lane) of an anti-HNF-4 antibody using primers corresponding to the proximal or the distal region of the apoM promoter, the proximal region (-256/-45) of the apoC-III promoter harboring a DR1 HRE (positive control) or the proximal region (-313/-185) of the RhoB promoter (unrelated region, negative control). Non-immunoprecipitated

chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are presented.

Binding of HNF-4 to the proximal apoM promoter *in vitro* was established using the DNA-affinity precipitation (DNAP) assay. For this purpose, two biotinylated overlapping PCR fragments covering the proximal apoM promoter region between nucleotides -241 to +42 were generated (-241/-81 and -105/+42) (Fig. 5A). As shown in Fig. 5B, endogenous HNF-4 present in nuclear extracts from HepG2 cells bound to the -105/+42 biotinylated promoter fragment (lane 4) but not to the -241/-81 biotinylated promoter fragment (lane 3). In control experiments it was shown that HNF-4 did not bind to the streptavidin Dynabeads (Fig. 5B lane 2, no probe) or to a double-stranded biotinylated oligonucleotide corresponding to the -76/-43 region of the human RhoB promoter that contains a previously characterized CAAT box (Fig. 5B, lane 5) (540). In competition DNAP assays, it was shown that binding of HNF-4 to the -105/+42 biotinylated promoter fragment could be competed out by increasing amounts of an non-biotinylated PCR promoter fragment corresponding to the -49/+42 region of the apoM promoter (Fig. 5C). This finding, taken together with the transactivation data of Fig. 3, strongly suggested that the HNF-4 binding site is located in the region defined by nucleotides -49 and -20.

The sequence of the proximal human apoM promoter contains a putative DR1 (direct repeat with one nucleotide spacing) hormone response element (HRE) spanning nucleotides -33 to -21 (Fig. 5A). As DR-1 elements are preferred binding sites for HNF-4 (541), we investigated whether HNF-4 binds to the apoM promoter via this DR-1 HRE. For this purpose, DNAP experiments were performed using endogenous HNF-4 from HepG2 nuclear extracts and a double-stranded biotinylated oligonucleotide corresponding to the -40/-14 region of the apoM promoter that includes the putative HRE. As shown in Fig. 5D (top), HNF-4 bound efficiently to the -40/-14 biotinylated oligonucleotide probe (lane 3). In line with a previous study (123), the -40/-14 apoM biotinylated probe bound Liver Receptor Homologue 1 (LRH-1) (Fig. 5D bottom lane 3). LRH-1 recognizes a 5' CAAGG 3' motif present in the -29/-25 region of the apoM promoter and LRH-1 binding

as well as LRH-1 mediated transactivation were abolished by a C/T substitution at the first position of this motif (123). To characterize further this HRE, a biotinylated oligonucleotide probe was used bearing 3 nucleotide substitutions in the two half repeats and the flanking region. As shown in Fig. 5D (top, lane 4), these mutations completely abolished the binding of HNF-4 to this oligonucleotide whereas they had no effect on binding of LRH-1 (Fig. 5D bottom, lane 4).

To exclude the presence of additional HNF-4 binding sites in the proximal apoM promoter, the same HRE mutations were introduced into the -105/+42 apoM promoter fragment (-105/+42mut). As shown in Fig. 5E (top lane 4), no binding of HNF-4 to the mutated -105/+42 apoM promoter fragment was observed. As expected, the mutations had no effect on the binding of LRH-1 to this promoter fragment (Fig. 5E bottom lane 4).

In order to investigate the functional importance of the proximal HRE for the HNF-4-mediated transactivation of the apoM promoter, the same mutations in the HRE that abolished the binding of HNF-4 (Fig. 5) were introduced into the (-950/+42)hapoM luciferase reporter plasmid (Fig. 6A). As shown in Fig. 6B, mutagenesis of this HRE decreased the basal activity of the (-950/+42)apoM promoter in HepG2 cells to 12% of the control indicating the importance of this element for apoM gene regulation in hepatic cells. In contrast, the HRE mutations did not affect the basal activity of the apoM promoter in HEK293T cells that lack endogenous HNF-4 (Fig. 6C). Furthermore, the HRE mutations abolished the HNF-4 mediated transactivation of the apoM promoter in both HepG2 (Fig. 6B) and HEK293T (Fig. 6C) cells. Moreover, expression of increasing amounts of shRNA targeting HNF-4 in HepG2 cells dose-dependently reduced the activity of the wild type promoter to 43% of control while it did not affect the activity of the mutant promoter (Fig. 6D). In agreement with the findings of Fig. 5, the HRE mutations did not abolish the transactivation of the apoM promoter by LRH-1 in HEK293T cells (Fig. 7A).

The combined findings of Fig. 5, 6 and **7** indicated that HNF-4 directly regulates apoM gene expression via a novel HRE located in the -33 to -21 region.

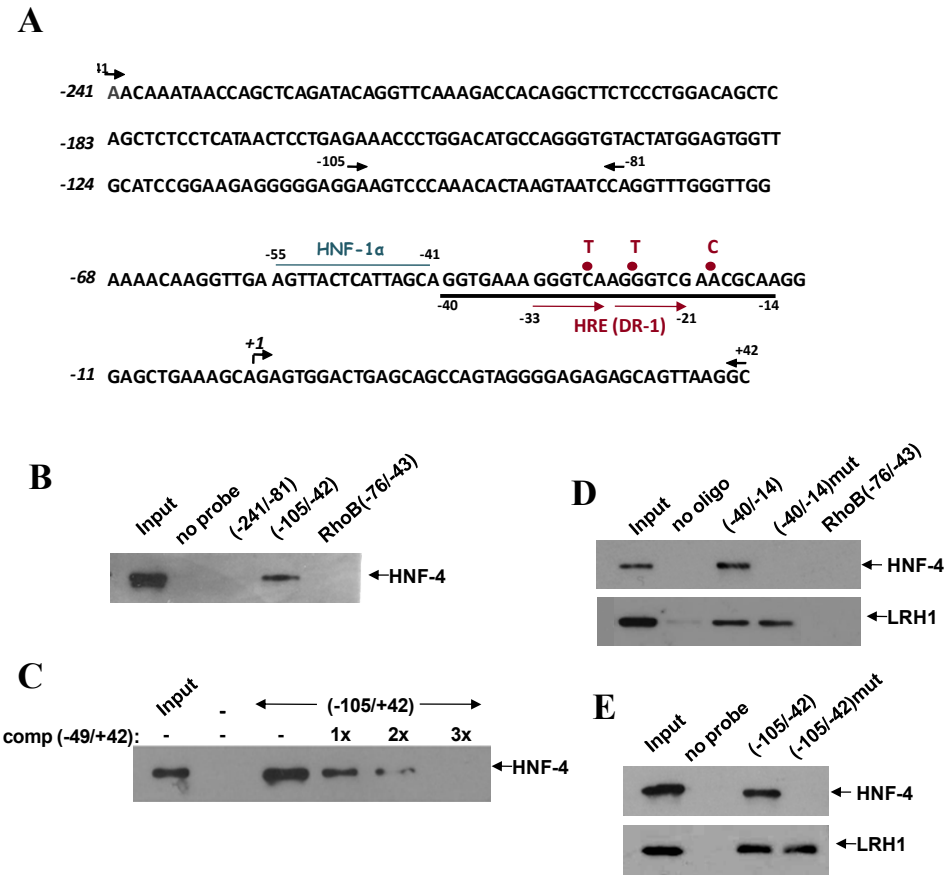


Figure 5. HNF-4α binds to a HRE located in the -33/-21 region of the proximal human apoM promoter. (A) Sequence of the proximal human apoM promoter region spanning nucleotides -241 to +42, showing the location of the previously characterized regulatory element that binds HNF-1α as well as the location of the DR1 HRE. The primer sets used for the amplification of the biotinylated promoter fragments -241/-81 and -105/+42 are indicated by arrows. Nucleotide substitutions in the two half repeats of the HRE of the apoM promoter are indicated with the black dots. (B) DNA-affinity precipitation using HepG2 nuclear extracts and biotinylated PCR fragments corresponding to the -241/-81 or the -105/+42 region of the human apoM promoter or no probe. A biotinylated oligonucleotide corresponding to the -76/-43 region of the human RhoB promoter that contains a CAAT box was used as a negative control. Oligonucleotide-bound HNF-4 was detected by Western blotting using a polyclonal anti-HNF-4 antibody. (C) DNA-affinity precipitation using HepG2 nuclear extracts and a biotinylated PCR fragment corresponding to the wild type -105/+42 region of the human apoM promoter in the presence of increasing amounts (1, 2 and 3 fold molar excess) of a competitor, non-biotinylated, PCR fragment corresponding to the -49/+42 region of the apoM promoter. Oligonucleotide-bound HNF-4 was detected by Western blotting using a polyclonal anti-HNF-4 antibody. (D) DNA-affinity precipitation experiments using HepG2 nuclear extracts and biotinylated oligonucleotides corresponding to the wild type -40/-14 region of the human apoM promoter, the -40/-14 region bearing mutations in the

HRE (mut), the -76/-43 region of the human RhoB promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-4 or LRH-1 was detected by Western blotting using a polyclonal anti-HNF-4 or anti-LRH-1 antibody respectively. (E) DNA-affinity precipitation using HepG2 nuclear extracts and biotinylated PCR fragments corresponding to the wild type or the mutated -105/+42 region of the human apoM promoter. Oligonucleotide-bound HNF-4 or LRH-1 were detected by Western blotting using polyclonal anti-HNF-4 or anti-LRH-1 antibodies. All experiments in Panels B-E were performed at least three times and representative images are presented.

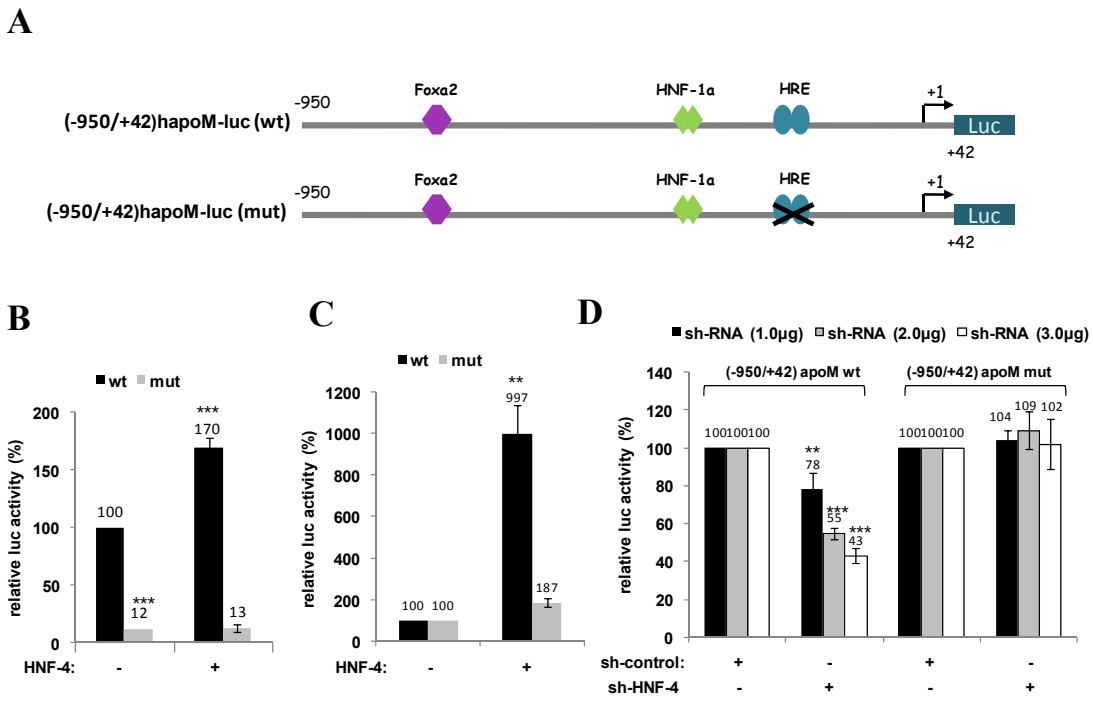


Figure 6. Mutations in the proximal HRE reduce basal apoM promoter activity and abolish HNF-4α-mediated transactivation. (A) Schematic representation of the wild type (-950/+42)hapoM-luc promoter construct and the corresponding construct bearing the mutations in the HRE that are shown in Fig. 4A [(-950/+42)hapoM-luc (mut)]. (B) HepG2 cells were transiently transfected with the wt or mutated (-950/+42)hapoM-luc reporter plasmid (1.0μg) along with the HNF-4α expression vector (1.0μg) and a β-galactosidase expression vector (1.0μg). (C) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the HNF-4α expression vector (1.0μg) and a β-galactosidase expression vector (1.0μg). (D) HepG2 cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with increasing concentrations of the sh-control or sh-HNF-4α producing vector (1.0μg, 2.0μg and 3.0μg) and a β-galactosidase expression vector (1.0μg). In Panels B-D, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average

(±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

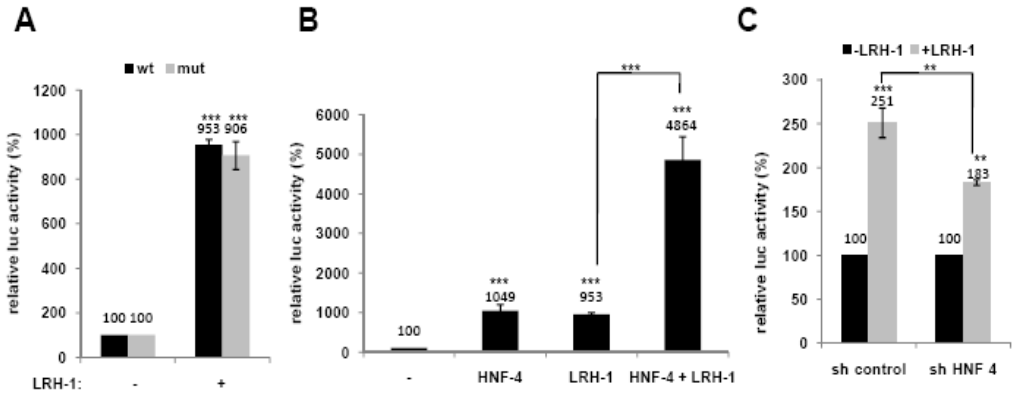


Figure 7. HNF-4 and LRH-1 synergistically transactivate the human apoM promoter. (A) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with an LRH-1 expression vector (1.0μg) and a β-galactosidase expression plasmid (1.0μg). Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. (B) HEK293T cells were transiently transfected with the wt (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the expression vectors for HNF-4α (1.0μg) or LRH-1 (1.0μg) or both and a β-galactosidase expression plasmid (1.0μg). (C) HepG2 cells were transiently transfected with the wt (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a sh-control or sh-HNF-4α producing vector (1.0μg) and a β-galactosidase expression vector (1.0μg). In Panels A-C, luciferase activity was normalized to β-galactosidase activity andpresented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

The novel HRE in the proximal apoM promoter binds ligand-dependent nuclear receptors

Previous studies have shown that hormone response elements present in the promoters of various apolipoprotein genes such as apoC-III, apoA-I, apoC-II, apoA-IV or apoA-II could be shared by many hormone nuclear receptors including HNF-4 and homodimers or heterodimers of Retinoid X Receptor alpha (RXRα) (241,542). Initially, the association of RXRα homodimers and its heterodimers with Retinoic Acid Receptor alpha (RARα), Peroxisome Proliferator Activated Receptor alpha (PPARα), Liver X Receptor alpha (LXRα), Thyroid Hormone Receptor beta (TRβ), Farnesoid X Receptor (FXR) as well as of the orphan nuclear receptor Chicken Ovalbumin Upstream Promoter Transcription Factor I (COUP-TFI or Ear-3) with the novel HRE in the proximal apoM promoter was examined by DNAP assays. As shown in Fig. 8A, RXRα homodimers (lane 1) and its heterodimers with RARα (lane 2), PPARα (lane 3), LXRα (lane 4) and TRβ1 (lane 5) bound to the wild type -40/-14 oligonucleotide. Binding of all heterodimers except RXRα/TRβ was abolished by mutations in the HRE (-40/-14 mut). No binding to the apoM HRE was detected by TRβ1 homodimers (lane 6), RXRα/FXRα heterodimers (lane 7) or Ear-3 (lane 8). Similar to HNF-4, RXRα homodimers and RXRα/RARα heterodimers bound to a larger apoM promoter fragment (-105/-42) but this binding was severely affected by the mutations in the HRE (Fig. 8B). In contrast, binding of RXRα/TRβ1 heterodimers to the above apoM promoter fragment was not affected by the HRE mutations (Fig. 8B) in agreement with the findings of Fig. 6A.

The recruitment of the RXRα homodimers and heterodimers to the apoM promoter *in vivo* was investigated by chromatin immunoprecipitation assays in HepG2 cells using antibodies against the corresponding nuclear receptors. These experiments (Fig. 8C) showed that nuclear receptors RXRα, RARα, PPARα, LXRα and TRβ are all recruited to the proximal apoM promoter region -241/+42 albeit with different affinities in agreement with the findings from the DNAP assays of Fig. 8A (strong binding of RXRα and RARα, less efficient binding of PPARα, LXRα and TRβ). The specificity of the above nuclear receptors for the apoM HRE was established using primers that

amplified the apoC-III and apoA-I promoters (positive controls) as well as the RhoB promoter (negative control).

Altogether, the findings of Fig. 8 indicated that in addition to HNF-4, homodimers of RXRα and heterodimers of RXRα with other ligand-dependenrt nuclear receptors can occupy the same HRE in the proximal apoM promoter with different affinities.

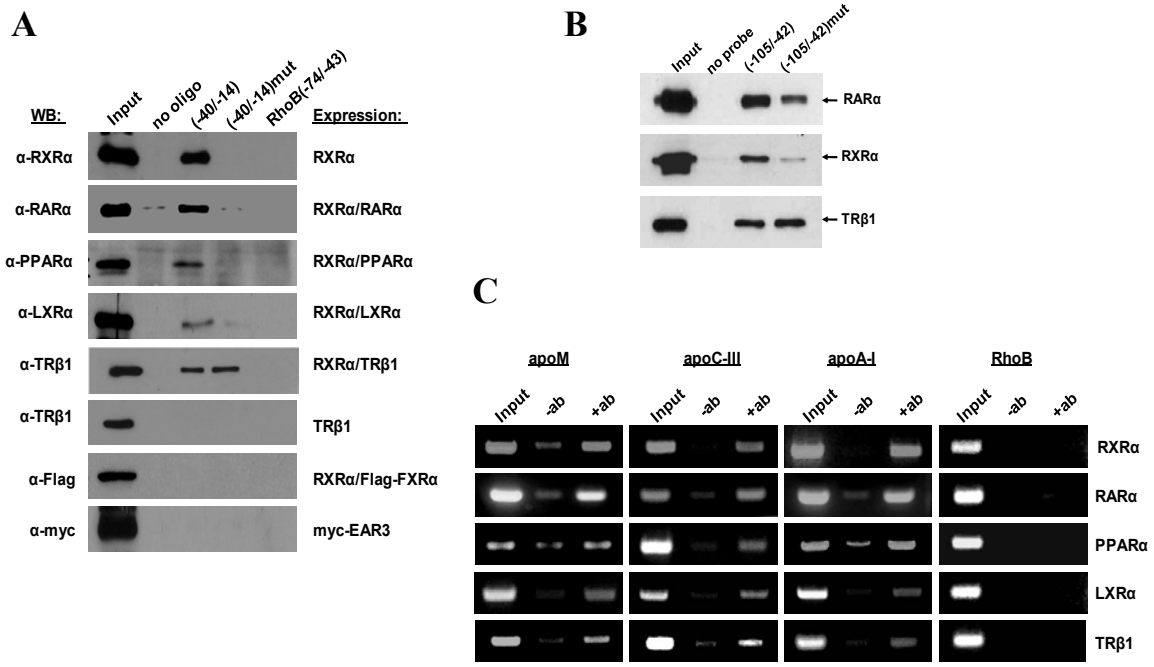


Figure 8. The proximal HRE in the apoM promoter binds ligand-dependent nuclear receptors (homo- and heterodimers of RXRα). (A) DNA-affinity precipitation experiments using nuclear extracts from HEK293Tcells transiently transfected with expression vectors for RXRα and its heterodimer partners or Ear-3 as indicated on the right and biotinylated oligonucleotides corresponding to the wild type -40/-14 region of the human apoM promoter, the mutated -40/-14 apoM promoter or the -76/-43 region of the human RhoB promoter. Oligonucleotide-bound nuclear receptors were detected by Western blotting using the corresponding antibodies shown on the left. All experiments were performed three times and representative images are shown. (B) HepG2 cells were subjected to chromatin immunoprecipitation in the absence (-ab) or the presence (+ab) of an anti-RXR, RAR, PPAR, LXR, or TRβ1 antibody as indicated on the right. Promoter occupancy was assessed by PCR amplification using primers corresponding to the proximal region of the apoM promoter (-241/+42), the proximal region of the apoC-III promoter (-256/-45) and of the apoA-I promoter (-315/+22) as positive controls or the

proximal region of the RhoB promoter (-313/-185) as negative control. Non immunoprecipitated chromatin was also included (input).

Regulation of apoM gene expression by 9-cis retinoic acid

In order to assess the functional relevance of the association of RXRα homo- and hetero-dimers with the novel HRE of the proximal apoM promoter, nuclear receptor signaling was stimulated in HepG2 cells by the administration of the corresponding ligands and their effect on apoM gene expression was evaluated using RT-PCR. As shown in Fig. 9A, treatment of HepG2 cells with 9-cis retinoic acid (9-cis RA) for different time periods (2-24h) resulted in a biphasic mode of apoM gene regulation with an early activation phase (at 2-4h) which was followed by a repression phase (at 6-24h). In order to investigate the mechanism behind this biphasic regulation of apoM gene transcription by 9-cis RA, we determined the mRNA levels of RXRα, HNF-4 and HNF-1, all of which have been shown previously to be regulated by RXR (297,298,308). HNF-4 and HNF-1 exhibited a similar response to retinoic acid treatment: their mRNA levels were increased at 2 to 6 h of treatment and returned to basal levels at 8-24h of treatment (Fig. 9A). In addition, a time-dependent down-regulation of the RXR mRNA levels was observed in the presence of 9-cis RA resulting in 70% repression relative to control at 8h of treatment (Fig. 9A). A strong reduction was also observed in the protein levels of RXRα in 9-cis RA-treated HepG2 cells (Fig. 9C). Surprisingly, when apoM protein levels were monitored, a significant increase was observed at 2h of 9-cis RA treatment that persisted for up to 24h (Fig. 9B) possibly due to a stabilizing effect of 9-cis RA on apoM protein given the fact that 9-cis RA is a ligand for apoM (89).

To investigate further the role of RXRα in the regulation of apoM gene expression in hepatic cells, a recombinant adenovirus expressing RXRα (Ad-RXR) was generated and used to infect HepG2 cells. As shown in Fig. 10A,B infection of HepG2 cells with Ad-RXRα in the presence of its ligand 9-cis RA led to a significant increase in both RXRα and apoM mRNA and protein levels (intracellular and secreted) compared to control Ad-GFP infected cells thus establishing RXRα as a positive regulator of apoM gene expression.

Transient overexpression of RXRα in HEK293T cells resulted in a strong transactivation of the wild type (-950/+42)apoM promoter in the presence of 9-cis RA (12.6 fold) whereas the mutations in the proximal HRE that abolished binding of RXRα to this element (Fig. 8A) reduced this transactivation to 3 fold (Fig. 10C).

Overexpression of RXRα along with its heterodimeric partner RARα in HEK293T cells was associated with a strong (24 fold) transactivation of the wild type (-950/+42)apoM promoter in the presence of 9-cis RA but not that of the promoter bearing mutations in the HRE (Fig. 10C and 13A and B). Using the apoM promoter deletion constructs, it was established that, similarly to HNF-4, deletion of the promoter region between nucleotides -49 and -20 abolished RXRα and RXRα/RARα-dependent transactivation of the apoM promoter (Fig. 10D and 13A and B).

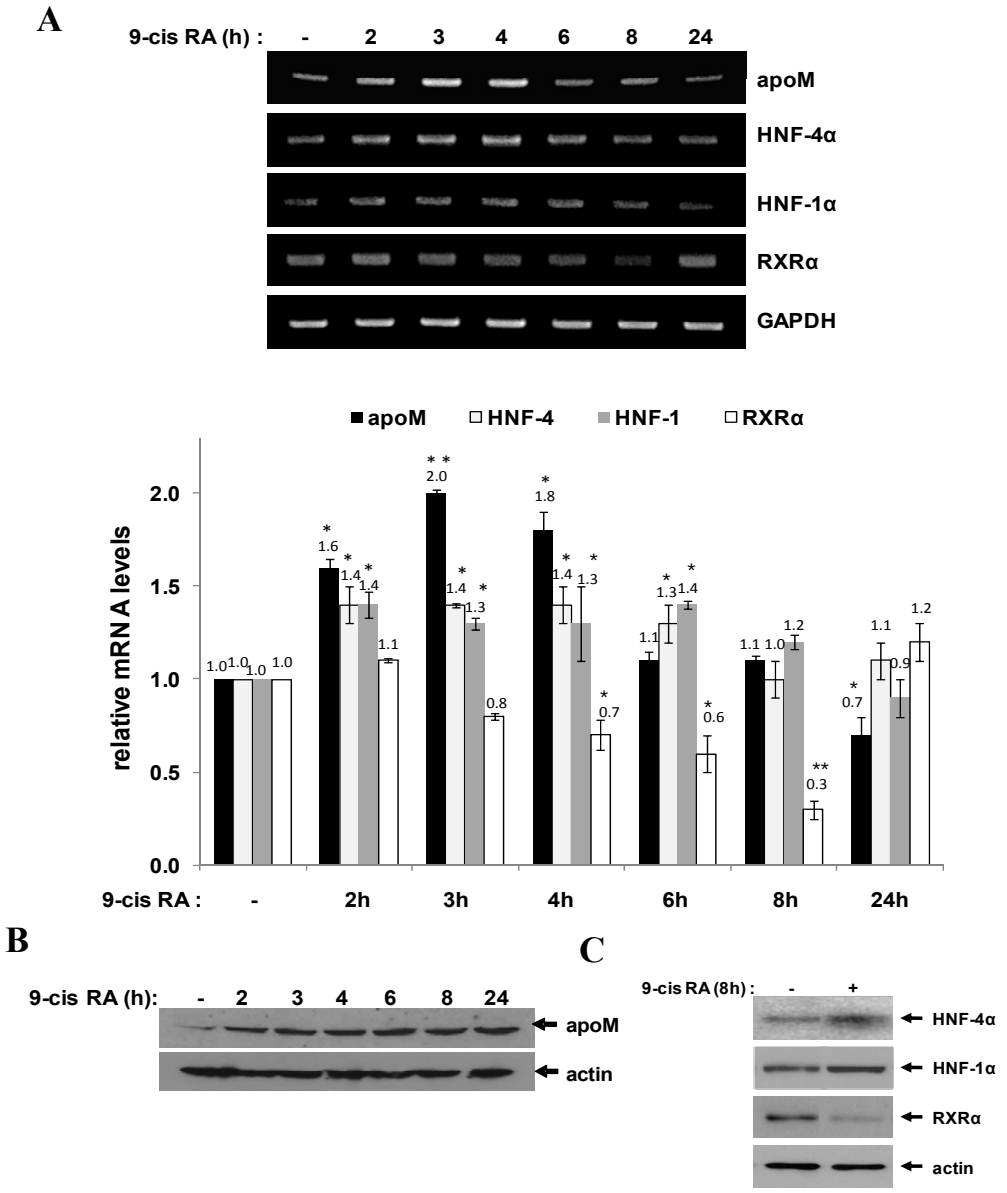


Figure 9. Regulation of apoM gene expression by 9-cis retinoic acid. (A) HepG2 cells were treated with 9-cis RA (1μM) for the indicated time periods or left untreated. Total RNA was extracted and apoM, HNF-4α, HNF-1α and RXRα mRNA levels were analyzed by RT-PCR, normalized relative to the mRNA levels of the GAPDH gene, quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were treated with 9-cis RA (1μM) for the indicated time periods or left untreated and the protein levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times and representative images are shown. (C) HepG2 cells were treated with 9-cis RA (1μM) for 8h (+) or left untreated (-) and the protein levels of HNF-4α, HNF-1α, RXRα and actin (loading control) were determined by

immunoblotting using the corresponding antibodies. The experiment was performed three times and representative images are shown.

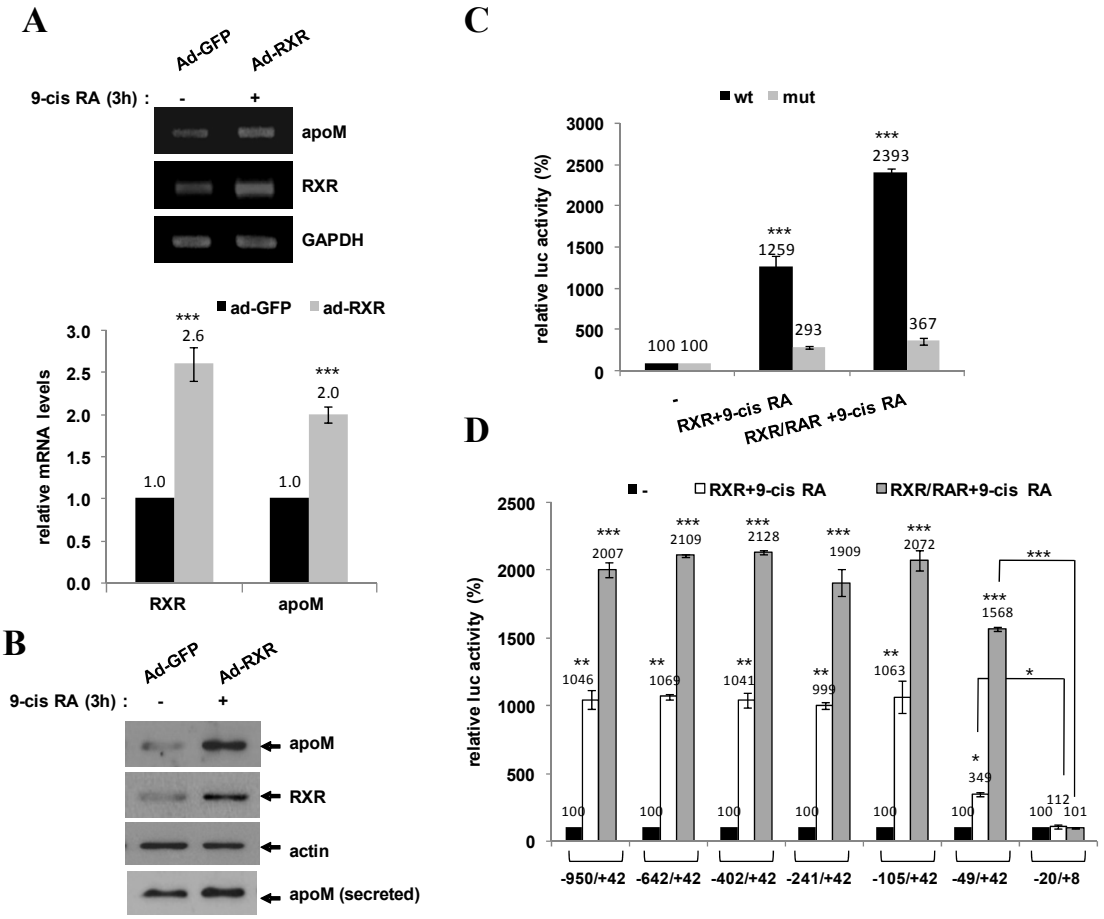


Figure 10: Induction of apoM gene transcription by retinoic acid receptors RXR and RAR. (A) HepG2 cells were infected with a control adenovirus expressing GFP (Ad-GFP) or with a recombinant adenovirus expressing RXRα (Ad-RXR) at an M.O.I. of 10 for 24h and treated with 9-cis RA (1μM) for 3h. Total RNA was extracted and the mRNA levels of apoM, RXRα and GAPDH genes were analyzed by RT-PCR. The normalized relative mRNA levels of the apoM and RXRα genes were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were infected for 24h with Ad-GFP or Ad-RXR at an M.O.I. of 10, treated with 9-cis RA (1μM) for 3h and the protein levels of intracellular apoM, RXR and actin (loading control) and of the secreted apoM were determined by immunoblotting. The experiment was performed three times and representative images are shown. (C)

HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with expression vectors for RXRα or both RXRα and RARα (1.0μg each). The β-galactosidase expression plasmid (1.0μg) was included in each sample for normalization of transfection variability. Following transfection, cells were treated with 9-cis RA (1μM) for 24h. Luciferase activity was normalized to β-galactosidase activity and presented with a histograph. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. (D) HEK293T cells were transiently transfected with the indicated apoM promoter deletion plasmids (1.0μg) along with expression vectors for RXRα (1.0μg) or both RXRα and RARα (1.0μg each) along with a β-galactosidase expressing plasmid (1.0μg). Following transfection, cells were treated with 9-cis RA (1μM) for 24h. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

Induction of apoM gene expression by fibrates, oxysterols and T3

To assess the functional contribution of the other RXRα heterodimers (RXRα/PPARα, RXRα/TRβ, RXRα/LXRα) in the regulation of the apoM gene, HepG2 cells were treated with the corresponding ligands for 3h and 24h and the expression levels of apoM were monitored by RT-PCR assays and immunoblotting. As shown in Fig. 11A, treatment of HepG2 cells with fibrates, 22(OH)C and T3 (ligands of PPARα, LXRα and TRβ) caused a 5.1-, 5.5- and 3.1-fold increase in apoM mRNA levels respectively compared to untreated cells. ApoM protein levels were also significantly increased after treatment of cells with the ligands for 3h (Fig. 11B). Induction of apoM mRNA and protein levels by ligands persisted for 24h (Fig. 11C, D). Significant increase in the protein levels of the secreted apoM was also observed in the media of HepG2 cells that had been treated with 9-cis RA, fibrates and 22(OH)C for 24h (Fig. 11E).

Overexpression of RXRα along with PPARα, LXRα, or TRβ1 in HEK293T cells that had been treated with the corresponding ligands was associated with a strong transactivation of the wild type (-950/+42)apoM promoter (28.4 fold , 46.5 fold and 38.9 fold respectively) while no significant effect was observed on the activity of the mutated apoM promoter (Fig. 12A). Strong transactivation by the RXR homo and heterodimers as well as by HNF-4 and LRH-1 was observed using the mouse apoM promoter (-761/-7) which contains the same HRE that is 100% homologous with the human (Fig. 14A,B). No transactivation could be observed by RXRα/FXRα heterodimers in the presence of chenodeoxycholic acid (CDCA) in accordance with the DNA binding data of Fig, 8A (data not shown). Finally, deletion analysis of the apoM promoter showed that the RXR/PPAR, RXR/LXR and RXR/TRβ heterodimers in the presence of their ligands transactivated strongly all apoM promoter fragments tested except the (-20/+8) reporter plasmid that lacks the proximal HRE (Fig. 12B) while they didn't have any effect on the residual apoM promoter activity upon insertion of the mutation in the apoM promoter deletion mutants up to nucleotide -105 (Fig. 13 C-E), establishing the contribution of the proximal HRE in the stimulation of apoM gene expression by fibrates, oxysterols and T3.

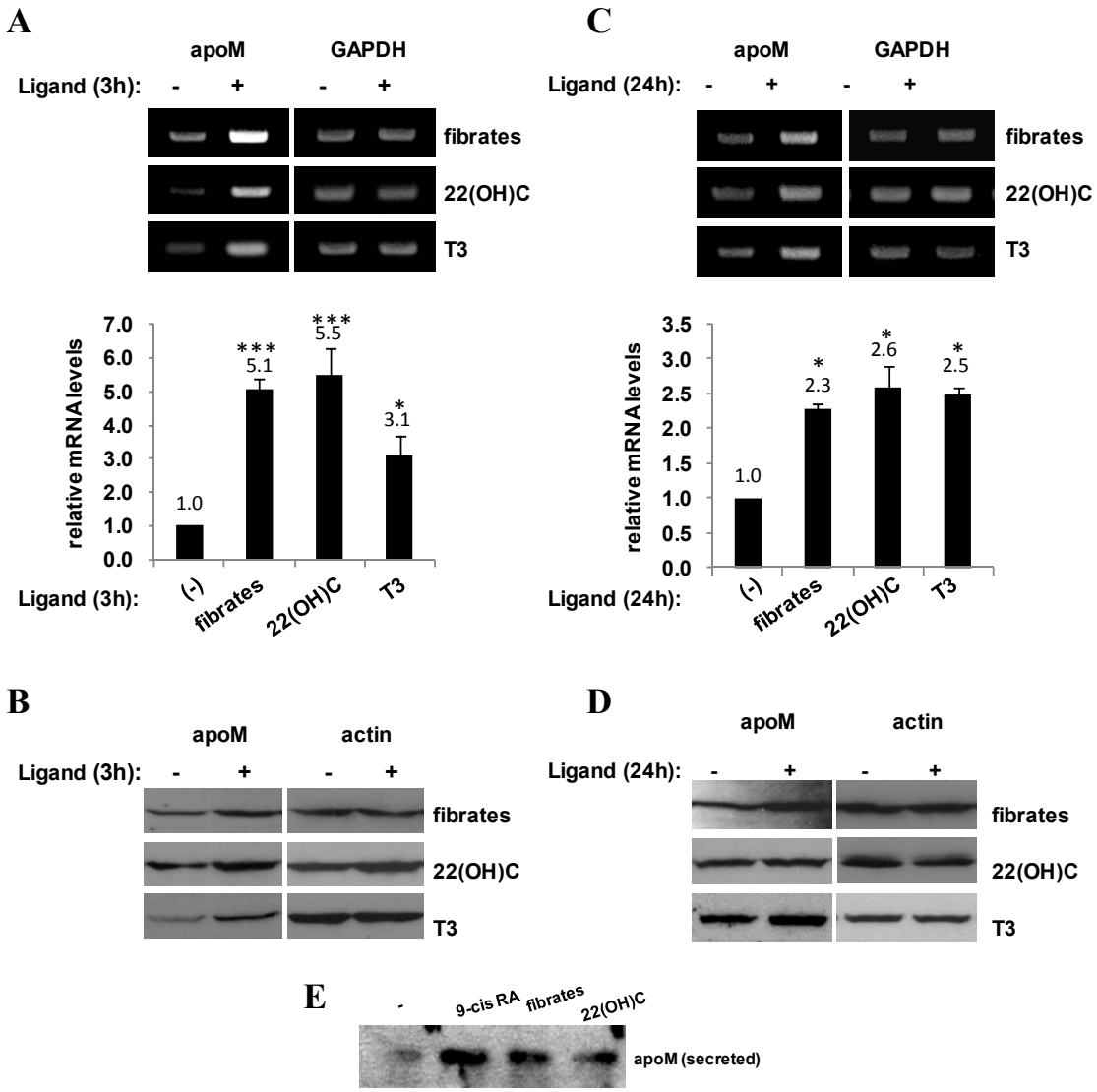


Figure 11. Induction of apoM gene expression by fibrates, oxysterols and T3. (A,C) HepG2 cells were treated with fibrates (250μM), 22(OH)C (1μM) or T3 (1μM) for 3h (A) or 24h (C). Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The normalized mRNA levels of the apoM gene were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B,D) HepG2 cells were treated with fibrates (250μM), 22(OH)C (1μM) or T3 (1μM) for 3h (B) or 24h (D) and the expression levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. Each experiment was performed three times and representative images are presented. (E) HepG2 cells were treated with 9-cis RA (1μM), fenofibrates (250 μM) or 22(OH)C (1 μM) for 24h (+) or left untreated (-). Equal amounts of culture medium was subjected to SDS-PAGE and immunoblotting using an anti-apoM polyclonal antbody. The experiment was performed three times and a representative image is presented.

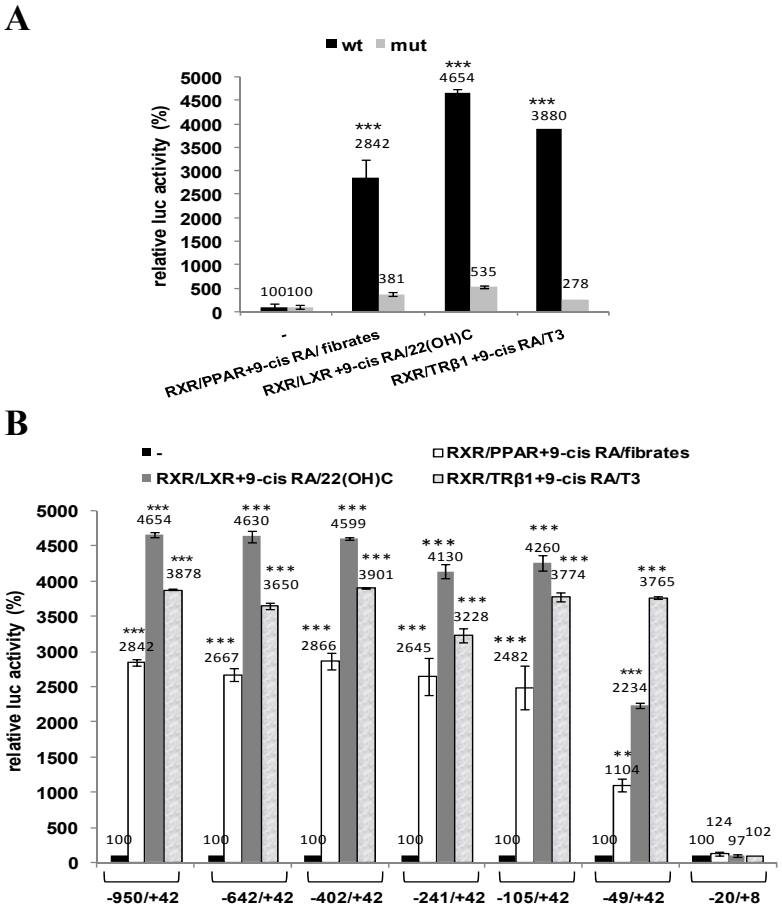


Figure 12. Induction of apoM promoter activity by fibrates, oxysterols and T3. (A) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with expression vectors for RXRα, PPARα, LXRα and TRβ1 (1.0μg each) at the combinations indicated and a β-galactosidase expressing plasmid (1.0μg). Following transfection, cells were treated with the appropriate ligands for 24h [9-cis RA (1μM), fibrates (100μM), 22(OH)C (1μM) or T3 (1μM)]. Luciferase activity was normalized to β-galactosidase activity and presented with a histograph. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. (B) HEK293T cells were transiently transfected with the indicated apoM promoter luciferase reporter plasmids (1.0μg) along with expression vector for RXRα, PPARα, LXRα or TRβ1 (1.0μg each) at the combinations indicated along with a β-galactosidase expressing plasmid (1.0μg). Following transfection, cells were treated with the appropriate ligands as in (A) for 24h. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

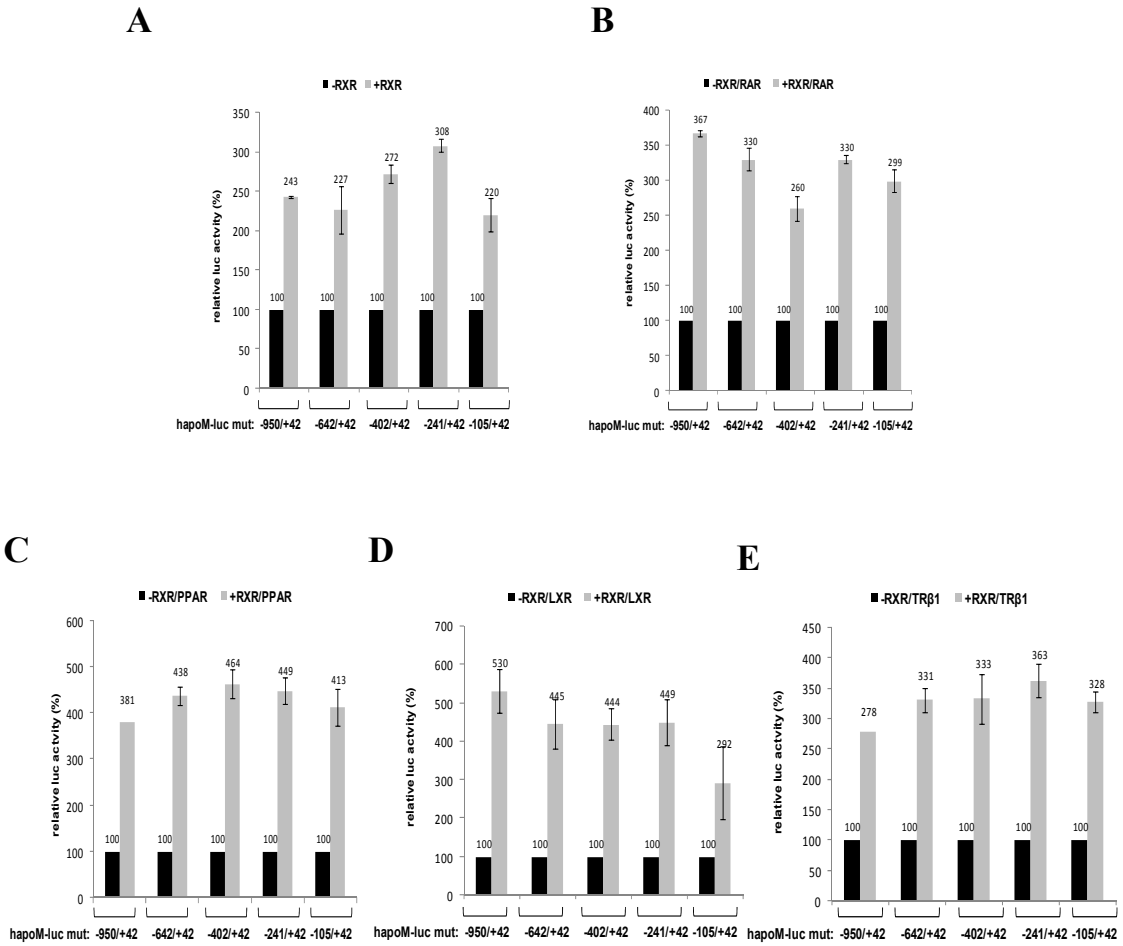


Figure 13. Retinoids, fibrates, oxysterols and T3 do not have additional effects on apoM promoter activity upon mutagenesis of the HRE. HEK293T cells were transiently transfected with the indicated mutated apoM promoter luciferase reporter plasmids (1.0μg) along with expression vectors for RXRα (A), RARα (B), PPARα (C), LXRα (D) and TRβ1 (E) (1.0μg each) at the combinations indicated and a β-galactosidase expressing plasmid (1.0μg). Following transfection, cells were treated with the appropriate ligands for 24h [9-cis RA (1μM), fibrates (100μM), 22(OH)C (1μM) or T3 (1μM)]. Luciferase activity was normalized to β-galactosidase activity and presented with a histograph. Each value represents the average (±SD) from at least three independent experiments performed in duplicate.

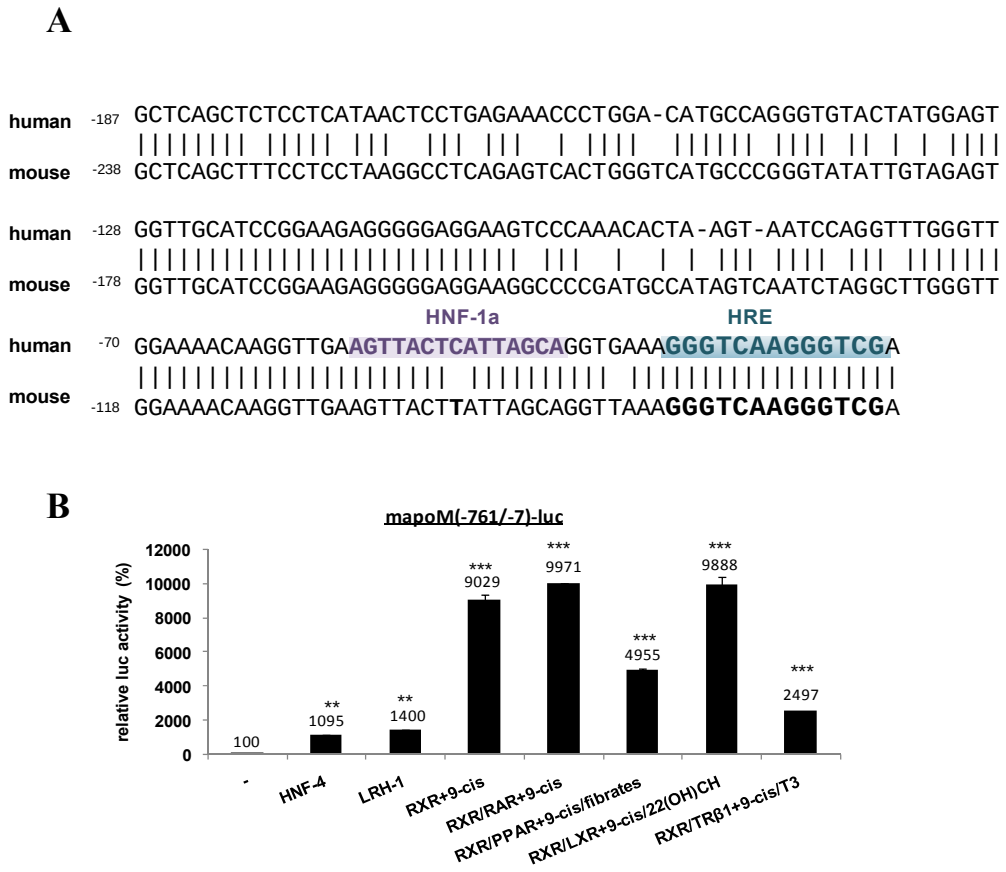
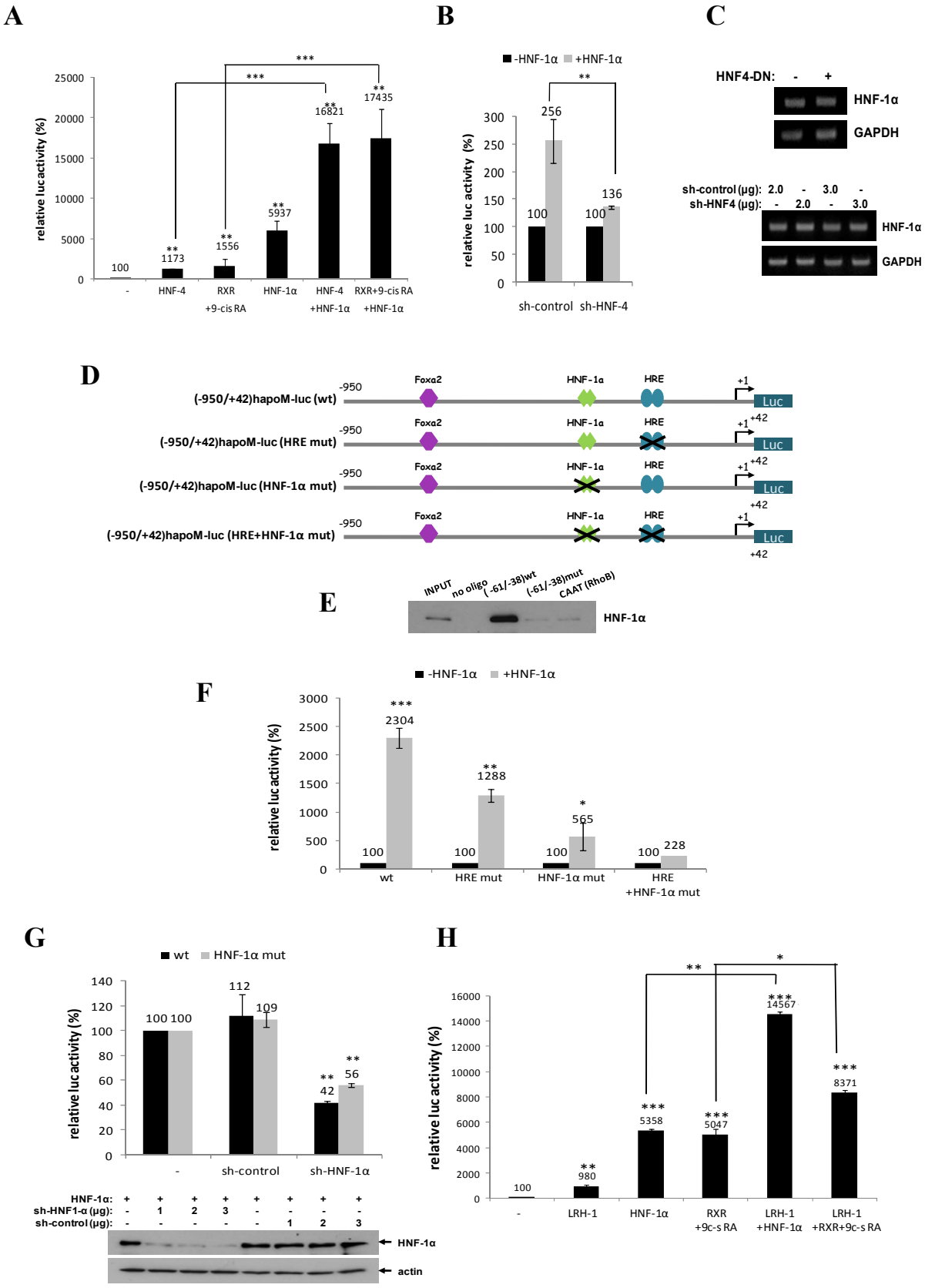


Figure 14. HNF-4 and RXR homodimers and heterodimers activate mouse apoM promoter activity. Alignment of the human and mouse apoM proximal promoter regions. Vertical lines indicate residues that are identical and purple and blue boxes the HNF-1α response element and the HRE respectively. The sequence identity between human and mouse apoM is 100% in the HRE and approximately 93% in the HNF-1α site. (B) HEK293T cells were transiently transfected with the (-761/-7)mouse apoM-luc reporter plasmid (1.0μg) along with expression vectors for HNF-4α, LRH-1, RXRα, RARα, PPARα, LXRα or TRβ1 (1.0μg each) and treated with the corresponding ligands as indicated. Each sample included a β-galactosidase expression plasmid (1.0μg) used for normalization of transfection variability. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

Synergism between nuclear receptors and HNF-1-α

Previous studies have shown that the liver-enriched transcription factors are part of a complex transcriptional network established by a number of autoregulatory and cross-regulatory pathways securing their balanced, high-level expression, and combinatorial protein interactions between hepatic factors for the achievement of transcriptional stimulation of most liver-specific genes including genes involved in lipoprotein metabolism (543-545). Based on the protein-DNA interactions of Fig 5 and 8 that identified the HRE at the proximal apoM promoter as the region that is responsible for nuclear receptor recruitment as well as on the transactivation data of Fig.3, 10D and 12B, potential synergism between the nuclear receptors and the transcription factor HNF-1α was investigated. Co-expression of HNF-4α and HNF-1α as well as RXRα and HNF-1α in HEK293T cells resulted in a synergistic transactivation of the apoM promoter (2.6 fold and 2.3 fold stronger than the sum of the transactivations obtained by individuals factors) (Fig. 15A) while silencing of HNF-4 gene expression by shRNA in HepG2 cells inhibited the HNF-1α -mediated transactivation of the apoM promoter by approximately 50% (Fig. 15B). In agreement with the known role of HNF-4 in the transcriptional regulation of HNF-1α during the early stages of hepatocyte differentiation (HNF-1α is absent in HNF-4 null fetal hepatocytes but unaffected in HNF-4 liver knock out adult hepatocytes), HNF-1α mRNA levels remained unaffected in HepG2 cells overexpessing the dominant negative mutant form of HNF-4 or a shRNA against HNF-4 (Fig. 15C). Mutagenesis of the proximal HRE, inhibited the HNF-1α mediated transactivation of the apoM promoter by 50% (Fig.15F) suggesting functional interactions between the two factors. Moreover, mutations in the HNF-1a binding site did not abolish the repression exerted by shRNA against HNF-1α on the activity of the apoM promoter (Fig.15G) nor HNF-1a-mediated apoM promoter activation, although the latter was severely affected and nearly abolished upon mutagenesis of both the HNF-1a responsive element and the HRE. Besides HNF-1, LRH-1 transactivated the apoM promoter synergistically with HNF-1 while the effects of RXR with LRH-1 on apoM promoter activity were rather additive (Fig.15H).


A
relative luc activity (%)
100
1173
1556
5937
16821
17435
-
HNF-4
RXR +9-cis RA
HNF-1α
HNF-4 +HNF-1α
RXR+9-cis RA +HNF-1α
B
-HNF-1α
+HNF-1α
relative luc activity (%)
100
256
100
136
sh-control
sh-HNF-4
C
HNF4-DN: - +
HNF-1α
GAPDH
sh-control (μg): 2.0 - 3.0 -
sh-HNF4 (μg): - 2.0 - 3.0
HNF-1α
GAPDH
D
(-950/+42)hapoM-luc (wt)
(-950/+42)hapoM-luc (HRE mut)
(-950/+42)hapoM-luc (HNF-1α mut)
(-950/+42)hapoM-luc (HRE+HNF-1α mut)
-950
Foxa2
HNF-1α
HRE
+1
+42
Luc
E
INPUT
no oligo
(-61/-38)wt
(-61/-38)mut
CAAT (RhoB)
HNF-1α
F
-HNF-1α
+HNF-1α
relative luc activity (%)
100
2304
1288
565
228
wt
HRE mut
HNF-1α mut
HRE +HNF-1α mut
G
wt
HNF-1α mut
relative luc activity (%)
100 100
112
109
42
56
-
sh-control
sh-HNF-1α
HNF-1α: + + + + + + + +
sh-HNF1-α (μg): - 1 2 3 - - - -
sh-control (μg): - - - - - 1 2 3
HNF-1α
actin
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relative luc activity (%)
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5047
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RXR +9c-s RA
LRH-1 +HNF-1α
LRH-1 +RXR+9c-s RA

Figure 15. Nuclear receptors and HNF-1α synergistically transactivate the human apoM promoter. (A) HEK293T cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression plasmid (1.0μg) and expression vectors for HNF-4α, RXRα, or HNF-1α (1.0μg). Cells transfected with RXRα were treated with 9-cis RA for 24h. (B) HepG2 cells were transiently transfected with the wt (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a sh-control or sh-HNF-4α producing vector (1.0μg), a β-galactosidase expression vector (1.0μg) and an expression vector for HNF-1α (1.0μg) as indicated. (C) HepG2 cells were transiently transfected with an expression vector for HNF-4α DN (10μg) (top) or increasing amounts (2.0 and 3.0μg) of sh-HNF-4α or sh-control producing vectors (bottom). 48h later, total RNA was extracted and HNF-1α mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (D) Schematic representation of the wild type (-950/+42)hapoM-luc promoter construct and the corresponding constructs bearing mutations in the HRE, the HNF-1α binding site or both (up). (E) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1α and biotinylated oligonucleotides corresponding to the wild type -61/-38 region of the human apoM promoter, the -61/-38 region bearing mutations in the HNF-1α site (mut), the -76/-43 region of the human RhoB promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α was detected by Western blotting using a polyclonal anti-HNF-1α antibody (middle). (F) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmids (1.0μg), along with the HNF-1α expression vector (1.0μg) and a β-galactosidase expression vector (1.0μg). (G) HepG2 cells were transiently transfected with the wild type or HNF-1α mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression plasmid (1.0μg) and the sh-control or sh-HNF-1α producing vectors (1.0μg) (top). HEK293T cells were transiently transfected with an HNF-1α expression vector along with increasing concentrations of the sh-HNF-1α or sh-control producing vector (0, 1.0, 2.0 and 3.0μg) and the protein levels of HNF-1α and actin (loading control) were determined by immunoblotting using the corresponding antibodies (bottom). (H) HEK293T cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression plasmid (1.0μg) and expression vectors for LRH-1, HNF-1α or RXRα (1.0μg), or both LRH-1 and HNF-1α or LRH-1 and RXRα (1.0μg each) as indicated. Following transfection, cells transfected with RXRα were treated with 9-cis RA for 24h. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at leastthree independent experiments performed in duplicate. Key: *$p<0.05$, **$p<0.01$, ***$p<0.001$

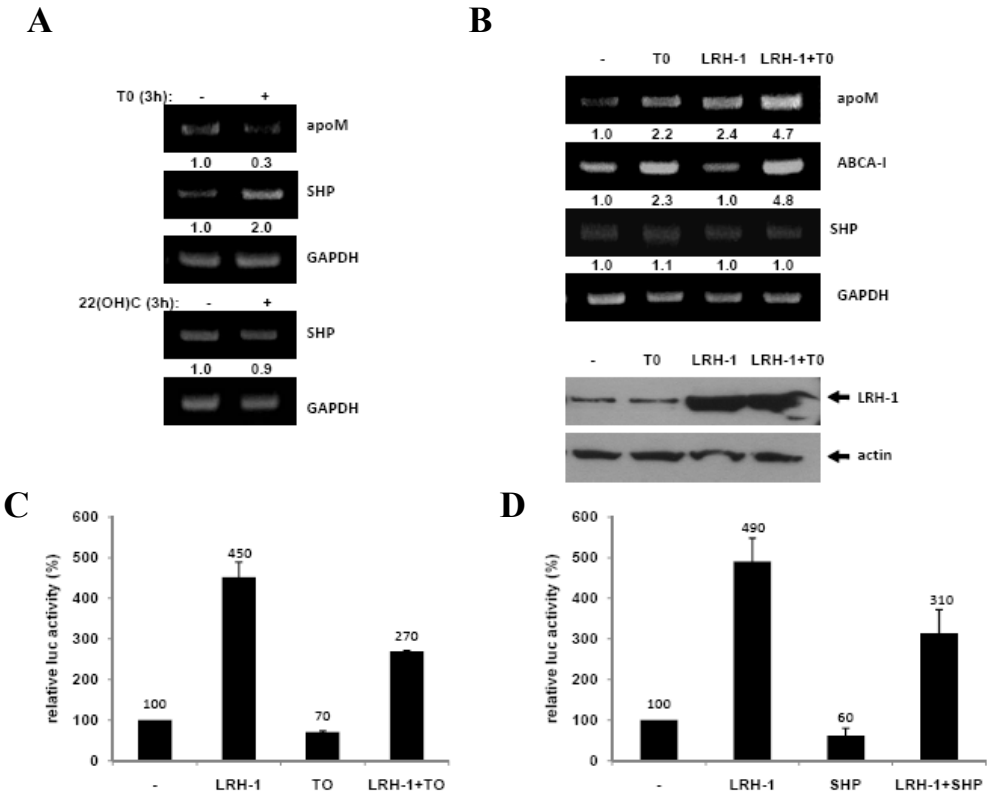

Figure 16. Effect of LXR ligands on the expression of apoM gene in hepatic and intestinal cells (see discussion). (A) HepG2 cells were treated with LXR ligands T0901317 or 22(OH)C (1μM) for 3h or with DMSO (vehicle). Total RNA was extracted and apoM, SHP and GAPDH mRNA levels were analyzed by RT-PCR. Normalized values (relative to GAPDH) represent the average from three independent experiments. (B) Caco-2 cells that had been transiently transfected with an LRH-1 expressing vector or a control vector (-) were treated with 10901317 (1μM) for 24 h or DMSO. Total RNA was extracted and apoM, ABCA1, SHP and GAPDH mRNA levels were analyzed by RT-PCR. Normalized values (relative to GAPDH) represent the average from three independent experiments. Expression of exogenous LRH-1 in transfected Caco-2 cells was monitored by immunoblotting using an anti-LRH-1 antibody. The experiment was performed three times and representative images are presented. (C) HepG2 cells were transiently transfected with the mutated (-105/+42)human apoM-luc reporter plasmid (1.0μg) along with an expression vector for LRH-1 (1.0 μg) and were either treated with the LXR ligand 10901317 (1μM) for 24h or DMSO. The β-galactosidase expression vector (1.0μg) was included for normalization purposes. (D) HepG2 cells were transiently transfected with the mutated (-105/+42)human apoM-luc reporter plasmid (1.0μg) along with expression vectors for LRH-1 and/or SHP (1.0 μg). The β-galactosidase expression vector (1.0μg) was included for normalization purposes. In Panels C and D, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each

value represents the average (±SD) from at least three independent experiments performed in duplicate.

Discussion

The impact of apoM on HDL metabolism has been studied mainly in mouse models. These studies indicated that apoM might affect the quality rather than the quantity of HDL and that apoM overexpression reduces the development of atherosclerosis in an atherosclerosis-prone setting (85,97) suggesting that exploring new ways of apoM gene upregulation may provide new therapeutic tools for the treatment of CAD.

In the present study we show that the apolipoprotein M gene is under the control of hormone nuclear receptors that have been shown to play pivotal roles in lipid and glucose metabolism such as HNF-4 and the receptors for retinoic acid, thyroid hormone, fibrates and oxysterols (241,542,546). Among these nuclear receptors, HNF-4 seems to play a major role in the regulation apoM gene expression in the liver. This is in agreement with a previous cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells which showed a 4.5 fold increase in apoM expression levels by overexpressed HNF-4 (536). HNF-1 knock out mice are characterized by the complete absence of apoM in plasma and abnormal HDL profiles (97). Previous studies had shown that HNF-1 and HNF-4 cross-regulate each other and that the two factors participate in a transcriptional network operating in hepatic cells that control the transcription of many liver-specific genes including genes involved in lipoprotein metabolism (543-545). The importance of HNF-4 and HNF-1 in lipid homeostasis is also supported by a recent genome wide association study which showed a statistical significant association between polymorphisms in the two genes and abnormalities in the HDL and LDL levels (290).

A second key finding of the present study is that apoM gene expression is subject to regulation by oxysterols which are the natural endogenous ligands of the LXRs. Previous studies had provided contradictory results regarding the effect of LXR ligands on apoM gene regulation. In one study, Zhang et al (165) showed that oral administration of the synthetic LXR agonist T0901317 in mice was associated with a reduction in basal apoM mRNA levels in the liver. In another study, Calayir et al (166) showed that T00901317 downregulated apoM gene expression in mouse liver but it upregulated apoM gene expression in mouse intestinal cells. Similar upregulation was observed when the

natural LXR ligand 22(OH)C was used (166). These contradictory findings should be evaluated in light of a previous study showing that T0901317 is a dual LXR/FXR agonist that activates FXR more efficiently than its natural ligand, the bile acid CDCA (chenodeoycholic acid) (547). Furthermore, Ventenclef et al (123) showed recently that bile acids suppress apoM gene expression in hepatic cells via an LRH-1 (Liver Receptor Homologue-1) binding site in the proximal apoM promoter and that this repression required SHP. SHP (Short Heterodimer Partner) is an inhibitory nuclear receptor activated by FXRs that interacts physically with many nuclear receptors including LRH-1 and interferes with their transactivation potential (548). The LRH-1 site reported by Venteclef et al (123) is located inside the proximal apoM HRE that was characterized in the present study. Interestingly, the C/T substitution in the first base of the LRH-1 binding site that was shown by Venteclef et al to abolish binding of LRH-1 and to inhibit the LRH-1 mediated transactivation of the apoM promoter (123) was not equally effective in our study. This controversy could possibly be accounted for by the two additional substitutions that we introduced to the apoM HRE which possibly restored LRH-1 binding to this element and LRH-1 mediated transactivation as shown in Fig. 5D and E and Fig. 7A.

Based on the above findings, we are tempted to speculate that the negative effect of T0901317 on apoM gene expression in liver cells reported previously (165,166) could be due to the activation of the FXR/SHP pathway that inhibits LRH-1 in hepatic cells. In agreement with this hypothesis we found that treatment of HepG2 cells with 1 μM T0901317 for 3h caused a 2 fold increase in SHP mRNA levels. In contrast, the SHP mRNA levels were not affected by 22(OH)C (Fig. 16A). Furthermore, T0901317 treatment or SHP overexpression inhibited the LRH-1-mediated transactivation of the -105/+42 apoM promoter bearing a mutation in the LXRE in HepG2 cells suggesting that in hepatic cells T0901317 suppresses apoM gene transcription via an SHP/LRH-1 pathway (Fig. 16C,D). In contrast to HepG2 cells, T0901317 failed to inhibit apoM gene transcription in intestinal Caco-2 cells where LRH-1 is expressed at low levels or in Caco-2 cells overexpressing LRH-1 suggesting that the T0901317/SHP pathway is not operating in these cells. As a result, T0901317 induced apoM gene expression in Caco-2

cells (Fig. 16B). Although the proximal apoM HRE resembles a DR-1 element (direct repeat with 1 nucleotide spacing) which is not favored by LXRα/RXRα heterodimers, a recent study showed that binding of LXR/RXR to such HREs is not prohibited (549).

Fig. 17 summarizes our current understanding of the regulation of apoM gene expression in the liver. Although it is possible that other key players are missing from this picture, strong evidence indicates that liver apoM gene expression is controlled by the interplay between liver-enriched factors such as HNF-1, HNF-4, LRH-1 and FOXA2/HNF-3β with ligand-dependent nuclear receptors such as homo and heterodimers of RXR. Thus, apoM is a novel target for ligands shown previously to have a beneficial effect on HDL levels (oxysterols, fibrates). Synergistic interactions between the above factors could be required for optimal apoM gene expression. In support of this hypothesis, we showed that HNF-4 transactivates the apoM promoter synergistically with LRH-1 (Fig. 7B,C) and HNF-1 (Fig.15).

HDL is an important atheroprotective molecule and many different HDL-based therapies of diabetes, obesity, metabolic syndrome coronary artery disease are currently under development. Having established the importance of apoM for HDL physiology and cholesterol homeostasis, it is anticipated that the detailed understanding of how this gene is regulated in health and disease will provide novel therapeutic and diagnostic tools for the above diseases that affect a large proportion of the population in Western countries.

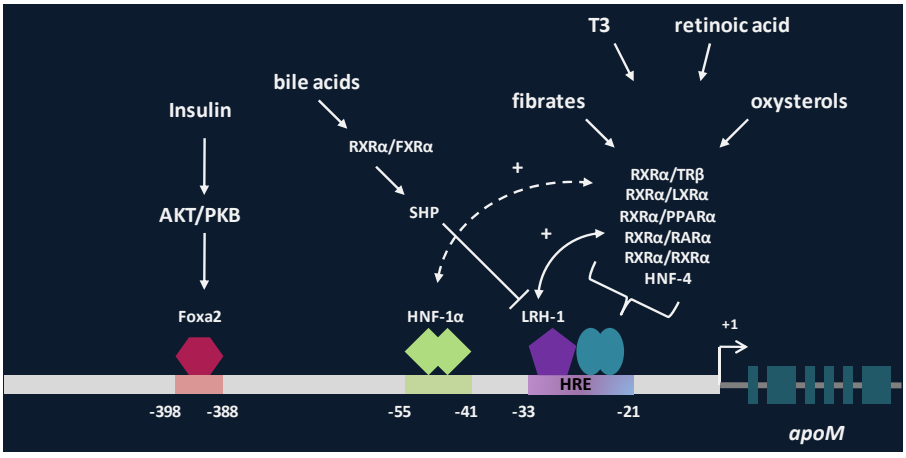

Figure 17. Summary of regulatory elements and transcription factors that participate in the control of human apoM gene transcription

PART II: Opposite regulation of the human apolipoprotein M gene by Hepatocyte Nuclear Factor 1 and Jun transcription factors

Signaling pathways that inhibit apoM gene transcription in hepatic cells via Jun proteins

Treatment of hepatoma cells with pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNF-α) was shown previously to significantly decrease apoM mRNA and proteins levels (155). In line with these observations, we show here that two members of the AP-1 family of transcriptional regulators, c-Jun and JunB, are essential for the downregulation of human apoM gene expression in response to TNF-α. As shown in Fig. 1A, treatment of human hepatoblastoma HepG2 cells with TNF-α for 24 h caused a significant reduction in the mRNA levels of the human apoM gene as well as in the human apoC-III gene which was also shown previously to be negatively regulated by TNF-α (550). Treatment of HepG2 cells with two different doses of TNF-α (100 and 200 ng/ml) was associated with a significant drop in the levels of the apoM protein (Fig. 1B). Importantly, inhibition of apoM gene transcription by TNF-α was completely abolished by overexpressing a combination of short hairpin (sh) RNAs targeting c-Jun and JunB but not by a control shRNA. In these experiments we monitored the levels of expression of the apolipoprotein C-III (apoC-III) gene which was shown previously to be negatively regulated by TNF-α (550) and we observed a similar effect. These findings suggested that Jun proteins are essential mediators of pro-inflammatory signaling pathways that modulate the expression of apolipoprotein genes in hepatocytes. The c-Jun and JunB shRNAs also abolished the negative effect of TNF-α on the activity of the -950/+42 apoM promoter in HepG2 cells (Fig. 1D). In control experiments, we showed that these shRNAs were very effective in silencing the corresponding genes as shown in Fig. 1E and F.

Besides pro-inflammatory cytokines, growth factors such as transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), epidermal growth factor (EGF) and hepatic growth factor (HGF), that can activate Jun factors (412), have been shown to decrease apoM expression levels (164). Similar to TNF-α treatment, treatment of cells with TGF-β reduced apoM mRNA and protein levels as well as apoM promoter activity in HepG2 cells and this reduction was counterbalanced upon overexpression of shRNAs targeting c-Jun and JunB gene expression (Fig. 2).

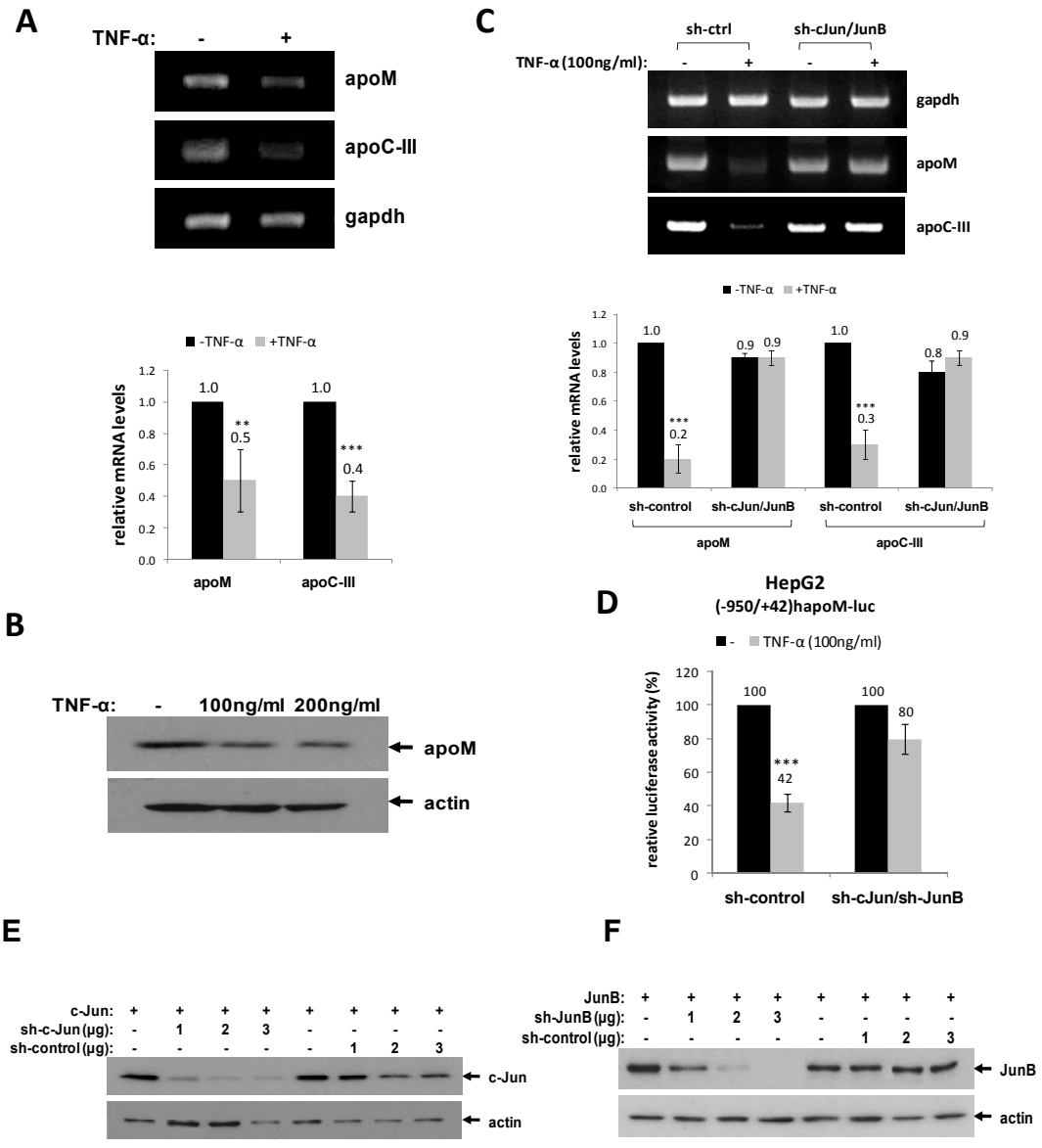

Figure 1. Jun proteins are essential for the down-regulation of apoM and apoC-III gene expression by Tumor Necrosis Factor α in HepG2 cells.

(A) HepG2 cells were treated with 100ng/ml TNF-α for 24h or left untreated. Total RNA was extracted and apoM and apoC-III mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM and apoC-III were quantified and are shown as a histograph. The experiment was performed three times and representative images are presented. (B) HepG2 cells were treated with increasing concentrations of TNF-α (100 and 200ng/ml) for 24h or left untreated and the protein levels of apoM and actin (loading control) were determined by immnublotting using the corresponding antibodies. The

experiment was performed three times and representative images are presented. (C) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0μg each). Following transfection, cells were treated with 100ng/ml TNF-α for 24h or left untreated. Total RNA was extracted and apoM and apoC-III mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM and apoC-III were quantified and are shown as a histograph. (D) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the sh-control or sh-c-Jun and sh-JunB producing vectors, (2.0μg each), as indicated, and a β-galactosidase expression vector (1.0μg). Following transfection, cells were treated with 100ng/ml TNF-α for 24h or left untreated. Luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **$p<0.01$, ***$p<0.001$. (E) HEK293T cells were transiently transfected with a c-Jun expression vector along with increasing concentrations of the sh-c-Jun or sh-control producing vector (0, 1.0, 2.0 and 3.0μg) and the protein levels of c-Jun and actin (loading control) were determined by immunoblotting using the corresponding antibodies. (F) HEK293T cells were transiently transfected with a JunB expression vector along with increasing concentrations of the sh-JunB or sh-control producing vector (0, 1.0, 2.0 and 3.0μg) and the protein levels of JunB and actin (loading control) were determined by immunoblotting using the corresponding antibodies.

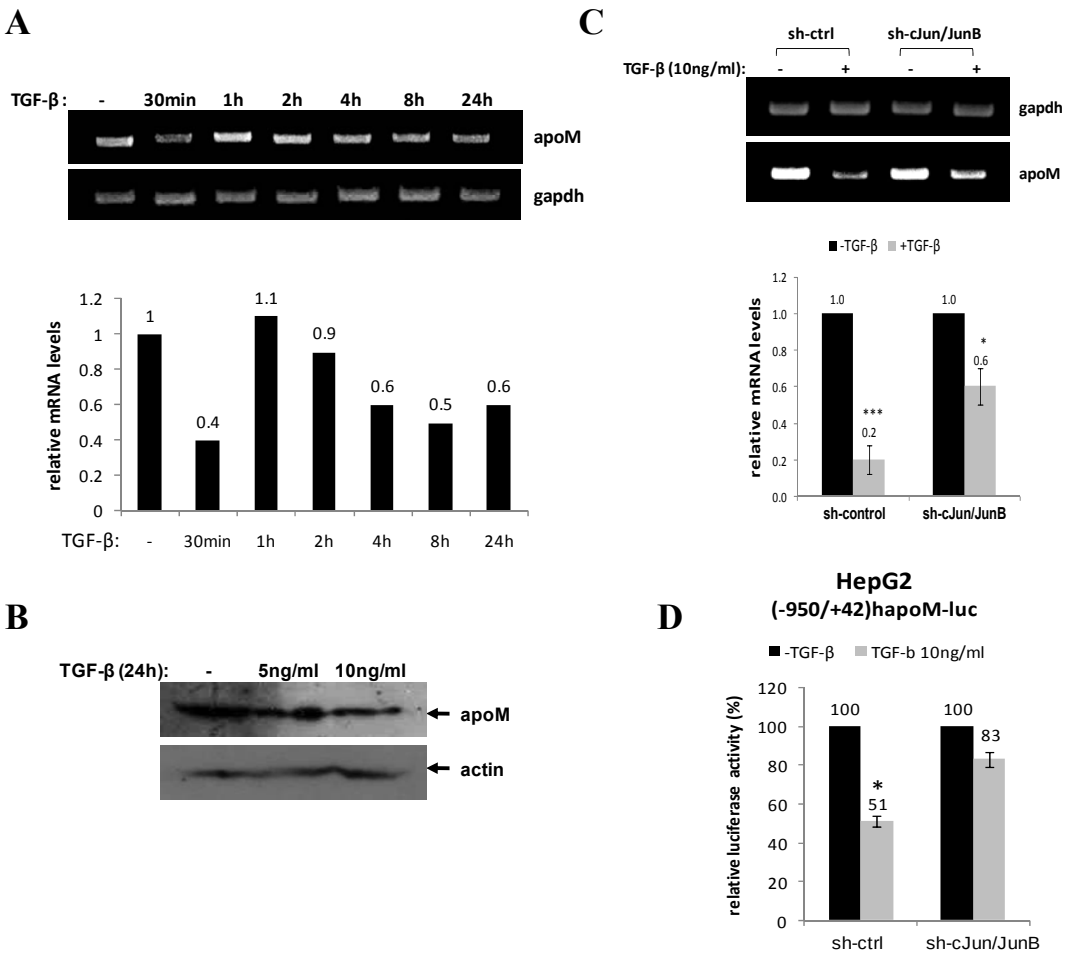

Figure 2. Jun proteins are implicated in the down-regulation of apoM gene expression by Transfroming Growth Factor β in HepG2 cells.

(A) HepG2 cells were treated with 5ng/ml TGF-β for the indicated time periods or left untreated. Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM gene were quantified and are shown as a histograph. (B) HepG2 cells were treated with increasing concentrations of TGF-β (5 and 10ng/ml) for 24h or left untreated and the protein levels of apoM and actin (loading control) were determined by immnublotting using the corresponding antibodies. (C) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0μg each). Following transfection, cells were treated with 10ng/ml TGF-β for 24h or left untreated. Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM gene were quantified and are shown as a histograph. (D) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the sh-control or sh-c-Jun and sh-JunB producing vectors, (2.0μg each), as indicated, and a β-galactosidase expression vector (1.0μg). Following transfection, cells were treated with 10ng/ml TGF-β for 24h or left

untreated. Luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *p<0.05, ***p<0.001.

To corroborate our findings regarding the negative role of Jun proteins on apoM gene transcription, we overexpressed different Jun proteins (c-Jun, JunB or JunD) in HepG2 cells and monitored the activity of the -950/+42 human apoM promoter in response to this overexpression by luciferase assays. Our results (Fig. 3A) showed that overexpression of c-Jun, JunB and JunD was associated with a potent inhibition of apoM promoter activity (to 52%, 42% and 43% respectively relative to the control) confirming that Jun proteins are negative regulators of apoM gene transcription. A dose-dependent inhibition of apoM promoter activity by c-Jun or JunB was observed (Fig. 3B).

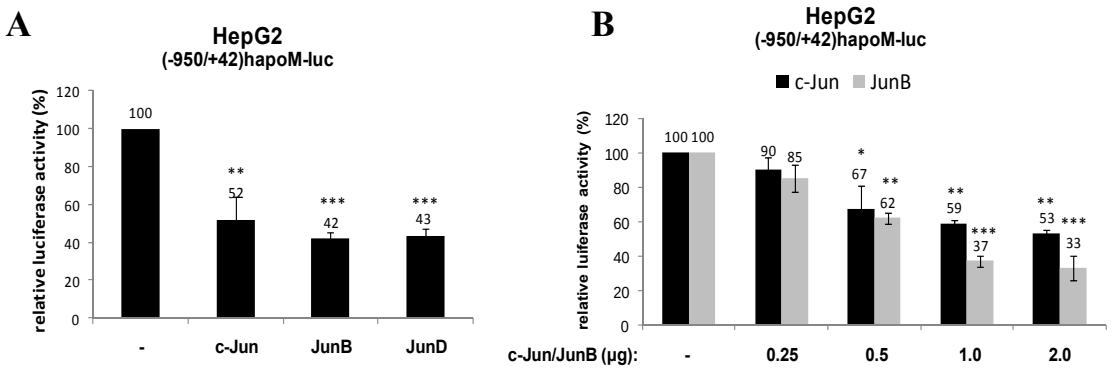

Figure 3. c-Jun and JunB are negative regulators of apoM promoter activity in HepG2 cells. (A) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0µg) along with expression vectors for c-Jun, JunB and JunD (1.0µg). An expression vector for β-galactosidase (1.0µg) was included in each sample for normalization purposes. (B) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0µg) along with increasing concentrations (0, 0.25, 0.5, 1.0 and 2.0µg) of a c-Jun or JunB expression vector as indicated. In panels A and B, an expression vector for β-galactosidase (1.0µg) was included in each sample for normalization purposes. Luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the

average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

Next, we used phorbol myristate acetate (PMA), a potent inducer of AP-1 gene transcription (551). As shown in Fig. 4A, treatment of HepG2 cells with 200 nM PMA for 18 h caused a potent induction of both c-Jun and JunB expression at the protein level. Kinetics experiments showed that the induction of c-Jun by PMA could be observed as early as 1h, peaked at 2h and was sustained for at least 18h (Fig. 5A and C). Induction of c-Jun and JunB by PMA was associated with a potent reduction in the protein levels of apoM, an effect that could be observed at 18h of treatment (Fig. 4A and Fig. 5C). PMA also caused a strong and time-dependent reduction in apoM mRNA levels (Fig. 4B and Fig. 5D). Importantly, this reduction was counterbalanced by overexpressing shRNAs targeting both c-Jun and JunB but not by a control shRNA indicating that the negative effect of PMA on apoM gene expression is mediated at least in part, by Jun proteins (Fig. 4C). Furthermore, PMA inhibited the activity of the -950/+42 apoM promoter and this inhibition was counterbalanced by overexpressing shRNAs targeting both c-Jun and JunB but not by a control shRNA (Fig. 4D).

Phorbol esters are potent activators of protein kinase C (PKC) (552) a family of at least 11 isozymes that are involved in many biological processes in different tissues and cell types such as hepatocytes, endothelial cells or smooth muscle cells (439). To investigate the potential role of PKC in apoM gene regulation in hepatic cells, we treated HepG2 cells with the PKCα/β-specific inhibitor Gö 6976 (553) and showed that this treatment abolished the inhibition of apoM gene expression by PMA both at the mRNA (Fig. 6A) and the protein (Fig. 6B) levels. The PKCα/β inhibitor also abolished the activation of c-Jun by PMA (Fig. 5B). Furthermore, overexpression of PKCα in HepG2 cells was associated with a 70% reduction in the mRNA levels of the apoM gene and this reduction was counterbalanced by shRNAs targeting c-Jun and JunB but not by a control shRNA (Fig. 6C).

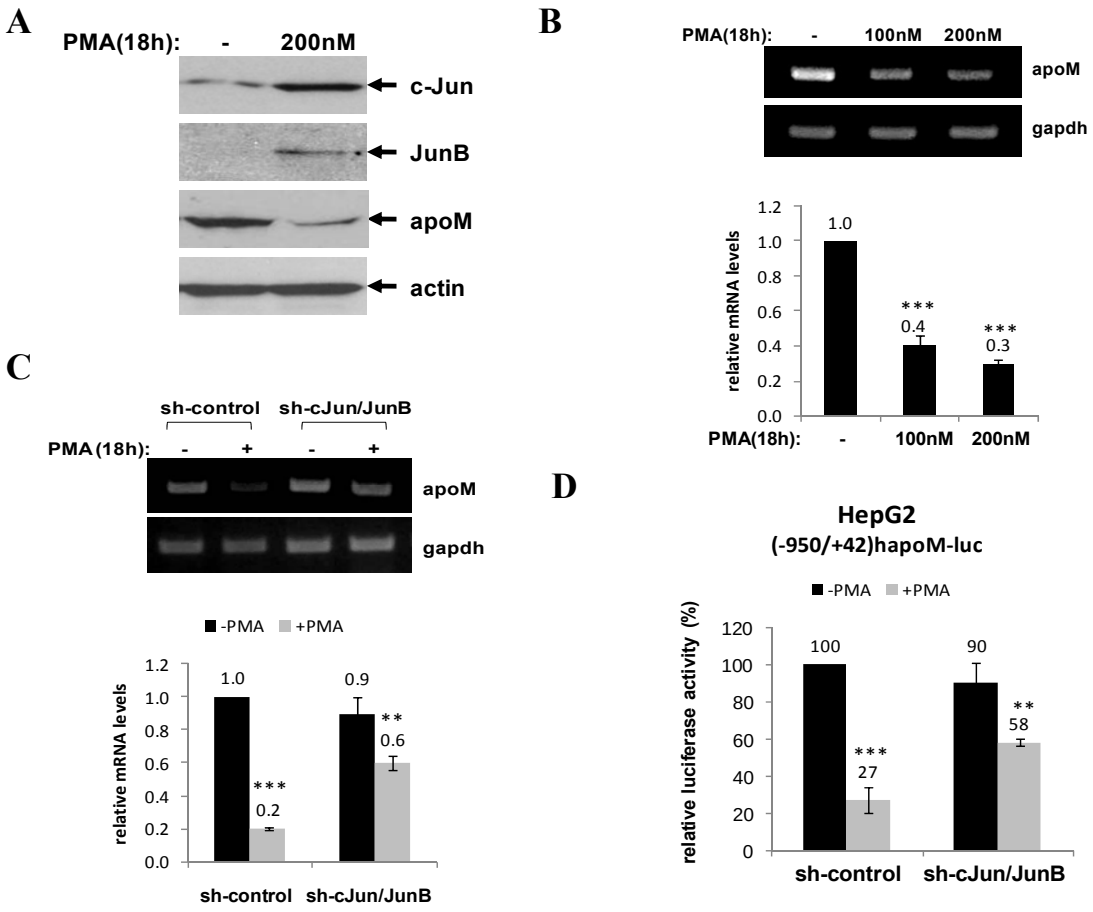

Figure 4. Inhibition of apoM gene expression by phorbol esters is mediated by Jun proteins. (A) HepG2 cells were treated with 200nM PMA for 18h or left untreated and the protein levels of c-Jun, JunB, apoM and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (B) HepG2 cells were treated with increasing concentrations of PMA (100 and 200nM) or left untreated. 18h later, total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (C) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0μg each). Following transfection, cells were treated with 200nM PMA for 18h or left untreated. Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. In panels B and C the relative mRNA levels of the apoM were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (D) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0μg each) and a β-galactosidase expression vector (1.0μg). Following transfection, cells were treated with 100nM PMA for 18h or left untreated.

Luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

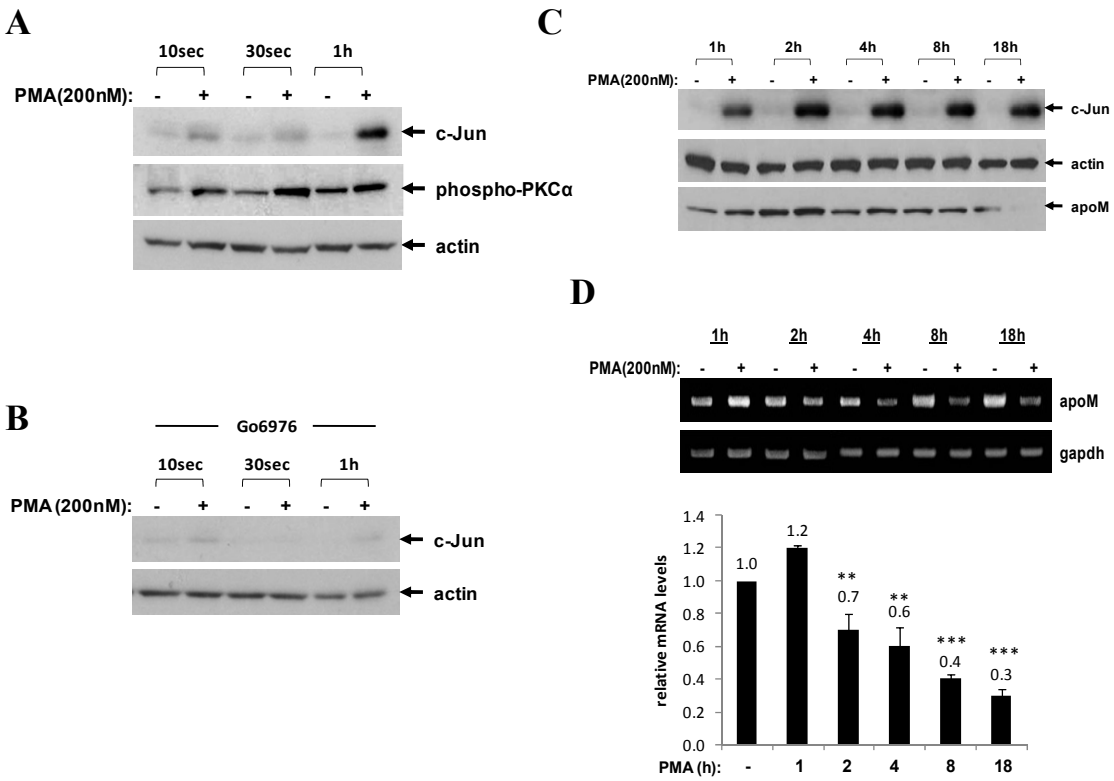

Figure 5. Time course of the PMA/PKCα-induced transcriptional down- regulation of the apoM gene. (A) HepG2 cells were treated with PMA (200nM) for the indicated time periods or left untreated and the protein levels of c-Jun, phospho-PKCα and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (B) HepG2 cells were treated with Go6976 (1μM) and PMA (200nM) for the indicated time periods or left untreated and the protein levels of c-Jun and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (C) HepG2 cells were treated with PMA (200nM) for the indicated time periods or left untreated and the protein levels of c-Jun, actin (loading control) and apoM were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (D) HepG2 cells were treated with PMA (200nM) for the indicated time periods or left untreated. Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM were

quantified and are shown as a histograph. Each value represents the average from three independent experiments. Key: **p<0.01, ***p<0.001

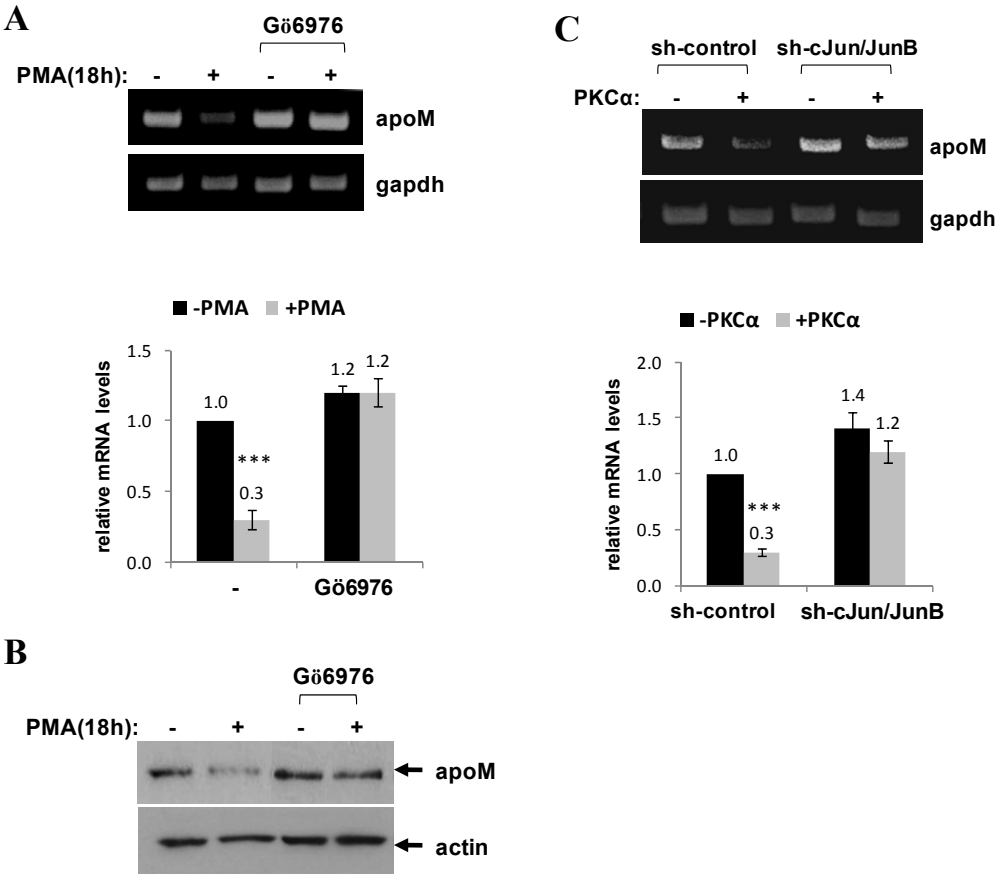

Figure 6. PMA-induced transcriptional donwregulation of the apoM gene is mediated by PKC. (A) HepG2 cells were treated with PMA (200nM) and/or the PKCα/β specific inhibitor Gö6976 (1μM) as indicated. Eighteen hours later, total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were treated with PMA (200nM) and/or Gö6976 (1μM) for 18h as indicated and the protein levels of apoM and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are presented. (C) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0μg each) along with an expression vector for PKCα

(2.0μg) as indicated. Total RNA was extracted and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM were quantified and are shown as a histograph. Each value represents the average from three independent experiments. Key: ***$p<0.001$

The -53/-47 region of the apoM promoter contains an AP-1 responsive element

In order to map the region of the apoM promoter that is responsible for the inhibition by Jun proteins, we utilized a panel of apoM promoter deletion mutants shown schematically in Fig. 7A. Transient transfections of HepG2 cells with these reporter plasmids in the absence or in the presence of expression vectors for c-Jun (Fig. 7B) or JunB (Fig. 7C) established that the minimal region of the apoM promoter that is required for Jun-mediated inhibition is defined by nucleotides -105 and -49.

Binding of endogenous c-Jun and JunB proteins to the apoM promoter *in vivo* was shown by chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig. 8B both c-Jun and JunB were recruited to the proximal (-214/-14) apoM promoter albeit with different affinities (top row, compare lanes 3 and 4). In contrast, binding of both proteins to the distal region of the apoM promoter (-950/-616) was not significant (Fig. 8B, middle row, lanes 3 and 4). As a control, we used primers that amplify the -313/-185 region of the promoter of the human RhoB gene and we found no recruitment of Jun proteins to this region (Fig. 8B, bottom row, lanes 3 and 4).

To identify more precisely the putative AP-1 element on the proximal apoM promoter, a series of DNA affinity precipitation (DNAP) assays was performed. As shown in Fig. 9B, both c-Jun and JunB proteins bound to a biotinylated -105/+42 apoM promoter fragment. In contrast, no binding of c-Jun or JunB could be observed using a biotinylated -241/-81 apoM promoter (Fig. 9B) or a control biotinylated oligonucleotide corresponding to the -76/-43 region of the human RhoB promoter (Fig. 9B).

A close inspection of the DNA sequence of the proximal apoM promoter region -241/+42 revealed the presence of a putative AP-1 element at position -53/-47 (Fig. 9A). This element contains the sequence 5’ TTACTCA 3’ and differs from the consensus AP-

1 site (5' TGACTCA 3') (554) by only one nucleotide at the second position (T instead of a G). Interestingly, this putative AP-1 element resides within a previously characterized binding site for Hepatocyte Nuclear Factor 1α (HNF-1α) which is located at position -55/-41 (Fig. 9A) (106).

First, we confirmed binding of c-Jun and JunB to this site by performing DNAP assays using a biotinylated oligonucleotide corresponding to the -61/-38 region of the apoM promoter (Fig. 9C). HNF-1α bound equally well to the same oligonucleotide as shown in Fig. 9D. To verify that the 5' TTACTCA 3' element is a true AP-1 binding site, we introduced mutations at positions 5, 6 and 7 of this element (5' TTAC*GGG* 3'). As shown in Fig. 9C, neither c-Jun nor JunB could bind to the mutated oligonucleotide (-61/-38)mut. As expected, these mutations also abolished the binding of HNF-1α (Fig. 9D).

Next, we introduced the same nucleotide substitutions into the corresponding positions of the -950/+42 apoM promoter (Fig. 10A) and examined the activity of the mutant versus the wild type promoter in HepG2 cells by luciferase assays. At basal conditions, the activity of the mutant -950/+42 apoM promoter was very low (8%) compared to the wild type promoter confirming that this element is essential for apoM gene expression in hepatic cells (Fig. 10B). Importantly, mutations in the AP1 element abolished the inhibition of apoM promoter activity by PMA (Fig. 10C) or by PKCα overexpression (Fig. 10D) strongly suggesting that this element, in addition to its importance for basal apoM expression, is essential for the inhibition of apoM gene expression by the PKC/Jun pathway.

We then showed that neither c-Jun nor JunB could repress the activity of the wild type -950/+42 apoM promoter in HEK293T cells that lack endogenous HNF-1α protein (Fig. 11A). HNF-1α caused a strong (40-fold) transactivation of the wild type but only a minor (4-fold) transactivation of the mutant apoM promoter (Fig. 11A). This finding is in agreement with the data of Fig. 9D which showed that the introduced mutations abolished the binding of HNF-1α to the apoM promoter. More importantly, both c-Jun and JunB inhibited the HNF-1α mediated transactivation of the wild type but not of the mutated apoM promoter (Fig. 11A) suggesting a negative cross talk between Jun proteins and HNF-1α on the activity of the apoM promoter.

In HepG2 cells that express endogenous HNF-1α, c-Jun and JunB inhibited the activity of the proximal -105/+42 apoM promoter that contains the dual HNF-1α/AP-1 element and inhibited the HNF-1α mediated transactivation of this promoter (Fig. 11B). In contrast, c-Jun and JunB could not suppress the activity of the -105/+42 apoM promoter in HepG2 cells in which the endogenous HNF-1α gene had been silenced by a shRNA targeting HNF-1α (Fig. 11C and D).

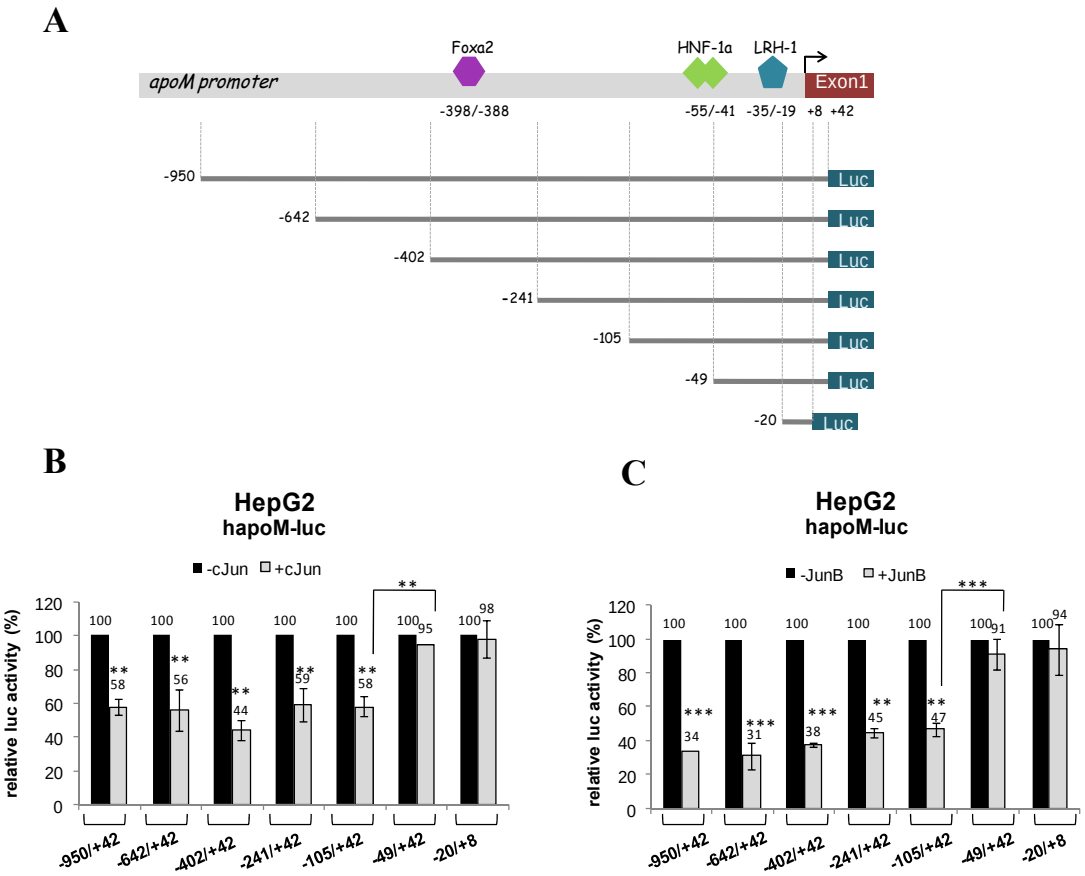

Figure 7. The proximal region -105/-49 of the human apoM promoter is required for transcriptional repression by c-Jun and JunB. (A) Schematic representation of the human apoM promoter fragments that were cloned upstream of the luciferase reporter gene and used in the transactivation experiments of panels B and C. The position of previously described regulatory elements and factors is also shown. (B) HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the bottom of the graph (1.0μg) along with a c-Jun expression vector (1.0μg) or an empty vector. (C)

HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the bottom of the graph (1.0μg) along with a JunB expression vector (1.0μg) or an empty vector (1.0μg). In panels B and C, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

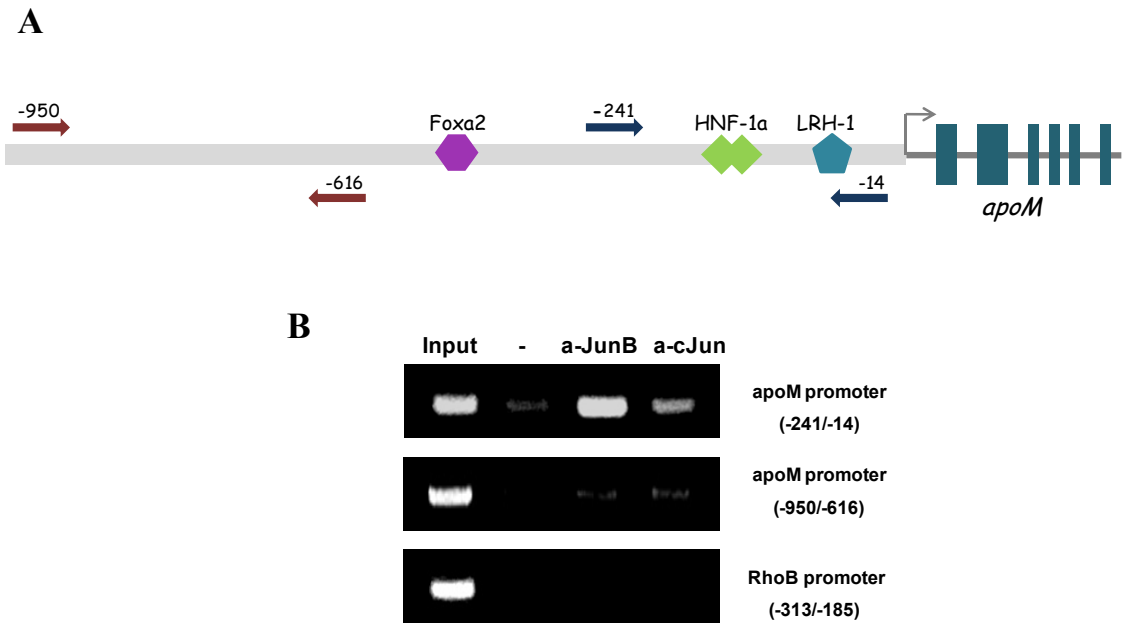

Figure 8. Chromatin immunoprecipitation assays established the recruitment of c-Jun and JunB to the proximal human apoM promoter in HepG2 cells. (A) Schematic representation of the human apoM promoter region, showing by arrows the location of the oligonucleotide primer sets (distal region: -950/-616, proximal region: -241/-14) that were utilized in the chromatin immunoprecipitation assays of panel B. The relative position of previously described regulatory elements and factors is also shown. (B) HepG2 cells were subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-JunB (third lane) or an anti-c-Jun (fourth lane) antibody using primers corresponding to the proximal region of the apoM promoter (top row), the distal region of the apoM promoter (middle row) or the proximal region (-313/-185) of the RhoB promoter (bottom row, negative control). Non-immunoprecipitated chromatin was included for comparison (first lane, input). The experiment was performed three times and representative images are presented.

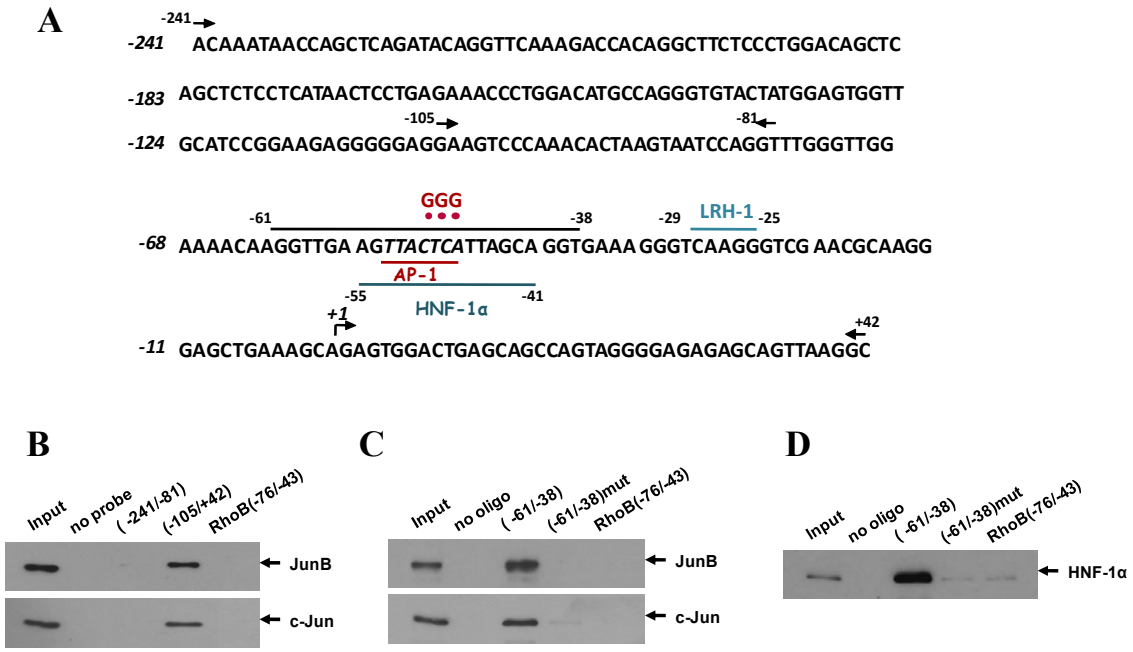

Figure 9. c-Jun and JunB bind to a novel AP-1 site located in the -53/-47 region of the proximal apoM promoter. (A) Sequence of the proximal human apoM promoter region spanning nucleotides -241 to +42, showing the location of the previously characterized regulatory elements that bind HNF-1α and LRH-1 as well as the location of the AP-1 response element. The primer sets used for the amplification of the biotinylated promoter fragments -241/-81 and -105/+42 are indicated by arrows. Nucleotide substitutions in the AP-1 site of the apoM promoter are indicated with the red dots. (B) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with expression vectors for JunB or c-Jun and biotinylated PCR fragments corresponding to the -241/-81 or the -105/+42 region of the human apoM promoter or no probe. A biotinylated oligonucleotide corresponding to the -76/-43 region of the human RhoB promoter that contains a CAAT box was used as a negative control. Oligonucleotide-bound JunB or c-Jun was detected by Western blotting using an anti-JunB or anti-c-Jun antibody respectively. (C) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with expression vectors for JunB or c-Jun and biotinylated oligonucleotides corresponding to the wild type -61/-38 region of the human apoM promoter, the -61/-38 region bearing mutations in the AP-1 site (mut), the -76/-43 region of the human RhoB promoter or no oligonucleotide (no oligo). Oligonucleotide-bound JunB or c-Jun was detected by Western blotting using an anti-JunB or anti-c-Jun antibody respectively. (D) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1α and biotinylated oligonucleotides corresponding to the wild type -61/-38 region of the human apoM promoter, the -61/-38 region bearing mutations in the AP-1 site (mut), the -76/-43 region of the human RhoB promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α was detected by Western blotting using a polyclonal

anti-HNF-1α antibody. All experiments in Panels B-D were performed at least three times and representative images are presented.

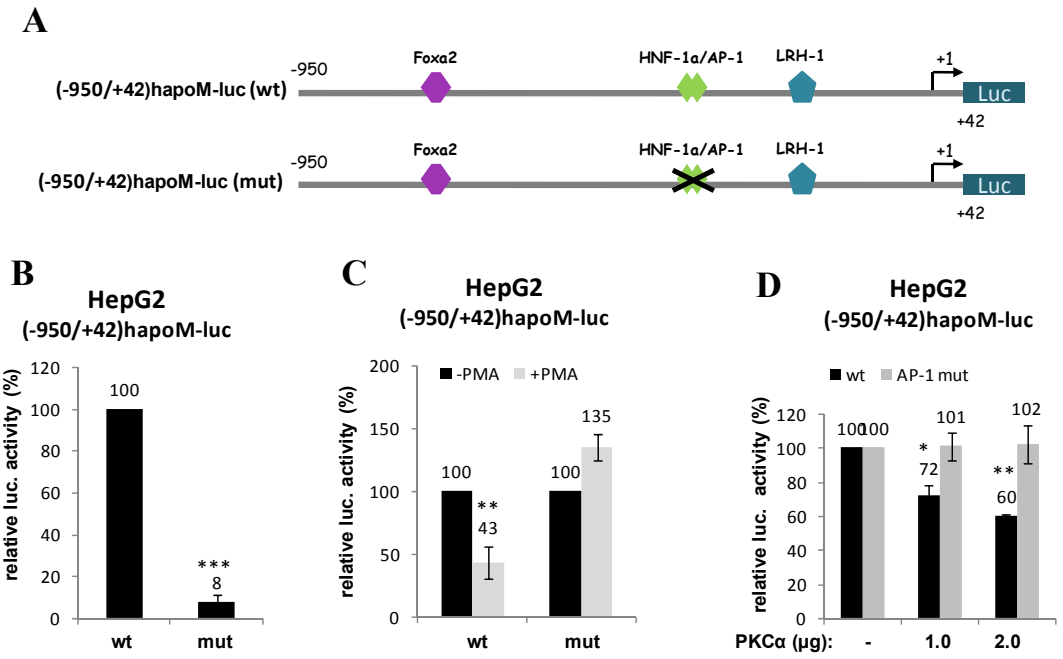

Figure 10. Mutations in the dual HNF-1α/AP-1 site abolished inhibition of apoM gene expression by PMA treatment or by overexpression of PKCα. (A) Schematic representation of the wild type (-950/+42)hapoM-luc promoter construct and the corresponding construct bearing the mutations in the HNF-1α/AP-1 site. (B) HepG2 cells were transiently transfected with the wt or mutated (-950/+42)hapoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg). (C) HepG2 cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg). Following transfection, cells were treated with PMA (100nM) for 18h or left untreated. (D) HepG2 cells were transiently transfected with the wt or mutated (-950/+42)hapoM-luc reporter plasmid (1.0μg) along with increasing concentrations (1.0 and 2.0 μg) of an expression vector for PKCα and a β-galactosidase expression vector (1.0μg). In panels B-D, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

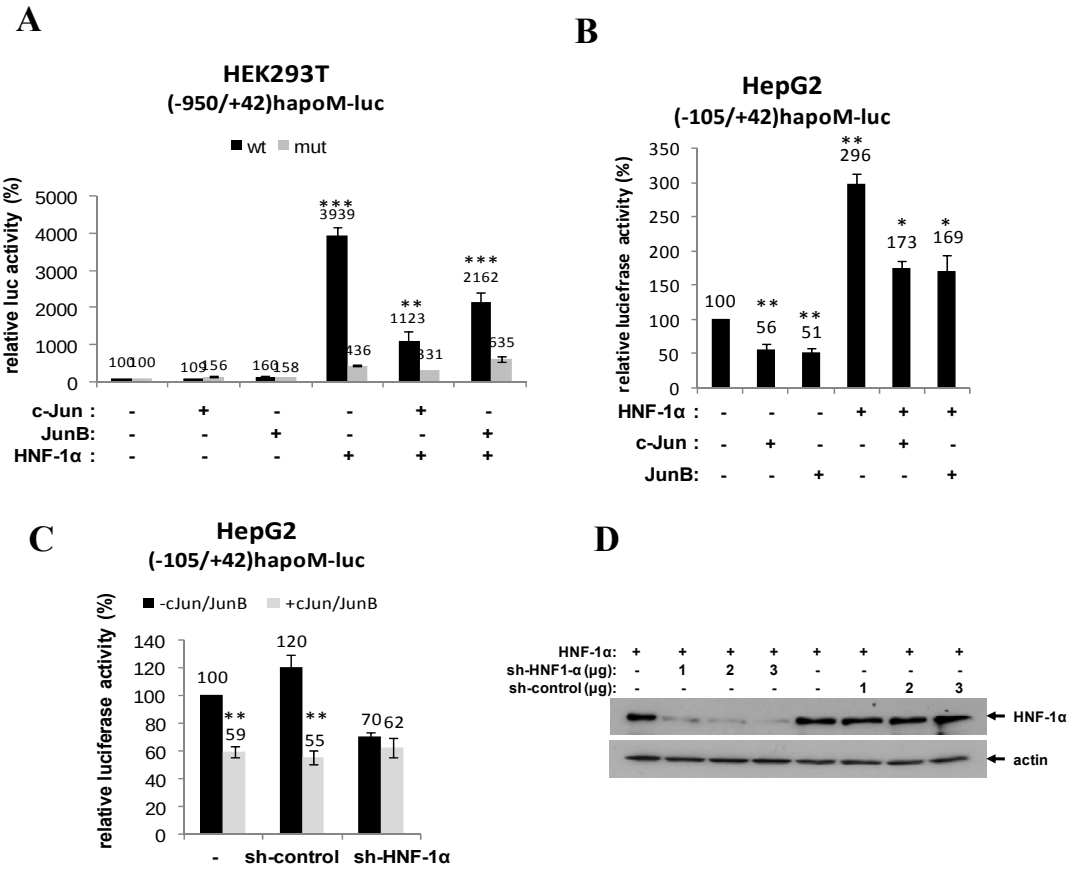

Figure 11. HNF-1α is required for the repression of apoM promoter activity by c-Jun and JunB. (A) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg) and expression vectors for HNF-1α, c-Jun or JunB (1.0μg) as indicated (B) HepG2 cells were transiently transfected with the (-105/+42)hapoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg) and expression vectors for HNF-1α, c-Jun or JunB (1.0μg) as indicated. (C) HepG2 cells were transiently transfected with the (-105/+42)hapoM-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg), the sh-control or sh-HNF-1α producing vectors (2.0μg) and expression vectors for c-Jun and JunB (1.0μg each) as indicated. In panels A-C, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. (D) HEK293T cells were transiently transfected with an HNF-1α expression vector along with increasing concentrations of the sh-HNF-1α or sh-control producing vector (0, 1.0, 2.0 and 3.0μg) and the protein levels of HNF-1α and actin (loading control) were determined by immunoblotting using the corresponding antibodies. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

The mouse apoM promoter does not contain a functional AP1 site

The findings of Fig. 9 to 11 indicated that the -55/-41 region of the human apoM promoter contains a dual HNF-1α/AP-1 responsive element that mediates activation or repression of apoM gene expression by HNF-1α and AP-1 factors respectively. Interestingly, the corresponding region in the mouse apoM promoter is highly conserved with the exception of a single nucleotide substitution at position 6 of the AP-1 element (5' TTACT*T*A 3') (Fig. 12A). Using DNAP assays we first showed that HNF-1α binds to both the mouse and the human elements with equal affinity (Fig. 12B). In contrast, c-Jun and JunB could not bind to the mouse element possibly due to the nucleotide substitution (Fig. 12B). In agreement with the DNA binding data of Fig. 12B, c-Jun and JunB could not inhibit the basal activity of the mouse apoM promoter in HepG2 cells (Fig. 12C).

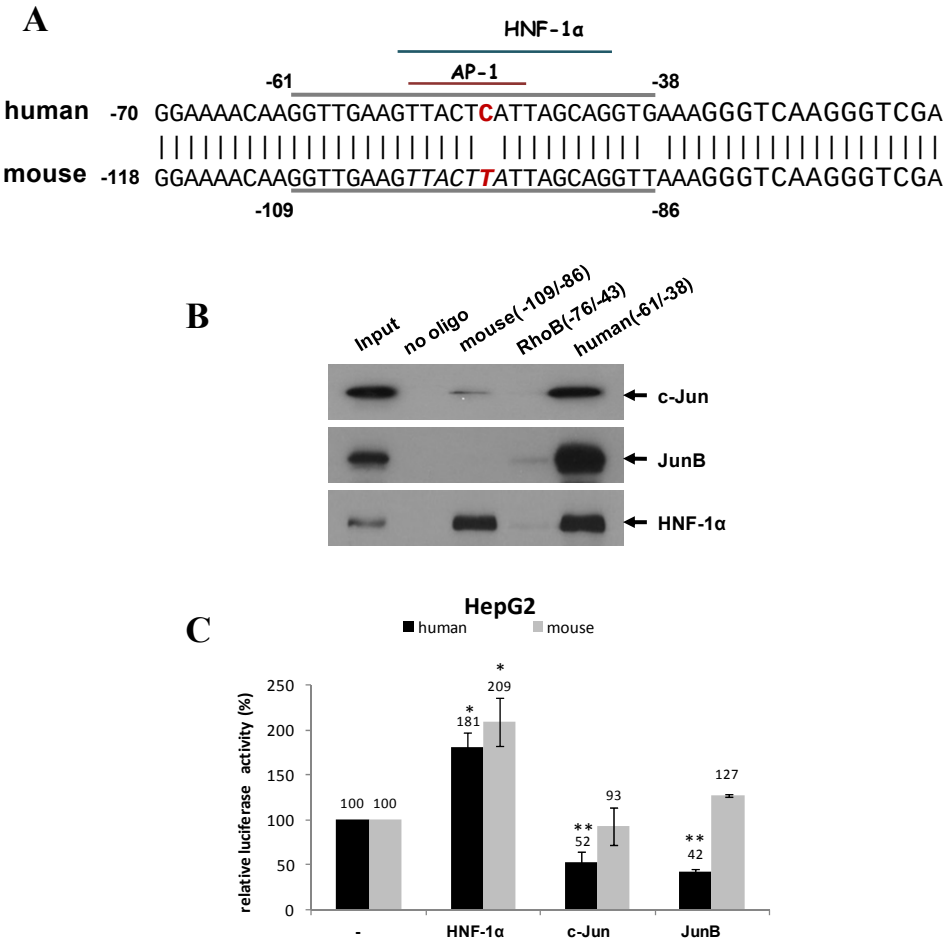

Figure 12. The mouse apoM promoter does not contain a functional AP-1 element. (A) Alignment of the human and mouse apoM proximal promoters in the region of the dual HNF-1α/AP-1responsive elements. Vertical lines indicate identical nucleotide residues. The non-homologous nucleotides inside the HNF-1α/AP-1 site are shown in red and italics. The overall homology between the human and the mouse apoM promoter sequences shown is 93%. (B) DNA-affinity precipitations using nuclear extracts from HEK293T cells transiently transfected with expression vectors for c-Jun, JunB or HNF-1α and biotinylated oligonucleotides corresponding to the -109/-86 region of the mouse apoM promoter, the -76/-43 region of the human RhoB promoter, the -61/-38 region of the human apoM promoter or no oligonucleotide (no oligo). Oligonucleotide-bound c-Jun, JunB or HNF-1α was detected by Western blotting using an anti-JunB, anti-c-Jun or anti- HNF-1α antibody respectively. (C) HepG2 cells were transiently transfected with the (-950/+42)human apoM-luc reporter plasmid (1.0μg) or the (-761/-7)mouse apoM-luc reporter plasmid (1.0μg) along with expression vectors for HNF-1α, c-Jun or JunB (1.0μg) and a β-galactosidase expression vector (1.0μg). Luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$

Binding of HNF-1α and AP1 factors to the proximal apoM promoter is mutually exclusive

Using chromatin immunoprecipitation assays, we sought to examine the recruitment of HNF-1α and AP-1 factors on the apoM promoter under basal conditions as well as under conditions of AP-1 activation. As shown in Fig. 13A and B, under basal conditions, HNF-1α bound strongly to the apoM promoter whereas binding of either c-Jun or JunB was barely detectable. Upon a 2h treatment with PMA, binding of HNF-1α to the apoM promoter was markedly reduced (Fig. 13A and B, first row, lane 7). In contrast, binding of both c-Jun (Fig. 13A, first row, compare lanes 4 and 8) and JunB (Fig. 13B, first row, compare lanes 4 and 8) to the proximal apoM promoter was enhanced upon PMA treatment. No binding of HNF-1α or Jun proteins was observed on the distal (-950/-616) apoM promoter or to the unrelated RhoB promoter (Fig. 13A, B second and third row respectively). By imunoblotting analysis, we showed that the 2h PMA treatment had no effect on the expression of the endogenous HNF-1α gene in HepG2 cells (Fig. 13C).

The findings of Fig. 13 suggested that the binding of HNF-1α and Jun proteins to the dual-specificity responsive element on the proximal apoM promoter is mutually exclusive. To investigate further this mechanism, we performed a series of *in vitro* DNAP assays. These experiments showed that binding of HNF-1α to the -61/-38 apoM oligonucleotide probe could be competed out by increasing concentrations of c-Jun (Fig. 14A, first row, compare lane 3 with lanes 6 and 9) or JunB (Fig. 14B, first row, compare lane 3 with lanes 6 and 9). Similarly, binding of c-Jun (Fig. 14C) or JunB (Fig. 14D) to the -61/-38 apoM oligonucleotide could be competed out by increasing concentrations of HNF-1α (second row, compare lane 3 with lanes 6 and 9). These findings provided additional evidence that binding of HNF-1α and Jun proteins to the -61/-38 region of the apoM promoter is mutual exclusive.

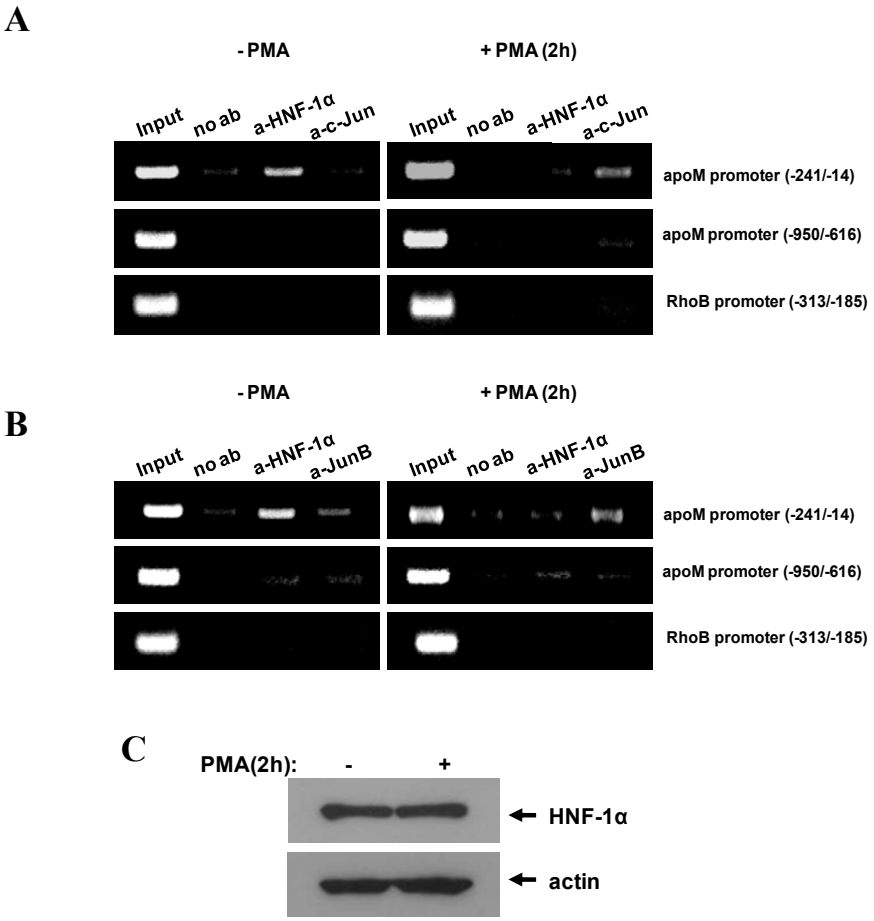

Figure 13. Chromatin immunoprecipitation analysis of HNF-1α, c-Jun and JunB binding to the apoM promoter following PMA treatment. (A) HepG2 cells were treated with 200nM PMA for 2h and then subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-HNF-1α (third lane) or an anti-c-Jun (fourth lane) antibody using primers corresponding to the proximal (-241/-14) or the distal (-950/-616) region of the apoM promoter or the proximal region (-313/-185) of the RhoB promoter. Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are shown. (B) HepG2 cells were treated with 200nM PMA for 2h and then subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-HNF-1α (third lane) or an anti-JunB (fourth lane) antibody using primers corresponding to the proximal (-241/-14) or the distal (-950/-616) region of the apoM promoter or the proximal region (-313/-185) of the RhoB promoter. Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are presented. (C) HepG2 cells were treated with 200nM PMA for 2h or left untreated and the protein levels of HNF-1α and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are shown.

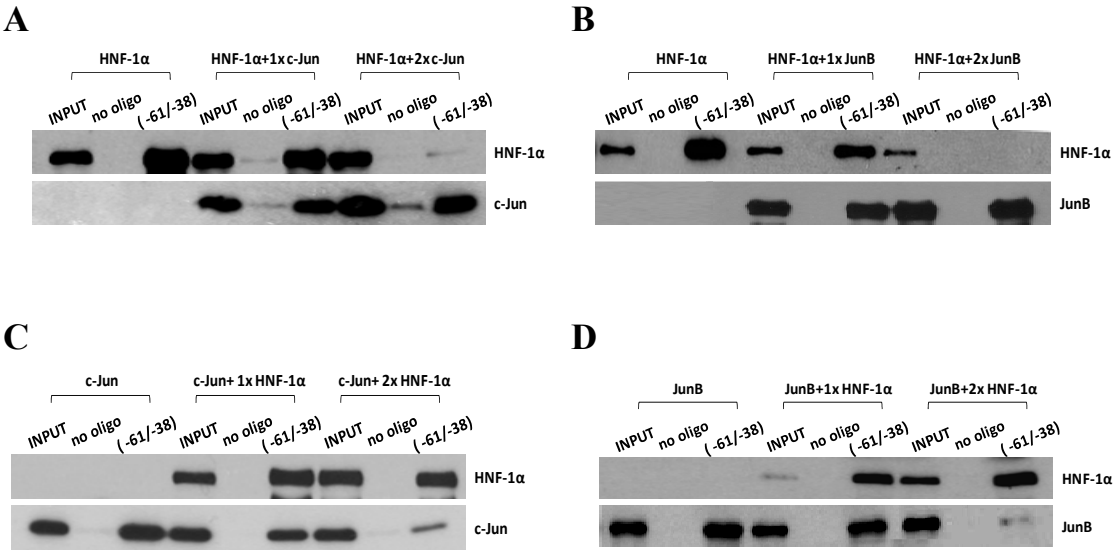

Figure 14. Mutually exclusive binding of c-Jun/JunB and HNF-1α to the -61/-38 region of the apoM promoter. (A) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1α and increasing amounts of c-Jun and biotinylated oligonucleotides corresponding to the -61/-38 region of the human apoM promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α and c-Jun was detected by Western blotting using an anti-HNF-1α or anti-c-Jun antibody respectively. (B) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1α and increasing amounts of JunB and biotinylated oligonucleotides corresponding to the -61/-38 region of the human apoM promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α and JunB was detected by Western blotting using an anti-HNF-1α or anti-JunB antibody respectively. (C) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for c-Jun and increasing amounts of HNF-1α and biotinylated oligonucleotides corresponding to the -61/-38 region of the human apoM promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α and c-Jun was detected by Western blotting using an anti-HNF-1α or anti-c-Jun antibody respectively. (D) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for JunB and increasing amounts of HNF-1α and biotinylated oligonucleotides corresponding to the -61/-38 region of the human apoM promoter or no oligonucleotide (no oligo). Oligonucleotide-bound HNF-1α and JunB was detected by Western blotting using an anti-HNF-1α or anti-JunB antibody respectively. All experiments were performed at least three times and representative images are presented.

Antagonistic interactions between HNF-1α and AP-1 factors control the transcription of the human apolipoprotein A-II gene

In a search for additional genes that could be regulated by a similar mechanism, we identified a putative AP-1 site within a previously identified HNF-1α binding site in the promoter of the human apolipoprotein A-II (apoA-II) gene (element H, -573/-554) (Fig. 15A) (555,556). First we showed that apoA-II mRNA levels in HepG2 cells were decreased following a 18h PMA treatment and this decrease was not observed upon overexpression of shRNA targeting c-Jun gene expression or in the presence of the PKCα/β inhibitor Gö 6976 (Fig. 15B and C respectively). Using chromatin immunoprecipitation assays we established that c-Jun, but not JunB, was recruited to the -649/-343 region of the apoA-II promoter that contains the putative AP-1 element under basal conditions (Fig. 15D). Overexpression of c-Jun in HepG2 cells was associated with a 50% reduction in the activity of the -911/+29 apoA-II promoter (Fig. 15E). DNAP assays established that the region of the apoA-II promoter between nucleotides -573 and -554 is a dual HNF-1/AP-1 site that binds both c-Jun and HNF-1α (Fig. 15F). Finally, using chromatin immunoprecipitations we showed that there is competition between c-Jun and HNF-1α for binding to the apoA-II promoter under conditions of c-Jun activation by PMA (Fig. 15G).

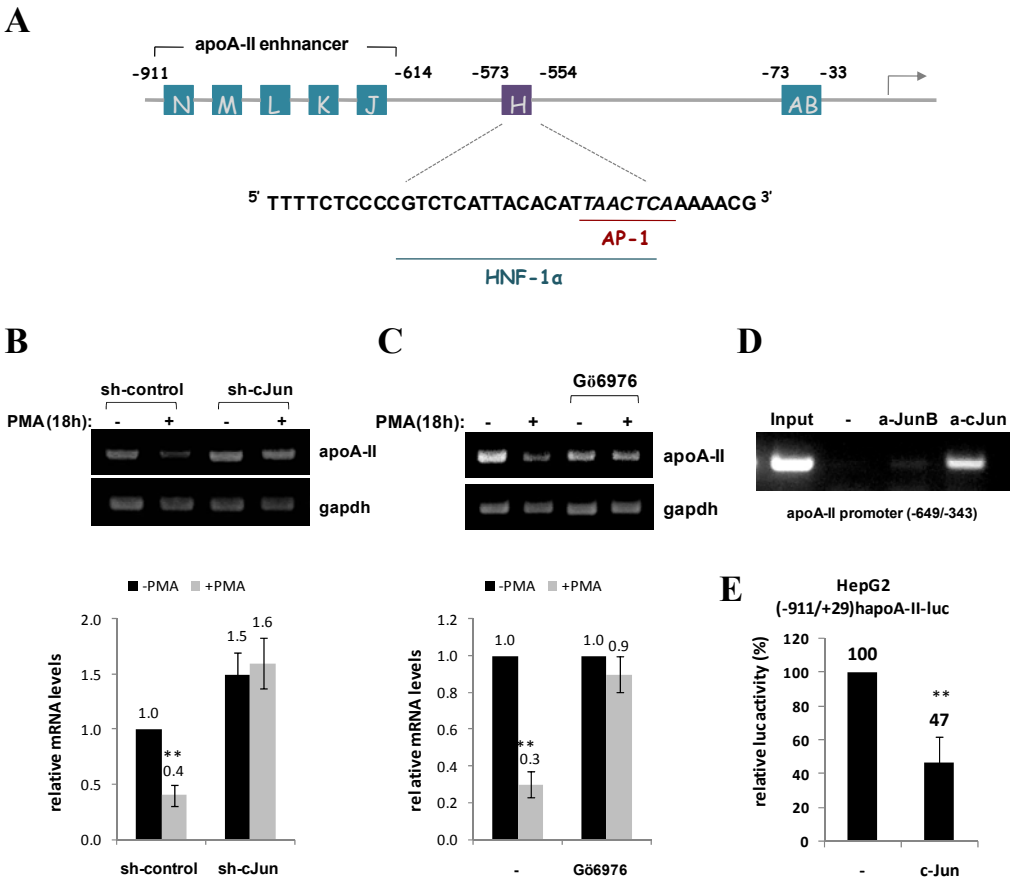
A
apoA-II enhnancer
-911
-614
-573
-554
-73
-33
N
M
L
K
J
H
AB
5' TTTTCTCCCCGTCTCATTACACATTAACTCAAAAACG 3'
AP-1
HNF-1α
B
sh-control
sh-cJun
PMA(18h):
apoA-II
gapdh
-PMA
+PMA
relative mRNA levels
1.0
0.4
1.5
1.6
sh-control
sh-cJun
C
Gö6976
PMA(18h):
apoA-II
gapdh
1.0
0.3
1.0
0.9
Gö6976
D
Input
a-JunB
a-cJun
apoA-II promoter (-649/-343)
E
HepG2
(-911/+29)hapoA-II-luc
relative luc activity (%)
100
47
c-Jun
F
Input
apoM(-61/-38)
comp (-573/-550) apoA-II:
comp (-76/-43) RhoB:
c-Jun
HNF-1α
G
- PMA
+ PMA (2h)
Input
no ab
a-HNF-1α
a-c-Jun
a-JunB
apoA-II promoter(-649/-343)

Figure 15. Role of PKC and AP-1 proteins in the regulation of apolipoprotein A-II gene transcription in hepatic cells. (A) Schematic representation of the human apoA-II promoter region spanning nucleotides -911 to -33, showing the location of the previously characterized regulatory elements as well as the location of the dual HNF-1α/AP-1 site (element H). (B) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun producing vector (2.0μg). Following transfection, cells were treated with 200nM PMA for 18h or left untreated. Total RNA was extracted and apoA-II mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (C) HepG2 cells were treated with PMA (200nM) and/or Gö 6976 (1μM) as indicated. 18h later, total RNA was extracted and apoA-II mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. In panels B and C the relative mRNA levels of the apoA-II were quantified and are shown as a histograph. Each value represents the average from three independent experiments. (D) Chromatin immunoprecipitation assay establishing the recruitment of c-Jun to the human apoA-II promoter region in HepG2 cells. HepG2 cells were subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-JunB (third lane) or an anti-c-Jun (fourth lane) antibody using primers corresponding to the -649/-343 region of the apoA-II promoter encompassing the AP-1 site. Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and a representative image is shown. (E) HepG2 cells were transiently transfected with the (-911/+29)human apoA-II-luc reporter plasmid (1.0μg) along with an expression vector for c-Jun (1.0μg) and β-galactosidase (1.0μg). Luciferase activity was normalized to β-galactosidase activity and presented with histograph. The value represents the average (±SD) from at least three independent experiments performed in duplicate. (F) DNA binding competition assays establishing that the -573/-550 region of the apoA-II promoter harbors a dual HNF-1/AP-1 element. The DNA-affinity precipitation was performed using nuclear extracts from HEK293T cells transiently transfected with expression vectors for c-Jun (top row) or HNF-1α (bottom row) and a biotinylated PCR oligonucleotide corresponding to the -61/-38 region of the human apoM promoter or no probe. Competition was performed in the presence of increasing amounts (1-3 fold molar excess) of non-biotinylated oligonucleotides corresponding to the -573/-550 region of the apoA-II promoter (lanes 4-6) or the -76/-43 region of the RhoB promoter (lanes 7-9). Oligonucleotide-bound c-Jun or HNF-1α were detected by Western blotting using the corresponding antibodies. (G) HepG2 cells were treated with 200nM PMA for 2h and then subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-HNF-1α (third lane), anti-c-Jun (fourth lane, top) or anti-JunB (fourth lane, bottom) antibody using primers corresponding to the -649/-343 region of the apoA-II promoter. Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are presented. Key: **$p<0.01$

Discussion

HDL is a heterogeneous population of lipoprotein particles in the plasma comprising larger spherical particles (such as HDL_2 and HDL_3) as well as smaller (preβ-HDL) discoidal particles. HDL particles in plasma are in a dynamic equilibrium that is governed by a continuous remodeling mediated by plasma enzymes, membrane transporter proteins and receptors (557). HDL undergoes significant changes in HDL composition, structure, antioxidative and anti-inflammatory activities during the acute phase response (558). Apolipoprotein M is a recently described apolipoprotein that is associated with HDL and has been shown to play an important role in HDL maturation and remodeling (97). The atheroprotective role of apoM was established by studies which showed that adenoviral apoM overexpresison in LDL-receptor deficient mice (animal model for premature atherosclerosis) or hepatic overexpression of apoM in apoM transgenic mice led to a reduction in atherosclerosis development (97). Thus, it could be anticipated that reduction in apoM synthesis in the liver could accelerate atheroscelrosis and coronary artery disease. Indeed, it was found that the expression of the apoM gene is under negative regulation during inflammation, a condition that favors atherosclerosis (155).

Hormone nuclear receptors play an important role in the regulation of HDL genes during inflammation. As it was described in Part I, the proximal apoM promoter harbors a multi-functional hormone response element (HRE) that serves as a binding site for a plethora of orphan and ligand-inducible nuclear receptors including Hepatocyte Nuclear Factor 4 (HNF-4), homodimers of retinoid X receptor α (RXRα) and heterodimers of RXRα with liver X receptor α (LXRα), retinoic acid receptor α (RARα), peroxisome proliferator activated receptor α (PPARα) and thyroid hormone receptor β (TRβ1). This site was shown to mediate apoM gene induction by ligands for the above nuclear receptors whereas mutagenesis of this site severely reduced both the basal and the inducible activity of the apoM promoter in hepatic cells. Furthermore, preliminary evidence for a cooperation between HNF-1 and the above hormone nuclear receptors was

obtained. These findings, combined with previous studies which had shown that TNF- α inhibits the transcriptional activity of nuclear receptor HNF-4α in hepatic cells via nuclear factor κB (NF-κB) activation (559) and preliminary studies that show repression of apoM promoter activity by NF-κB (data not shown) suggested that regulation of apoM gene transcription during inflammation may be complex and mediated by multiple signaling pathways and molecular cross talks.

We showed here that Jun factors are essential for the down-regulation of apolipoprotein C-III (apoC-III) gene expression by TNF-α in HepG2 cells (Fig. 1). This finding is in support of previous studies which had shown that overexpression of different Jun proteins (c-Jun, JunB and JunD) in HepG2 cells was associated with a potent reduction in the activity of the apoC-III promoter and that this reduction required the distal apoC-III enhancer region (560). In the case of apoC-III, the mechanism of Jun-mediated down-regulation of this gene was not studied further but it was hypothesized that Jun proteins interfere with the communication between promoter and the enhancer which is facilitated by HNF-4 and Sp1 transcription factors (560,561).

To study in detail the mechanism by which Jun proteins inhibit apoM gene transcription in hepatic cells, we used phorbol esters as Jun inducing agents. In agreement with previous studies (551), we found that PMA induces the expression of the c-Jun and JunB genes and that this induction requires an active Protein Kinase C (Fig. 6). We found that PMA treatment as well as PKC overexpression had a strongly negative impact on the expression of the apoM gene both at the mRNA and the protein level (Fig. 4). These findings established PKC as a negative modulator of apoM gene transcription. PKC comprises a family of serine/threonine kinases that play a role in many stages of atherosclerotic plaque development. By downregulating apoM gene expression in the liver, activated PKC is expected to influence the maturation of HDL particles in the plasma and as a consequence, HDL structure and functions including atheroprotective functions on the arterial wall and the reverse cholesterol transport pathway. Thus, selective regulators of PKC isozymes may be promising candidates for the prevention or even the regression of atherosclerosis via HDL particles (439).

We report here the identification of a dual specificity regulatory element in the proximal apoM promoter that mediates activation or repression of apoM promoter activity by HNF-1 and Jun proteins respectively in hepatic cells. The experimental evidence that supports the dual functionality of this element of the apoM promoter is provided by the present study and is as follows: a) Jun proteins repress apoM promoter activity only in cells expressing HNF-1α. Jun-mediated repression was abolished in HepG2 cells in which endogenous HNF-1α expression had been silenced by specific shRNAs (Fig. 11); b) mutations in the AP-1 element abolished the binding of HNF-1α and the HNF-1α-mediated transactivation of the apoM promoter (Fig. 9-11); c) the mouse apoM promoter does not contain a functional AP-1 site due to single nucleotide difference but it retains a functional HNF-1α responsive element (Fig. 12); d) competition experiments showed that binding of Jun proteins and HNF-1α to the apoM promoter is mutually exclusive e) chromatin immunoprecipitation assays established that AP-1 activation leads to the recruitment of c-Jun and Jun B proteins to the proximal apoM promoter with the simultaneous displacement of HNF-1 (Fig. 13); f) a similar mechanism of Jun-mediated repression of promoter activity via dual specificity AP-1/HNF-1 responsive element operates on the promoter of the human apolipoprotein A-II gene, the expression of which is also under negative regulation during the acute phase response (562).

A previous study has shown that administration of lipopolysacharide (LPS) to mice (model of bacterial infection) was associated with a marked reduction in the expression levels of HNF-1α in the liver (156). The same group also showed that both apoM and HNF-1α genes are downregulated in the kidney of LPS-treated mice (155). We show here that the expression of HNF-1α in HepG2 cells was not affected by phorbol ester treatment (Fig. 13C) suggesting that a mechanism involving a direct effect of Jun on HNF-1α gene expression in hepatic cells could be excluded.

The question that remains is why recruitment of Jun proteins to the apoM and apoA-II promoters and the displacement of HNF-1α is associated with transcriptional repression. We could hypothesize that Jun proteins bind to the AP-1 elements of the apoM and apoA-II promoter without activating transcription. Active repression by Jun

could be excluded in light of the findings that both c-Jun and JunB failed to repress the activity of the apoM promoter in HEK293T cells that lack hepatocyte nuclear factors (Fig. 11A). The inability of Jun proteins to activate the apoM promoter could be either due to the absence of cooperating factors in the vicinity of the AP-1 element or due to the deviation of the apoM AP-1 element from the consensus sequence (Fig. 9). Transcriptional repression by Jun and other AP-1 proteins has been reported in many cases including the genes encoding for c-Fos, osteocalcin, adipocyte P2, creatinine kinase, myoD, major histocompatibility class I, chorionic gonadotropin α and β, apoC-III and insulin (560,563-570). However, in the majority of the above cases, the molecular mechanisms by which AP-1 proteins inhibit transcription are not understood.

In summary, our model described here provides a mechanistic explanation for the inhibitory effect of pro-inflammatory cytokines or other factors on the expression of acute phase HDL genes such as apoM and apoA-II (Fig.16). HDL is a lipoprotein particle with multiple but still not fully characterized atheroprotective properties. Understanding how the HDL genes are controlled during health and disease will be essential for the development of novel therapeutic and diagnostic tools for diseases such as coronary artery disease and diabetes that affect a large proportion of the population in Western countries.

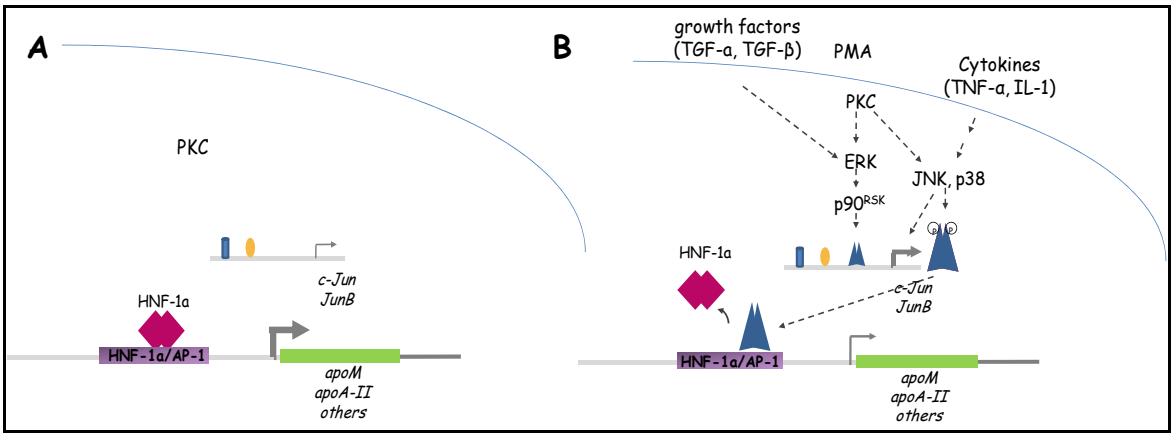

Figure 16. Proposed model for the inhibitory effect of pro-inflammatory cytokines on the expression of apoM and apoA-II genes.

Part III: Regulation of ABCA1 gene expression by c-Jun

Inhibition of ABCA1 gene expression by the PMA/PKC signaling pathway in hepatic cells

Previous studies by others and our lab (E.Thymiakou, PhD thesis, University of Crete 2009) have shown that the expression of the ATP-binding cassette lipid transporter A1 (ABCA1) , a major regulator of HDL metabolism, is induced upon differentiation of human monocyte THP-1 cells to macrophages by phorbol myristate acetate (PMA) (263). In the present study the role of PMA in the regulation of ABCA1 gene expression in hepatic cells was investigated.

Initially, HepG2 cells were treated with increasing concentrations of PMA, 100 and 200nM for 18h and its effect on ABCA1 gene expression was evaluated using RT-PCR. As shown n Fig.1A, PMA-stimulation of HepG2 cells dose-dependently reduced ABCA1 mRNA levels up to 80% compared with control cells. Kinetic experiments showed that this reduction was observed as early as 2h and was sustained for up to 18h (Fig.1B) Strong reduction was also observed in ABCA1 protein levels in PMA-treated HepG2 cells (Fig.1C and 1D) as well as in the activity of a luciferase reporter construct containing the -668/+33 ABCA1 promoter region (Fig.1E).

As most PMA effects are mediated by PKC, a PKCα/β-specific inhibitor Gö 6976 and a more general inhibitor of PKCα/β/γ/δ/ε isozymes bisindolylmaleimide I was used to treat cells along with PMA. As shown in Fig.1F both inhibitors were equally effective in abolishing the inhibition of ABCA1 gene expression by PMA while overexpression of PKCα in HepG2 cells dose dependently reduced ABCA1 promoter activity up to 50% (Fig.1G). In summary, the findings of Fig. 1 show that in hepatic cells PMA, in contrast to macrophages, negatively regulates ABCA1 gene expression and indicate PKC as the downstream mediator in the PMA signaling pathway.

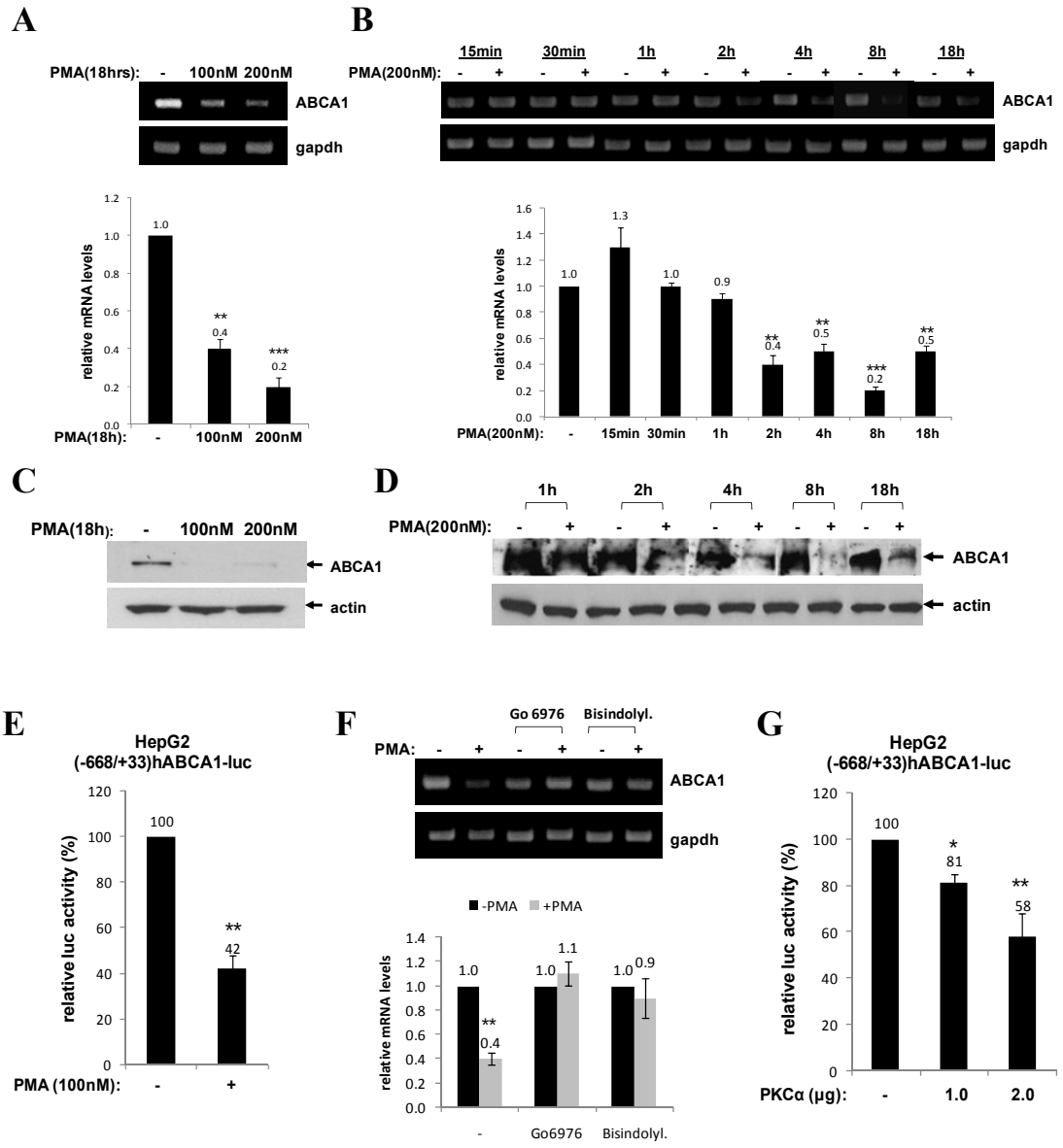

Figure 1. Inhibition of ABCA1 gene expression in hepatic cells by a PMA/PKC signaling pathway. (A) HepG2 cells were treated with increasing concentrations of PMA (100 and 200nM) or left untreated. 18h later, total RNA was extracted and ABCA1 mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (B) HepG2 cells were treated with PMA (200nM) for the indicated time periods or left untreated. Total RNA was extracted and ABCA1 mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (C) HepG2 cells were treated with increasing concentrations of PMA (100 and 200nM) for 18h or left untreated and the protein levels of ABCA1 and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are shown. (D) HepG2 cells were treated with PMA (200nM) for the indicated

time periods or left untreated and the protein levels of ABCA1 and actin (loading control) were determined by immnublotting using the corresponding antibodies. The experiment was performed three times and representative images are shown. (E) HepG2 cells were transiently transfected with the (-668/+33) human ABCA1-luc reporter plasmid (1.0μg) along with a β-galactosidase expression vector (1.0μg). Following transfection, cells were treated with PMA (100nM) for 18h or left untreated. (F) HepG2 cells were treated with PMA (200nM) and/or Go6976 (1μM) or bisindolymaleimide I (100nM) as indicated. 18h later, total RNA was extracted and ABCA1 mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. (G) HepG2 cells were transiently transfected with the (-668/+33)ABCA1-luc reporter plasmid (1.0μg) along with increasing concentrations (1.0 and 2.0 μg) of an expression vector for PKCα and a β-galactosidase expression vector (1.0μg). In panels E and G, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. In panels A,B and F the relative mRNA levels of the ABCA1 were quantified and are shown as a histograph. Each value represents the average from three independent experiments. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

Inhibition of ABCA1 gene expression by the PMA/PKC signaling pathway in hepatic cells is mediated by c-Jun

PMA is a potent inducer of AP-1 gene transcription (551). As already shown in Part II (Fig. 4), treatment of HepG2 cells with 200nM PMA for 18h caused a potent induction of c-Jun and JunB at the protein level while kinetic experiments showed that the induction of c-Jun by PMA could be observed as early as 1h, peaked at 2h, when the ABCA1 expression levels started to decline, and was sustained for at least 18h (Part II, Fig. 5). Therefore the putative involvement of AP-1 factors in the PMA/PKC-induced repression of ABCA1 gene expression was examined.

Overexpression of c-Jun in HepG2 cells strongly inhibited the activity of the -668/+33 ABCA1 promoter to a degree similar to that observed with PMA, while JunB overexpression had a minor effect on ABCA1 promoter activity (19% reduction) (Fig.2A). Furthermore, short hairpin RNA (shRNA)-mediated silencing of c-Jun gene expression increased the activity of the -668/+33 ABCA1 promoter in HepG2 cells by 1.5-fold and counterbalanced the PKC-induced repression of ABCA1 promoter activity (Fig.2B) or the PMA-induced downregulation of ABCA1 mRNA levels (Fig.2C) suggesting that c-Jun is essential for the PMA/PKC-induced inhibition of ABCA1 gene expression.

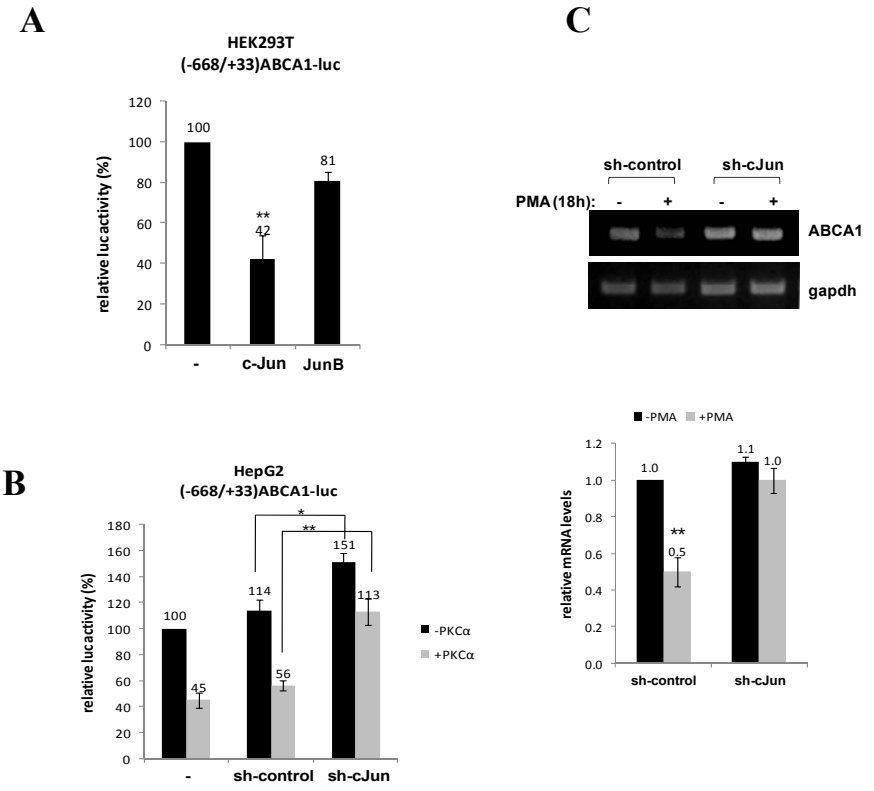

Figure 2. PMA/PKC-induced inhibition of ABCA1 gene expression in hepatic cells is mediated by c-Jun. (A) HEK293T cells were transiently transfected with the (-668/+33) ABCA1-luc reporter plasmid (1.0μg) along with expression vectors for members of the Jun family, c-Jun and JunB (1.0μg). An expression vector for β-galactosidase (1.0μg) was included in each sample for normalization purposes. (B) HepG2 cells were transiently transfected with the (-668/+33) ABCA1-luc reporter plasmid (1.0μg) along with the sh-control or sh-c-Jun producing vector (2.0μg), a PKCα expression vector (2.0μg) and a β-galactosidase expression vector (1.0μg). (C) HepG2 cells were transiently transfected with the sh-control or sh-c-Jun producing vector (2.0μg). Following transfection, cells were treated with 200nM PMA for 18h or left untreated. Total RNA was extracted and ABCA1 mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the ABCA1 were quantified and are shown as a histograph. Each value represents the average from three independent experiments. In panels A and B luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: *, $p<0.05$, **$p<0.01$, ***$p<0.001$

The -122/-116 region of the ABCA1 promoter contains an AP-1 responsive element

Having established a negative role of c-Jun in the transcriptional regulation of the ABCA1 gene, in silico analysis of the ABCA1 regulatory region was performed in order to identify putative AP-1 response elements. This analysis revealed two putative AP-1 binding sites: one putative site is located upstream of the first exon, at position -122/-116 (5'-TGACTGA-3') and the other putative site is located upstream of the second exon (within the first intron) at position -145/-139 (5'-TGACTCA-3') (Fig.3A). We first examined the binding of c-Jun to the ABCA1 upstream promoter using chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig.3B c-Jun was recruited to the proximal -205/+204 ABCA1 promoter region that contains the putative AP-1 element under basal conditions but not to the promoter of the human RhoB gene that was used as a control. No recruitment of JunB protein to the ABCA1 promoter was found in agreement with the transactivation data of Fig.2B. Upon a 2h treatment with PMA, binding of c-Jun to the ABCA1 promoter, as expected, was enhanced while no binding was observed to the RhoB promoter (Fig.3C).

Binding of c-Jun to the ABCA1 promoter was then further confirmed in vitro using the DNA-affinity precipitation assay (DNAP). For this purpose, two biotinylated overlapping PCR fragments covering the ABCA1 promoter between nucleotides -535 to +205 were generated (-535/-94 and -118/+205) (Fig 3A). As shown in Fig 3D, c-Jun bound strongly to the -535/-94 biotinylated promoter fragment but less efficiently to the -118/+205 containing the half AP-1 site. In control experiments it was shown that c-Jun did not bind to the streptavidin Dynabeads (Fig 3D, lane2, no probe) or to a double stranded biotinylated oligonucleotide corresponding to the human RhoB promoter that contains a previously characterized CAAT box (Fig. 3D, lane 5)

To investigate the functional importance of this AP-1 site for ABCA1 promoter inhibition, mutations that are supposed to destroy the AP-1 site were introduced into the (-668/+33)ABCA1 luciferase reporter plasmid (Fig. 4A). Mutagenesis of this site increased the basal activity of the (-668/+33)ABCA1 promoter in HepG2 cells to 489 %

of control indicating that this element plays an inhibitory role in ABCA1 gene regulation in hepatic cells.

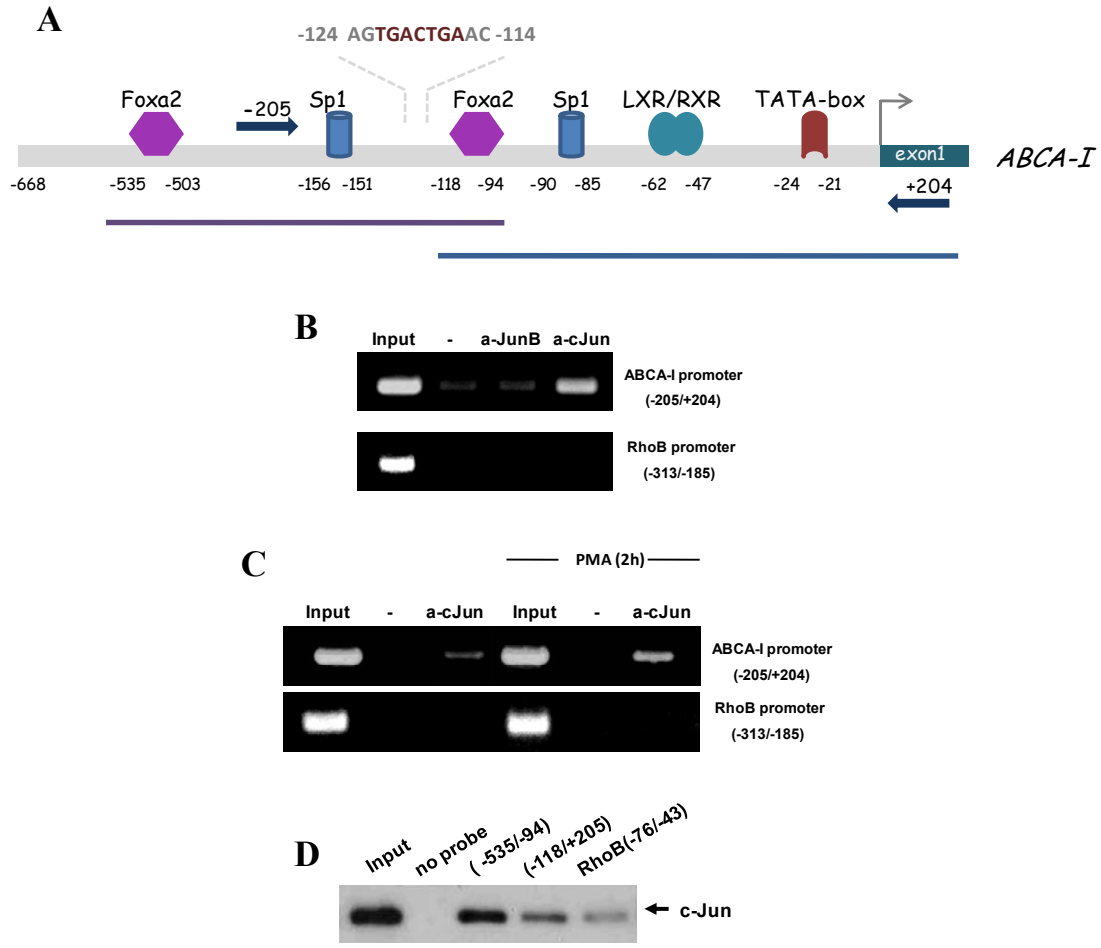

Figure 3. c-Jun is recruited to the proximal human ABCA1 promoter in HepG2 cells. (A) Schematic representation of the human ABCA1 promoter region, showing the location of the previously characterized regulatory elements and factors as well as the location of the putative AP-1 response element. The primers used in the chromatin immunoprecipitation assays are indicated by arrows while purple and blue lines indicate the biotinylated promoter fragments -535/-94 and -118/+205 that were utilized in DNAP assays. (B) HepG2 cells were subjected to chromatin immunoprecipitation in the absence (second lane) or presence of an anti-JunB (third lane) or an anti-c-Jun (fourth lane) antibody using primers corresponding to the proximal region (-205/+204) of the ABCA1 promoter or the proximal region (-313/-185) of the RhoB promoter (unrelated region,

negative control). Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are shown. (C) HepG2 cells were treated with 200nM PMA for 2h or left untreated and then subjected to chromatin immunoprecipitation in the absence (second lane) or presence (third lane) of an anti-c-Jun antibody using primers corresponding to the -205/+204 region of the ABCA1 promoter. Non-immunoprecipitated chromatin was included as a positive control (first lane, input). The experiment was performed three times and representative images are shown. (D) DNA-affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for c-Jun and biotinylated PCR fragments corresponding to the -535/-94 or the -118/+205 region of the human ABCA1 promoter or no probe. A biotinylated oligonucleotide corresponding to the -76/-43 region of the human RhoB promoter that contains a CAAT box was used as a negative control. Oligonucleotide-bound c-Jun was detected by Western blotting using an anti-c-Jun antibody.

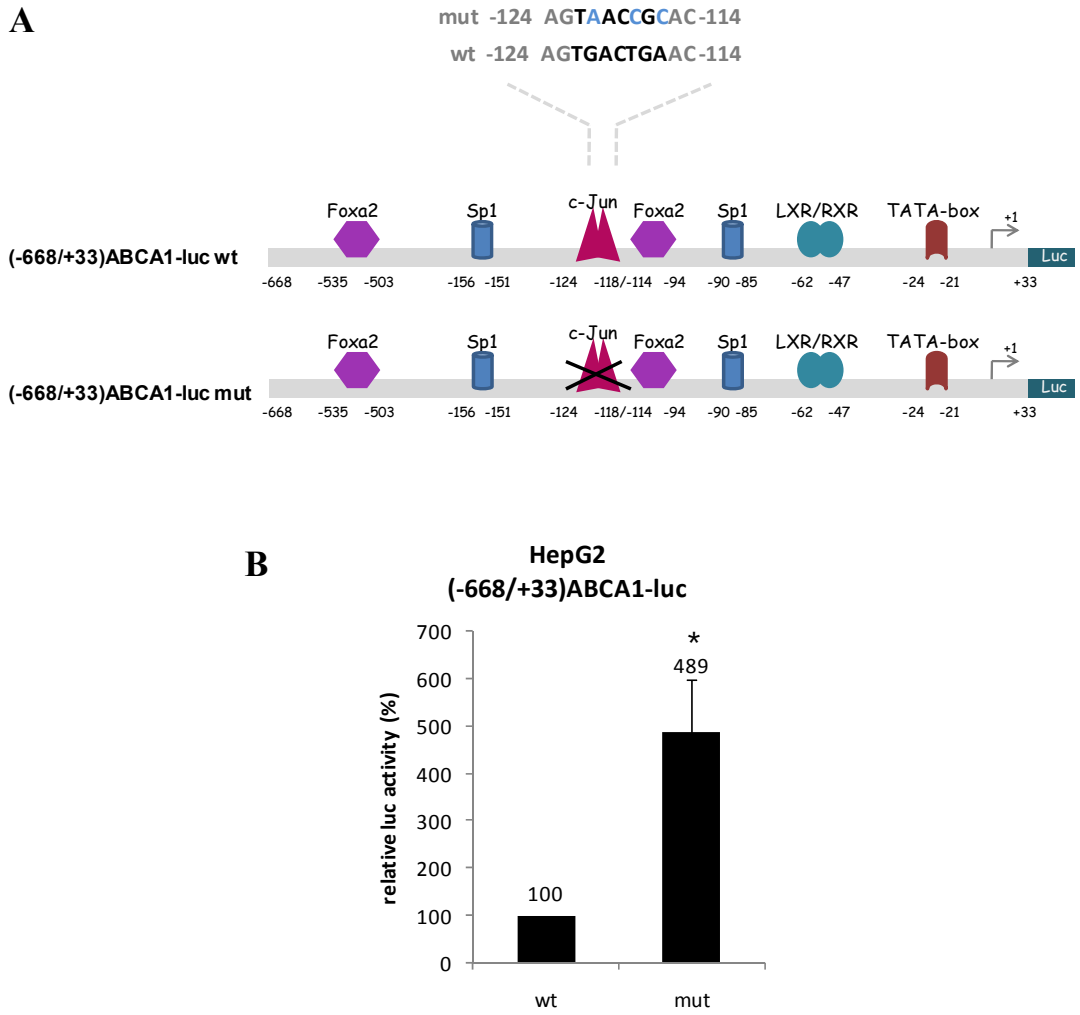

Figure 4. Mutations in the putative AP-1 binding site increase basal ABCA1 promoter activity in HepG2 cells. (A) Schematic representation of the wild type (-668/+33)ABCA1-luc promoter construct and the corresponding construct bearing mutations in the putative AP-1 binding site. Nucleotide substitutions are indicated in blue. (B) HepG2 cells were transiently transfected with the wt or mutated (-668/+33)ABCA1-luc reporter plasmid (1.0μg) along a β-galactosidase expression vector (1.0μg). Luciferase activity was normalized to β-galactosidase activity and presented with histographs. The value represents the average (±SD) from three independent experiments performed in duplicate. Key: *, $p<0.05$

Discussion

The lipid transporter ABCA1 is a key player in the reverse cholesterol transport pathway promoting cholesterol efflux from peripheral cells and thus attenuating macrophage foam cell formation and the development of atherosclerosis. Inflammatory factors that influence the activity of many proteins that are implicated in HDL biogenesis and maturation such as the apolipoprotein A-I, apolipoprotein M, and the remodeling enzymes LCAT, CETP and hepatic lipase (562) have also been reported to affect ABCA1 gene expression levels. In particular, LPS and the proinflammatory cytokines TNF-α, IL-1β and IFN-γ have been shown to downregulate ABCA1 mRNA and protein levels in macrophages and murine peritoneal macrophage-derived foam cells (274, 475) as well as in cultured intestinal cells supporting the concept that inflammatory mediators modulate reverse cholesterol transport and macrophage foam cell formation at least in part by directly acting on the expression levels of ABCA1.

The TNF-α-mediated suppression of ABCA1 gene expression has been shown to be partially reversed by preventing activation of the nuclear factor κB pathway (571). In the present study, the possible involvement of the pro-inflammatory Jun transcription factors in ABCA1 gene regulation was investigated. In contrast to monocytes (263) and in agreement with a previous study showing an inhibitory role for the PMA/PKC signaling pathway on ABCA1 gene expression through PKCα-mediated suppression of LXR-mediated transactivation (572), PMA decreased ABCA1 mRNA and protein levels in hepatic cells. This suppression was alleviated by a PKC inhibitor or overexpression of shRNA targeting c-Jun, suggesting a dual mechanism of action for PKC. The effect was specific for c-Jun transcription factor as JunB could not inhibit ABCA1 promoter activity and seemed to be direct as an AP-1 consensus site located in the -124/-114 proximal ABCA1 promoter region was identified, capable of recruiting c-Jun. Mutagenesis of this site increased basal ABCA1 promoter activity further supporting the negative regulatory role of c-Jun on ABCA1 transcriptional activation.

In summary, these findings of Part III combined with the data presented in Part II and previous studies by other groups indicated that PKC and Jun transcription factors

negatively regulate the expression of several genes involved in HDL metabolism. In all cases, AP1 responsive elements were identified that recruit Jun factors upon stimulation by phorbol esters. These findings suggest that inhibition of PKC activity or Jun proteins is expected to have a positive effect on HDL metabolism and could exploited further as strategies to treat atherosclerosis in patients with coronary heart disease.

Part IV: Regulation of apolipoprotein E gene expression by let-7b

Involvement of let-7b in the LPS-induced repression of apoE gene expression in macrophages

Previous studies have indicated an opposite effect of lipopolysaccharide (LPS) and of a dominant negative mutant c-Jun (dn-cJun) on apolipoprotein E (apoE) and let-7 expression levels. More specifically, apoE gene expression was shown to be repressed by LPS in macrophages (573) and to be induced upon overexpression of dn-cJun in mice via adenovirus-mediated gene transfer (574). The expression of let-7b was downregulated by dn-cJun overexpression (506) and let-7e was upregulated by LPS (532). As miRNAs are negative regulators of gene expression, we speculated that let-7b may target apoE gene expression in macrophages in response to LPS stimulation.

To assess this hypothesis, the effect of LPS on apoE gene expression in macrophages was initially investigated. For this purpose, RAW 264.7 mouse macrophages and THP-1 human monocytes (that served as control) were treated with 1μg/ml LPS for 18h and the apoE expression levels were determined by RT-PCR. As shown in Fig. 1, apoE expression was decreased up to 70% in macrophages in the presence of LPS while in monocytes apoE gene expression was induced 3-fold (Fig. 1), confirming previous reports of apoE suppression in macrophages upon inflammation (573).

To investigate the potential involvement of let-7b in the repression of apoE gene expression in response to LPS, RT-PCR analysis was performed following treatment of RAW 264.7 macrophages with let-7b antisense inhibitor molecule (as-let-7b) or a control scrambled molecule (scr-miR) in the presence or absence of LPS. Inhibition of let-7b expression by as-let-7b ameliorated the inhibitory effect of LPS on apoE gene expression resulting in 20% repression of the apoE expression levels in comparison to the 60% repression observed in cells treated with as-control (Fig. 2) indicating a role for let-7b in the LPS-induced apoE downregulation. In the absence of LPS as-let-7b had no effect on apoE gene expression (Fig.2).

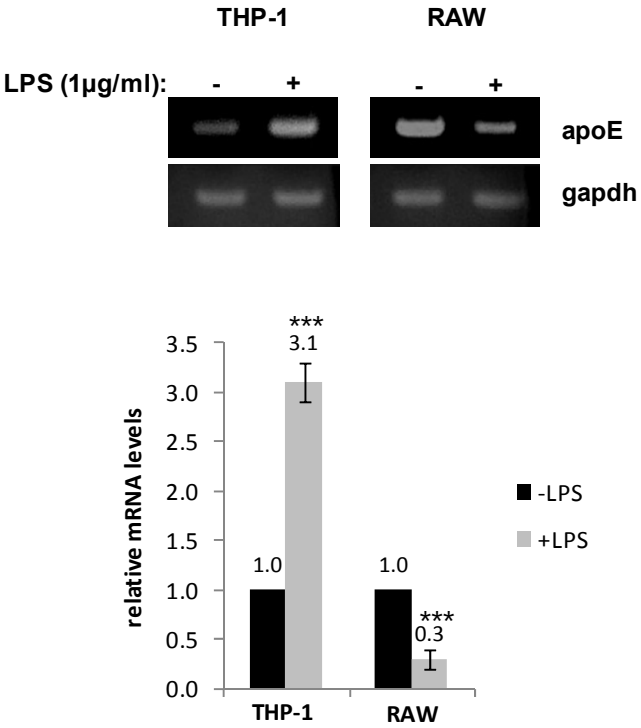

Figure 1. Modulation of apoE gene expression by LPS in monoytes and macrophages. Human monocytes THP-1 and mouse macrophages RAW 264.7 were incubated with 1µg/ml LPS for 18h and apoE mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoE gene are shown as a histograph. Each value represents the average from three independent experiments. Key: ***p<0.001

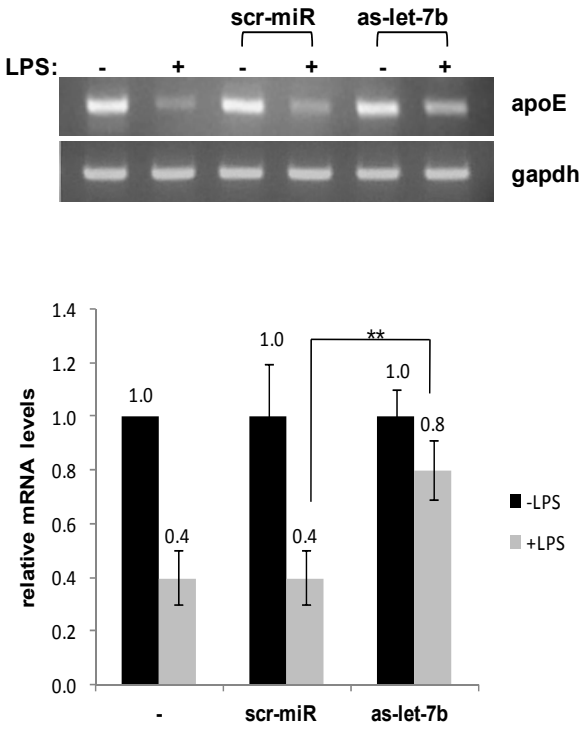

Figure 2. Partial reversal of LPS-induced downregulation of apoE gene expression upon inhibition of let-7b. RAW 264.7 were transfected with 100nM as-let-7b, 100nM scr-miR or left untransfected. Following transfection, cells were starved for 3h and subsequently treated with 1μg/ml LPS for 18h as indicated. Total RNA was extracted and apoE mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoE gene are shown as a histograph. Each value represents the average from three independent experiments. Key: **p<0.01

Inhibition of apoE gene expression by let-7b in hepatic cells

The regulatory role of let-7b on apoE gene expression was then evaluated in hepatic cells. For this purpose, HepG2 cells that do not express endogenously let-7 miRNAs at high levels were transfected with a double-stranded RNA that mimics the mature let-7b or a scrambled double-stranded RNA molecule and the expression levels of apoE were monitored. As shown in Fig. 3, overexpression of let-7b reduced apoE mRNA (A) and protein (B) levels by approximately 50% relative to cells overexpressing the scrambled miRNA. The mRNA levels of apoC-III remained unaffected indicating that let-7b-mediated downregulation is specific to apoE.

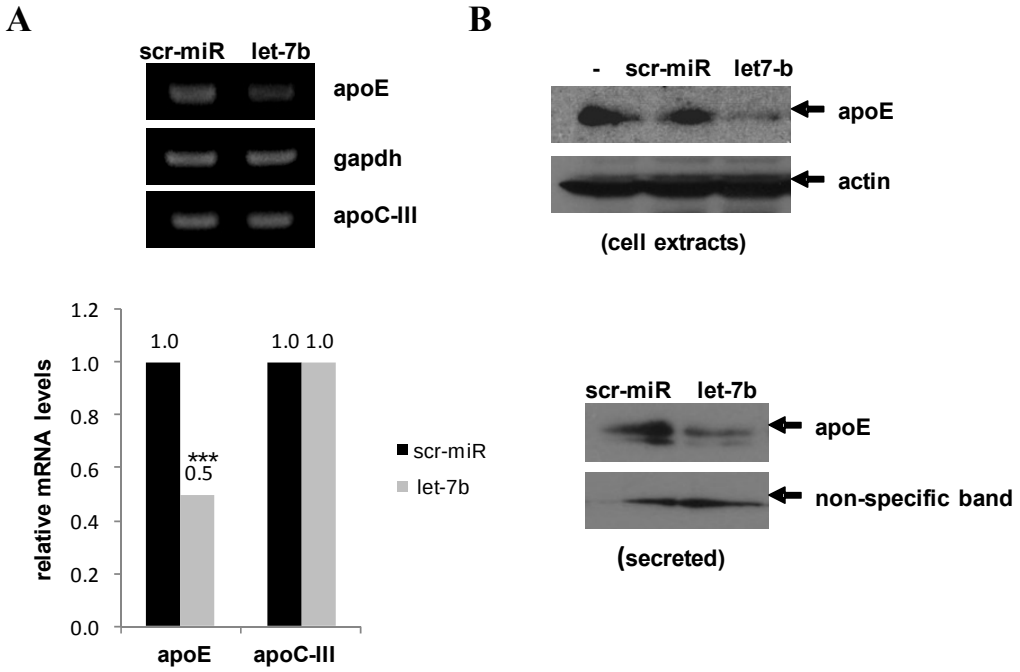

Figure 3. Downregulation of apoE gene expression by let-7b overexpression in hepatic cells. (A) HepG2 cells were transfected with 50nM let-7b or scr-miR. 48h after transfection, total RNA was extracted and apoE and apoC-III mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoE and apoC-III genes are shown as a histograph. Each value represents the average from three independent experiments. Key: ***$p<0.001$. (B) HepG2 cells were transfected with 50nM let-7b, 50nM scr-miR or left untrasfected and the intracellular protein levels of apoE and actin (loading control) as well as of the secreted apoE were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times and representative images are shown.

Having identified let-7b as a negative modulator of apoE gene expression in hepatic cells, the potential role of other isoforms of the let-7 family in the apoE gene regulation was examined. For this purpose, HepG2 cells were transfected with synthetic double-stranded mimics for several different members of the let-7 family (let-7a, b, c, d and f) as well as a scrambled double-stranded RNA molecule and the apoE mRNA levels were analyzed by RT-PCR. Similar to the results obtained from the previous experiment, let-7b overexpression was associated with a 40% reduction in apoE mRNA levels. However, none of the other let-7 isoforms examined had any significant effect on apoE gene expression. A slight reduction in apoE mRNA levels was observed upon overexpression of let-7a and let-7d that was not statistical significant suggesting that only the let-7b isoform of this family has a regulatory function on apoE gene expression. (Fig. 4)

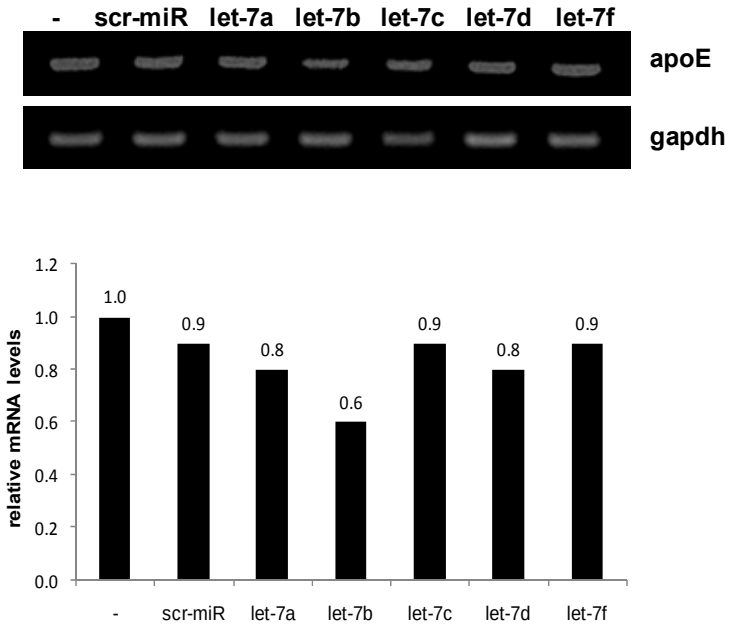

Figure 4. Effect of different let-7 isoforms on apoE gene expression in hepatic cells. HepG2 cells were transfected with 50nM let-7a, let-7b, let-7c, let-7d, let-7f or scr-miR as indicated. 48h after transfection, total RNA was extracted and apoE mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoE gene are shown as a histograph.

apoE is a direct target of let-7b

In silico analysis by Dr. D. Iliopoulos (Harvard Medical School) revealed comple188mentarity of let-7b with apoE predicting a potential binding site for let-7b in the position 33-53 of the 3'UTR of apoE (Fig. 5A). To confirm the in silico prediction of a direct interaction between let-7b and the apoE 3'UTR, a 153bp sequence corresponding to the 3'UTR of apoE including the let-7b binding site was cloned downstream of the luciferase gene, driven by the SV40 promoter, in the pGL3-promoter vector and was used in transient transfection assays. As shown in Fig. 5B, the luciferase apoE 3'UTR reporter was expressed at lower levels (30% reduction) in hepatic HepG2 cells, human embryonic kidney (HEK293T) cells and RAW 264.7 mouse macrophages as compared with the luciferase vector alone. Mutations in the seed sequence (in the second and fourth position) of the predicted let-7b binding site in the apoE-3'UTR reporter abolished suppression of luciferase expression in the three cell lines resulting in even a slight activation in HepG2 and HEK293T cells. Treatment of RAW 264.7 cells with LPS, that increases the endogenous levels of let-7b, further suppressed the wild type apoE-3'UTR reporter but not the corresponding mutant construct (Fig. 6). Together, these findings suggest that apoE is a direct target of let-7b.

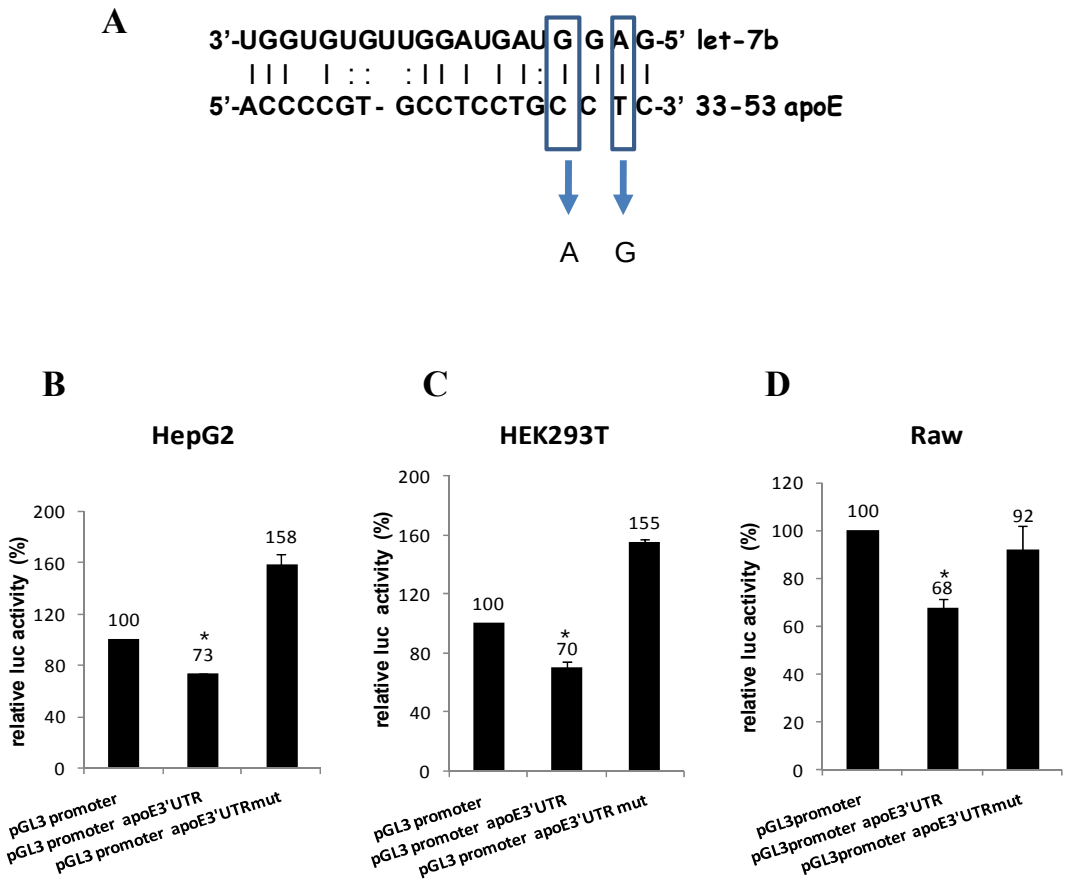

Figure 5. Let-7b target sthe 3' UTR of apoE. (A) Target sequence in the 3' UTR of apoE for let-7b. Nucleotide substitutions in the seed base-pairing sequence of the predicted let-7b target site generated in the apoE-3'UTR reporter are indicated by arrows. (B-D). HepG2 (B), HEK293T (C) or RAW 264.7 (D) cells were transiently transfected with the wild type apoE3'UTR-luc reporter plasmid, the mutated apoE3'UTR-luc reporter plasmid or the empty reporter vector (1.0µg) along with a β-galactosidase expression vector (1.0µg). Lucifearse activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments perfomed in triplicate. Key: *p<0.0.05.

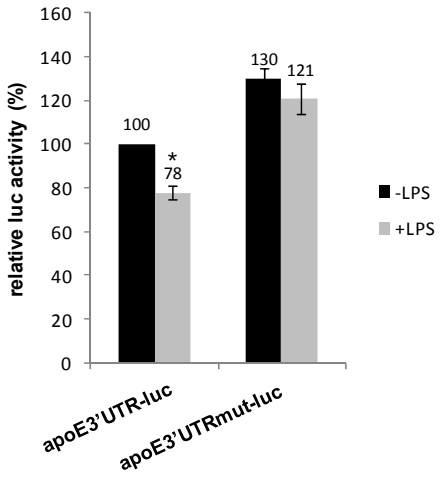

Figure 6. Effect of LPS on the activity of the luciferase reporter construct linked to the wild type or mutated 3' UTR of apoE in macrophages. RAW 264.7 macrophages were transiently transfected with the wild type apoE3'UTR-luc reporter plasmid or the mutated apoE3'UTR-luc reporter construct (1.0μg) along with a β-galactosidase expression vector (1.0μg). Following transfection, cells were starved for 3h and subsequently treated with 1μg/ml LPS for 18h as indicated. Lucifearse activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments perfomed in triplicate. Key: *p<0.0.05.

Discussion

ApoE is mainly expressed in the liver, kidney, brain and various cell types such as macrophages and plays an important role in lipid metabolism facilitating low-density lipoprotein clearance and enhancing reverse cholesterol transport. Studies in transgenic and knock out mice have revealed the significance of apoE secretion by macrophages as it facilitates the efflux of cholesterol from foam cells, inhibiting the formation of the atherosclerotic plaque. During infection and inflammation that promote atherogenic plaque formation, the expression of apoE in macrophages is reduced. Treatment of mouse macrophages RAW264.7 with lipopolysaccharide (LPS) that mimics infection has been shown to represses apoE gene expression via signaling pathways that involve the upstream kinases Tpl-2 and MEKK1, the intermediate mitogen-activated protein kinases ERK and JNK, and the downstream transcription factors AP-1 and NF-kB that inhibit the apoE promoter activity via distinct regions (573) (Fig.7). The effects of LPS on apoE gene expression, however, are not consistent in different cell types as upregulation of apoE gene expression has been reported in murine bone marrow-derived monocytic precursors that do not synthesize apoE (575) or astrocytes (576), suggesting that apoE gene regulation by LPS depends on the cellular context. In agreement with this, apoE was differentially modulated by LPS treatment in monocytes and macrophages with a significant downregulation in RAW 264.7 macrophages.

With the aim to identify new mechanisms of apoE gene regulation and based on recent studies that have revealed LPS-mediated stimulation of let-7 microRNA expression (532), we investigated the potential role of let-7b in apoE gene expression and its potential involvement in the LPS-induced repression of apoE gene expression in macrophages.

For this purpose, silencing of let-7b expression was performed in RAW 264.7 macrophages with the use of as-let-7b prior to LPS stimulation. It was shown that apoE downregulation by LPS was partially reversed in the presence of as-let-7b indicating that in addition to the signaling pathways of Tpl2 and MEKK1 kinases, let-7b is also involved in LPS-mediated apoE suppression (Fig. 7). The lack of apoE upregulation by let-7b

inhibition in the absence of LPS, suggests that let-b is expressed at low levels under basal (serum-starved) conditions in macrophages and confirms the LPS-induced upregulation of let-7b, similar to other let-7 isoforms. However, in contrast to the let-7e and let-7i isoforms which were recently shown to directly target the LPS receptor TLR-4 in macrophages and cholangiocytes ,respectivey (532,533), let-7b does not seem to target TLR4 because if this was the case enhanced repression of apoE gene expression would be expected upon inhibition of let-7b due to unimpaired LPS signaling. Let-7b and let-7e are the most divergent miRNAs among the let-7 family members differing in four nucleotides which might explain their different function on TLR4.

The amelioration of apoE repression by LPS upon inhibition of let-7b could be attributed to two potential functions of let-7b: a) an indirect, through let-7b targeting of a positive regulator of apoE gene expression b) direct, through let-7b targeting apoE 3'UTR. To assess the validity of the second hypothesis, let-7b overexpression experiments were performed in hepatic cells under basal conditions. It was found that apoE expression levels were reduced in hepatic cells overexpressing let-7b and the reduction was comparable at both the mRNA and protein levels suggesting that let-7b targets apoE at the transcriptional level. Other members of the let-7 family, let-7a (that belongs to the same gene cluster with let-7b), let-7c, let-7d and let-7f, failed to suppress apoE gene expression indicating that the interaction was specific for apoE and the let-7b isoform.

The functional interaction between let-7b and apoE was confirmed by luciferase reporter assays using reporter constructs containing the in silico predicted let-7b target site in the apoE 3'UTR linked to the 3'end of the luciferase gene. Basal luciferase expression was suppressed by the apoE 3'UTR and this suppression was further enhanced in the presence of LPS in macrophages while mutations at the seed sequence abolished both basal and LPS-induced suppression identifying apoE as a direct target of let-7b. The low, however, suppressive effect of apoE 3'UTR on basal luciferase activity of the reporter vector may be due to the low endogenous levels of let-7b in the cell lines tested as well as due to the single predicted target site in the 3'UTR as most miRNAs are known to act through multiple target sites. Although, let-7b has imperfect complementarity with

apoE suggesting regulation of apoE at the protein level, we obtained evidence for a pre-translational mode of action of let-7b on apoE gene expression.

Taken together, the above findings suggest that let-7b inhibits the expression of human and mouse apoE by targeting the apoE 3'UTR for mRNA degradation and imply its potential contribution in enhanced atherogenesis. Let-7b is mainly known to work in a tumor-suppressor role as low levels of let-7b is a poor prognostic indicator of survival in serous ovarian cancer, acute lymphoblastic leukemia and melanoma (577-579). However, the functional consequences of the let-7b-driven downregulation of apoE mRNA levels have not been tested. Given the important functions of apoE in lipid metabolism and protection from atherosclerosis and the need for identification of new mechanisms to treat atherosclerosis-associated diseases, the potential roles of let-7b on reverse cholesterol transport, triglyceride clearance and atherosclerosis development need to be explored.

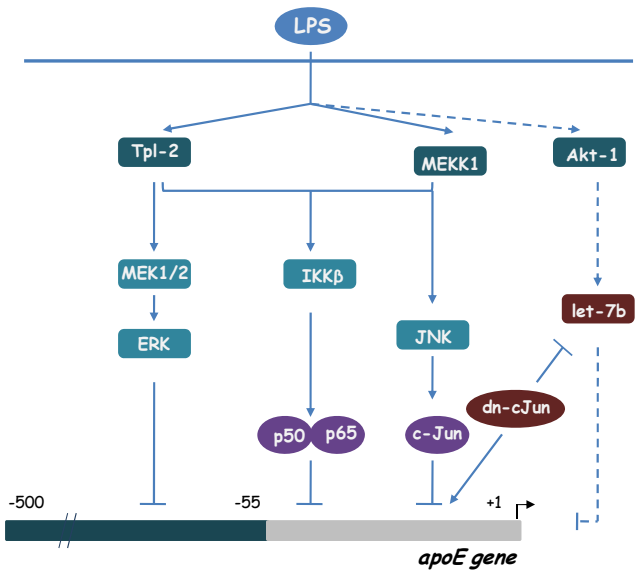

Figure 7. Schematic model of apoE gene regulation by LPS in macrophages. apoE dwon-regulation induced by LPS in macrophages is mediated by two upstream kinases (Tpl-2 and MEKK1), leading to the activation of intermediate kinases (IKKβ, JNK, MEK and ERK) that lead to the activation of NF-kB and AP-1 complexes and possibly other factors acting on different regions of the apoE promoter. Additionally, let-7b (potentially activated by Akt1) could mediate the LPS-induced downregulation of apoE through targeting the apoE 3'UTR for mRNA degradation.

Part V: Reglation of lipid-associated gene expression by miR-122

With the aim to elucidate new mechanisms governing the expression of genes associated with cholesterol and triglyceride metabolism in the liver, the role of miR-122, an abundant liver-specific miRNA, in the post-transcriptional regulation of their expression was investigated.

For this reason, hepatic HepG2 cells as well as HEK293T cells that do not express endogenously miR-122 at high levels were transiently transfected with a double-stranded RNA that mimics the mature miR-122 or a scrambled double-stranded RNA molecule and the expression levels of various genes involved in lipid metabolism, such as apolipoproteins and transcription factors regulating lipid-associated genes were analyzed by RT-PCR. As shown in Figure 1, overexpression of miR-122 in HepG2 cells upregulated apolipoprotein B (apoB) gene expression by 3.5-fold while it downregulated the expression of cationic amino acid transporter (CAT-1), a well known direct target gene of miR-122 by 50% indicating that miR-122 regulation is specific. The expression of the other apolipoprotein genes tested, apoA-I, apoC-III, apoA-IV and apoM were not affected by miR-122 (Fig.1)

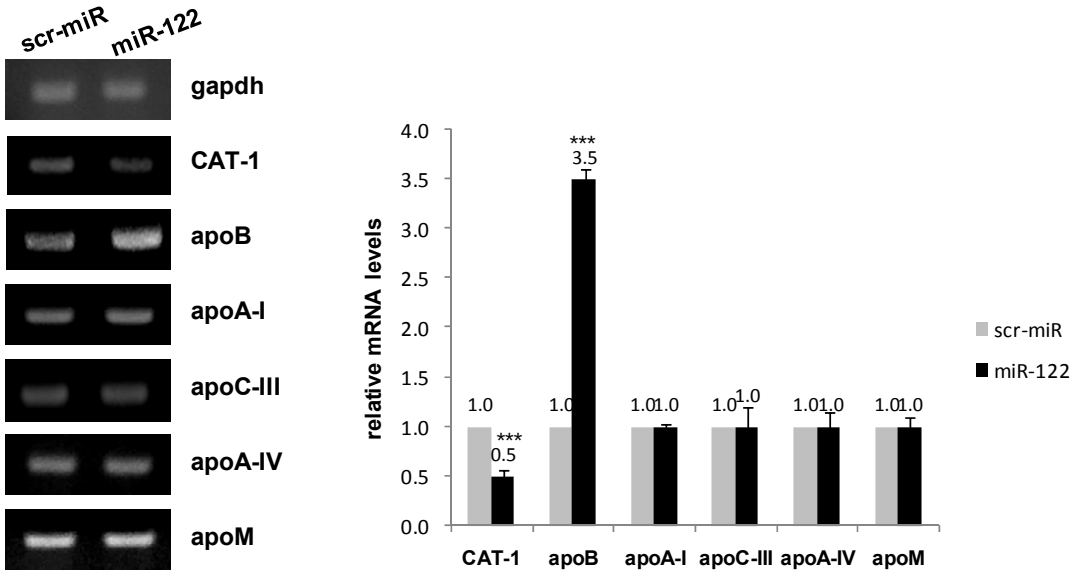

Figure 1. Effect of miR-122 on apolipoprotein gene expression in hepatic cells. HepG2 cells were transiently transfected with 100nM miR-122 or scr-miR. 48h after transfection, total RNA was extracted and apolipoprotein mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apolipoprotein genes are shown

as a histograph. Each value represents the average from three independent experiments. Key: ***p<0.001.

Analysis of the expression levels of two genes encoding for major proteins involved in the reverse cholesterol transport pathway, ABCA1 and SR-BI, revealed a 2.4 upregulation of the ABCA1 transporter upon overexpression of miR-122 as compared to cells overexpressing the scrambled miRNA and no change in the expression of the SR-BI gene (Fig. 2). In HEK293T cells, similar upregulation of ABCA1 by miR-122 was observed (Fig. 5).

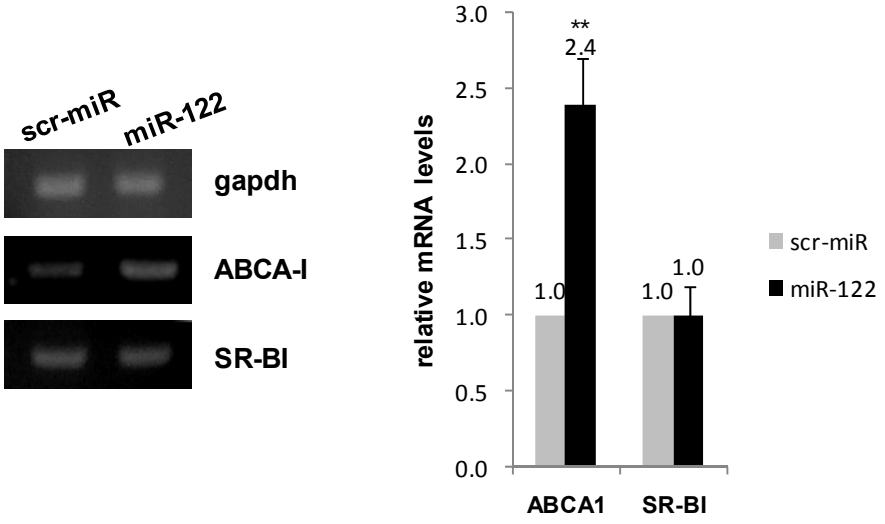

Figure 2. Effect of miR-122 on the expression of genes involved in RCT in hepatic cells. HepG2 cells were transiently transfected with 100nM miR-122 or scr-miR. 48h after transfection, total RNA was extracted and ABCA1 and SR-BI mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the ABCA1 and SR-BI genes are shown as a histograph. Each value represents the average from three independent experiments. Key: ***p<0.01.

Among the sterol regulatory element binding protein (SREBP) family of transcription factors involved in fatty acid and cholesterol synthesis, all three isoforms, (SREBP-1a, SREBP-1c and SREBP-2) were upregulated in cells transfected with the exogenous miR-122 as compared to scrambled transfected cells with the strongest activation exerted on SREBP1-a (3.1 fold) (Fig.3). SREBP-1c and SREBP-2 were

upregulated 2-, 3- and 1.9- fold respectively. Furthermore, the expression of stearoyl-coenzyme A desaturase-1 (SCD-1) involved in lipogenesis was increased 3.5- fold (Fig. 3). Similar results were obtained in HEK293T cells (Fig. 5)

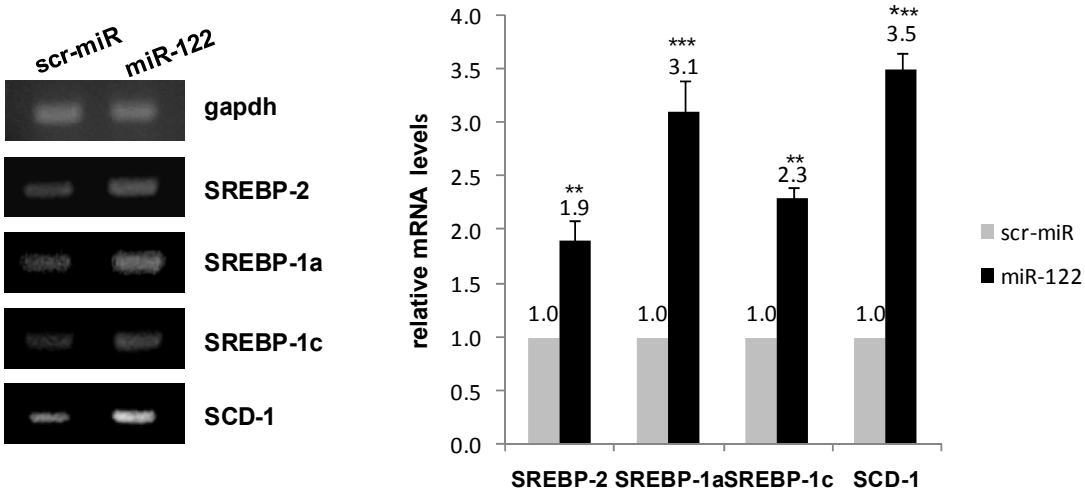

Figure 3. Effect of miR-122 on SREBP gene expression in hepatic cells. HepG2 cells were transiently transfected with 100nM miR-122 or scr-miR. 48h after transfection, total RNA was extracted and SREBP-2, SREBP-1a, SREBP-1c as well as Scd-1 mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the above genes are shown as a histograph. Each value represents the average from three independent experiments. Key: **p<0.01, ***p<0.01.

Finally, the expression of HNF-4, the key transcription factor in the liver and of its coactivator PGC-1α was examined. Overexpression of miR-122 was associated with a slight increase of HNF-4 gene expression up to 1.8 fold while it had no affect on PGC-1α gene expression levels (Figure 4).

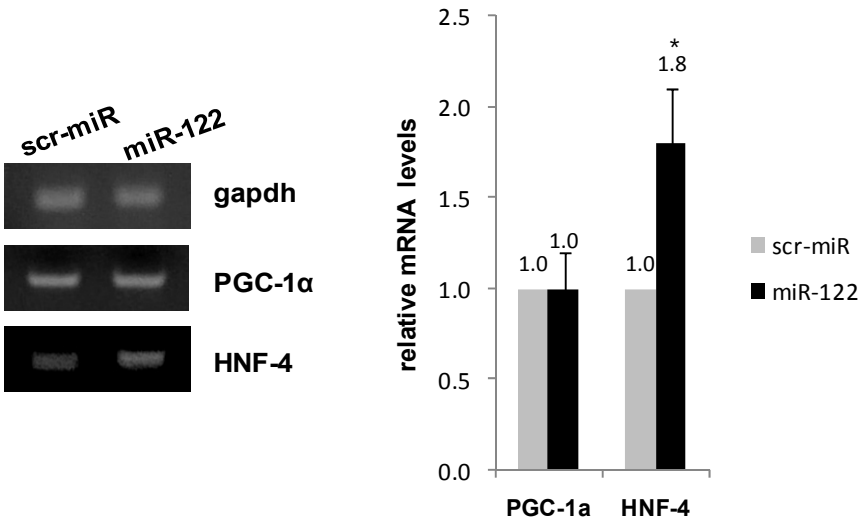

Figure 4. Effect of miR-122 on HNF-4 and PGC-1a gene expression in hepatic cells. HepG2 cells were transiently transfected with 100nM miR-122 or scr-miR. 48h after transfection, total RNA was extracted and HNF-4 and PGC-1a mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the HNF-4 and PGC-1a genes are shown as a histograph. Each value represents the average from three independent experiments. Key: *p<0.05.

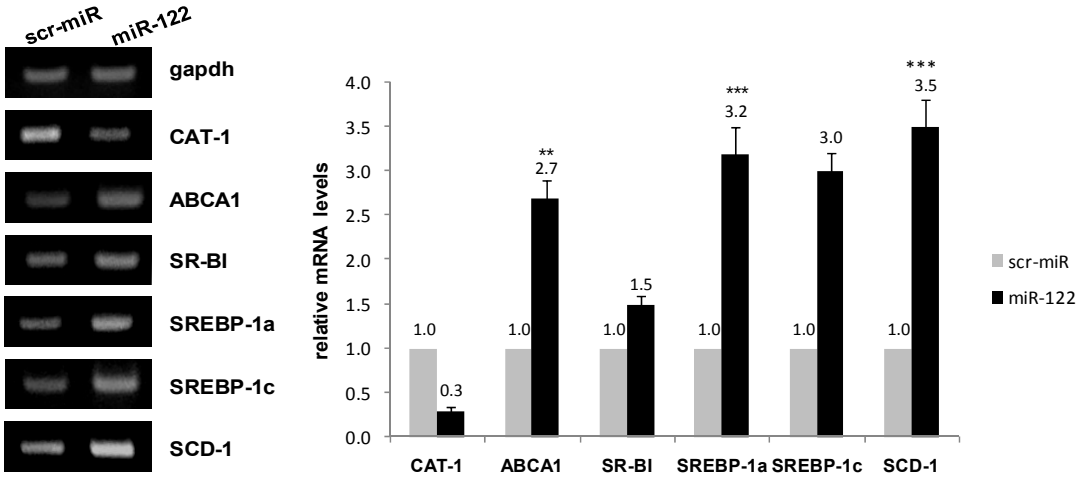

Figure 5. Effect of miR-122 on the expression of genes associated with lipid metabolism in HEK293T cells. HEK293T cells were transiently transfected with 100nM miR-122 or scr-miR. 48h after transfection, total RNA was extracted and the mRNA levels of CAT-1, ABCA1, SR-BI, SREBP-1a, SREBP-1c and SCD-1 genes were analyzed by RT-PCR.

The normalized (relative to GAPDH) mRNA levels of the above genes are shown as a histograph. Each value represents the average from three independent experiments. Key: *$p<0.05$.

Discussion

MiR-122 is the predominant miRNA in the liver and has been demonstrated to be important for normal lipid metabolism. Overexpression and knockdown studies in mice have shown a positive association between miR-122 expression levels and plasma cholesterol and triglyceride levels, hepatic cholesterol levels and lipid accumulation, as well as fatty acid and cholesterol synthesis (511,512). On the other hand, an inverse correlation of miR-122 with β-oxidation rate was observed. Despite its role in lipid metabolism, a direct target gene of miR-122 associated with lipid metabolism has not been identified while it was shown to be an indirect modulator of genes involved in fatty acid and cholesterol synthesis, such as acyl-CoA carboxylase -1 (ACC-1), fatty acid synthase (FAS), phosphomevalonate kinase (PMVK) and 3-hydroxy-3-methylglutaryl-CoA-reductase (HMG-CoA reductase) (511).

To further elucidate the role of miR-122 in cholesterol and triglyceride metabolism in the liver, we investigated the effect of its overexpression on additional-lipid associated genes, including apolipoproteins, the ABCA1 transporter and the SRB1 receptor playing essential roles in the reverse transport of cholesterol, the SREBP family of transcription factors that regulate the expression of genes involved in fatty acid and cholesterol biosynthesis as well as the central transcription factor in the liver, HNF-4.

Among apolipoproteins, apolipoprotein B involved in VLDL synthesis and secretion was the only gene affected by miR-122 overexpression in line with a previous study showing decreased apoB protein in the plasma of mice treated with antisense oligonucleotide (ASO) inhibition of miR-122 (511) while the expression of the genes of the apoA-I/apoC-III/apoA-IV cluster or apoM remained unaffected. Furthermore, this the expression of the ABCA1 transporter that is essential for cholesterol efflux was significant increased in both hepatic and non-hepatic cells. The reduced plasma cholesterol levels reported upon inhibition of miR-122 (512) could therefore be also attributed to reduced expression of ABCA1 and HDL biogenesis.

SREBP-2, SREBP-1a and SREBP-1c transcription factors, involved in cholesterol and fatty acid synthesis were all induced upon miR-122 overexpression in both hepatic and non-hepatic cells as well as SCD-1, the rate limiting enzyme in the synthesis of fatty acids. SCD-1 is regulated by SREBP-1 and in turn affects FAS suggesting that SREBP-1 lies upstream in the array of genes modulated by miR-122. During the performance of these experiments, a similar study was reported showing increased expression of SREBP-1c as early as 24h after miR-122 overexpression while FAS responded later to miR-122 treatment, 48-72h. In addition, silencing of SREBP-1c inhibited the miR-122 mediated upregulation of FAS (506). In contrast, SREBP-2 was found not to be affected while another study by Cheung et al. showed, in agreement with our results, upregulation of SREBP-2 (515).

Interestingly, SREBP2 and SREBP1 were recently found to be the host genes for two miR-33 isoforms, miR-33a and miR-33b, that target the expression of ABCA1(509,510). According to this, miR-122 mediated induction of SREBP1 and 2 is expected to lead to increased miR-33a/b levels and reduced ABCA1 expression through targeting its 3' UTR which is in contrast to the increased ABCA1 expression we previously observed. Probably miR-122 affects positively and negatively ABCA1 gene expression through different mechanisms and the net result is an increase in ABCA1 expression levels.

Taken together, these data are in line with previous studies showing increased lipogenesis upon upregulation of miR-122 in the liver as well as with the recently published observation of miR-122-mediated induction of SREBP-1c and add SREBP-1a ABCA1 and possibly HNF-4 to the list of genes indirectly regulated by miR-122. Based on these data, a regulatory role of miR-122 on the expression of SR-BI, PGC-1a, apoA-I, apoC-III, apoA-IV and apoM could be excluded. In search of a direct target of miR-122 that would account for the increased expression of lipogenic genes, a negative regulator of LXR seems a good candidate, as LXR is a common critical regulator of both SREBP and ABCA1 genes and was found to be upregulated by miR-122 (506). Initial experiments showed a reduction in c-Jun protein levels upon miR-122 overexpression in HepG2 cells suggesting that either c-Jun itself represents a direct target gene of miR-122

or even PKC could be implicated. In favor of the latter hypothesis is the fact that PKC negatively regulates LXR transcriptional activity (572) while it activates c-Jun, thus representing an interesting upstream target gene for miR-122. Furthermore, overexpression of dn-cJun via adenovirus-mediated gene transfer in the liver of mice resulted in increased levels of miR-122 suggesting that c-Jun may be associated with miR-122 both as a regulator of miR-122 expression and as a mediator of its downstream effects on lipogenic genes.

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Regulation of Human Apolipoprotein M Gene Expression by Orphan and Ligand-dependent Nuclear Receptors*[S]

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Apolipoprotein M (apoM) plays an important role in the biogenesis and the metabolism of anti-atherogenic HDL particles in plasma and is expressed primarily in the liver and the kidney. We investigated the role of hormone nuclear receptors in *apoM* gene regulation in hepatic cells. Overexpression via adenovirus-mediated gene transfer and siRNA-mediated gene silencing established that hepatocyte nuclear factor 4 (HNF-4) is an important regulator of *apoM* gene transcription in hepatic cells. *apoM* promoter deletion analysis combined with DNA affinity precipitation and chromatin immunoprecipitation assays revealed that HNF-4 binds to a hormone-response element (HRE) in the proximal *apoM* promoter (nucleotides −33 to −21). Mutagenesis of this HRE decreased basal hepatic *apoM* promoter activity to 10% of control and abolished the HNF4-mediated transactivation of the *apoM* promoter. In addition to HNF-4, homodimers of retinoid X receptor and heterodimers of retinoid X receptor with receptors for retinoic acid, thyroid hormone, fibrates (peroxisome proliferator-activated receptor), and oxysterols (liver X receptor) were shown to bind with different affinities to the proximal HRE *in vitro* and *in vivo*. Ligands of these receptors strongly induced human *apoM* gene transcription and *apoM* promoter activity in HepG2 cells, whereas mutations in the proximal HRE abolished this induction. These findings provide novel insights into the role of apoM in the regulation of HDL by steroid hormones and into the development of novel HDL-based therapies for diseases such as diabetes, obesity, metabolic syndrome, and coronary artery disease that affect a large proportion of the population in Western countries.

Numerous epidemiological studies have shown that levels of high density lipoprotein (HDL) cholesterol in plasma are inversely related to atherosclerosis susceptibility in humans (1–4). The atheroprotective properties of HDL involve cholesterol removal from macrophages of the arterial wall, anti-oxidation, anti-inflammation, and anti-thrombotic functions as well as protection of endothelial cells from apoptosis (5–8). Biogenesis of HDL involves the interaction of apolipoprotein A-I (apoA-I), which is synthesized and secreted by the liver, with the cholesterol and phospholipid membrane transporter ATP-binding cassette transporter A1 (ABCA1) (9–14). The premature HDL particles thus formed (called pre-β-HDL) are subsequently remodeled by various plasma enzymes to form the mature spherical αHDL particles that are catabolized by membrane receptors such as the scavenger receptor class B, type I (9–14).

Apolipoprotein M (apoM)[2] has been shown recently to participate in HDL maturation in plasma. ApoM is a 26-kDa glycoprotein that belongs to the lipocalin protein superfamily and has been shown to bind lipophilic ligands in its hydrophobic binding pocket (15–18). ApoM is secreted by the liver and associates with HDL through its retained N-terminal signal peptide (19, 20). ApoM is also secreted by the kidney and is involved in the recycling of small lipophilic ligands via the multiple ligand receptor megalin (15).

Studies in humans and in mice overexpressing or lacking apoM have shown a positive association between plasma apoM levels and total as well as HDL and LDL cholesterol concentrations (21–23). Wolfrum *et al.* (24) demonstrated that the lack of apoM expression in transcription factor-1α/hepatocyte nuclear factor-1α (TCF-1α/HNF-1α) knock-out mice or in apoM small interfering RNA-injected mice leads to formation of larger size HDL1 particles and the disappearance of pre-β-HDL particles in plasma suggesting that apoM may play a role in HDL remodeling, particularly with regard to metabolism of pre-β-HDL. Adenoviral apoM overexpression in LDL receptor-deficient mice (animal model for premature atherosclerosis) or hepatic overexpression of apoM in apoM transgenic mice led to a reduction in atherosclerosis development (23, 24). ApoM was also found to be positively associated with pre-β-HDL formation in type 2 diabetes subjects (22). ApoM-containing HDL particles isolated from human plasma and apoM transgenic mice have been shown to be more resistant to oxidation and more efficient in protecting against LDL oxidation as well as stimulating cholesterol efflux from macrophage foam cells (23, 25).

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[2] The abbreviations used are: apoM, apolipoprotein M; SHP, small heterodimer partner; DNAP, DNA-affinity precipitation; HRE, hormone-response element; RXR, retinoid X receptor; RAR, retinoid acid receptor; TR, thyroid hormone receptor; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; 9-*cis*-RA, 9-*cis*-retinoic acid; FXR, farnesoid X receptor; m.o.i., multiplicity of infection; T_3, triiodothyronine; 22(OH)C, 22(*R*)-hydroxycholesterol.

Genetic association studies in Chinese populations have associated two single-nucleotide polymorphisms located in the apoM proximal promoter region (single-nucleotide polymorphisms T-778C and SNP T-855C) with the development of coronary artery disease (26, 27). The T-778C polymorphism was also found to be associated with types 1 and 2 diabetes in Chinese populations supporting the possible involvement of apoM in the pathogenesis of diabetes as suggested by the reduced apoM expression levels found in animal models of diabetes and some patients with diabetes and metabolic syndrome (22, 28–34).

ApoM is expressed mainly in the liver and kidney (35), but the regulatory mechanisms that control human *apoM* gene transcription are not well understood. In the liver, apoM transcription has been shown to be controlled mainly by TCF-1α/HNF-1α, liver receptor homolog-1 (LRH-1), and Forkhead box A2 (FOXA2) transcription factors (33, 36, 37). Small heterodimer partner (SHP) has been shown to inhibit *apoM* gene expression in response to bile acids by interfering with the function of LRH-1, which binds to the proximal *apoM* promoter (36). Treatment of cells with lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or interleukin 1 (IL-1) decreased *apoM* mRNA levels and the secretion of apoM, although in humans with acute bacterial infections or chronic HIV infection, serum apoM levels were decreased (38).

In this study, we demonstrate that hepatocyte nuclear factor-4α (HNF-4α) positively regulates *apoM* gene expression via a hormone-response element in the proximal promoter adjacent to the previously characterized HNF-1α-binding site. HNF-4α is an orphan member of the hormone nuclear receptor gene superfamily (39, 40) and a key transcription factor in the liver, regulating numerous target genes involved in lipoprotein metabolism, including apolipoproteins, cholesterol synthesis enzymes, and bile acid transporters (41). Mice lacking HNF-4α expression in the liver have altered lipid metabolism exhibiting lipid accumulation in the liver and greatly reduced plasma total and HDL cholesterol levels (42). Recent genome-wide association studies identified a common variant in the coding region of *HNF-4α* gene (rs1899861) associated with low HDL concentrations in humans (43). A cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells showed a 4.5-fold increase in apoM expression levels (44). Using siRNA-mediated gene silencing and adenovirus-mediated gene transfer, we show here that HNF-4 is an important regulator of *apoM* gene expression in hepatic cells. HNF-4 binds to a hormone-response element (HRE) present in the proximal *apoM* promoter. The same HRE mediates induction of *apoM* gene transcription by ligands of other members of the hormone nuclear receptor superfamily such as homodimers of retinoid X receptor α (RXRα) and heterodimers of RXRα with retinoic acid receptor (RAR), thyroid hormone receptor β (TRβ), peroxisome proliferator-activated receptor (PPAR). and liver X receptor (LXR).

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, and trypsin/EDTA for cell culture were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Charcoal-stripped serum was prepared after treatment of FBS with charcoal and dextran. Restriction enzymes and T4 DNA ligase were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). Go *Taq*DNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega Corp. (Madison, WI). 9-*cis*-Retinoic acid (9-*cis*-RA), fenofibrates, 22(*R*)-hydroxycholesterol, T_3, poly(dI/dC), *o*-nitrophenyl β-D-galactopyranoside, PMSF, aprotinin, and benzamidine were purchased from Sigma. T0901317 was purchased from Cayman Chemicals (Ann Arbor, MI). QuikChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). Protein G-Sepharose were purchased from GE Healthcare. TRIzol reagent for RNA extraction, SuperScript RNase H-reverse transcriptase, random hexamers, Opti-MEM, Lipofectamine 2000, and Dynabeads M-280 streptavidin were purchased from Invitrogen. The SuperSignal West Pico chemiluminescent substrate was purchased from Pierce. Anti-HNF-4α (C-19), anti-RXRα (D-20), anti-RARα (C-20), anti-LXR (H-144), anti-TRβ1 (J51), and anti-PPARα (H-98) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Myc (9E-10), anti-FLAG M2 (F-3165), and the anti-goat peroxidase-conjugated secondary antibody were purchased from Sigma. Anti-LRH-1 (ab18293) antibody was purchased from Abcam (Cambridge, MA). Anti-apoM (NB100-57088) was purchased from Novus Biologicals (Littleton, CO). Anti-actin and anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Chemicon International Inc. (Temecula, CA). Biotinylated oligonucleotides were synthesized at VBC Biotech (Vienna, Austria). All other oligonucleotides were synthesized at the microchemical facility of the Institute of Molecular Biology and Biotechnology (Heraklion, Greece).

Plasmid Construction—The human *apoM* promoter constructs (−950/+42, −642/+42, −402/+42, −241/+42, −105/+42, and −49/+42)h*apoM*-luc were generated by PCR amplification of the corresponding fragments using human genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector (Promega Corp.). The *apoM* promoter construct (−20/+8)h*apoM*-luc was generated by ligation of a double-stranded oligonucleotide corresponding to the −20/+8 region of the human *apoM* promoter into the KpnI-HindIII sites of the pGL3basic vector. The (−950/+42)mut h*apoM*-luc, which bears mutations in the HRE, was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mouse *apoM* promoter construct (−761/−7)m*apoM*-luc was generated by PCR amplification of the corresponding fragment using mouse genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector. The sh-HNF-4- and sh-control-producing vectors were generated by ligation of a double-stranded oligonucleotide that contained the siRNA-expressing sequence targeting HNF-4 or a scrambled sequence (si-control) into the BglII-HindIII sites of the pSuper.GFP/neo vector (Oligoengine, Seattle). The sequence of all oligonucleotides is shown in supplemental Table 1. The expression vectors pcDNA3-myc-LRH-1 and pCMV-SHP were kindly provided by Dr. Ioannis

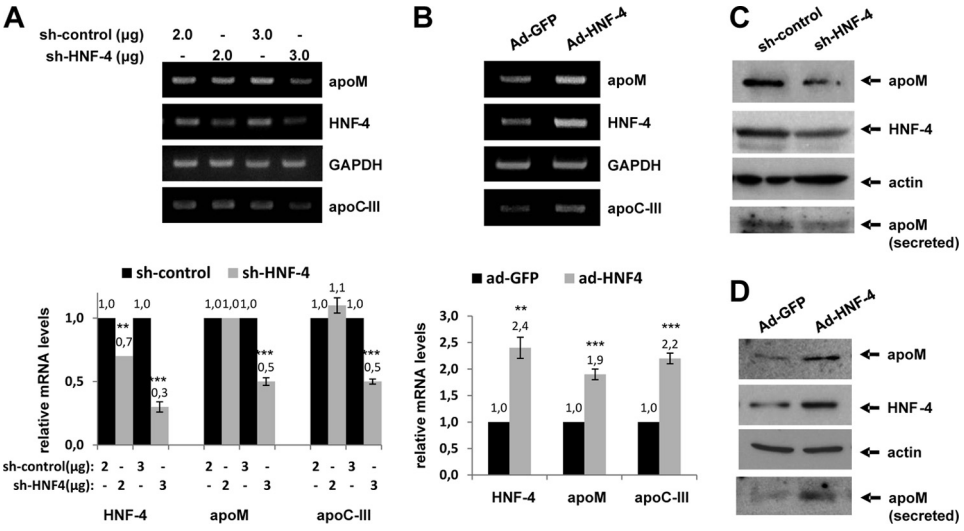

FIGURE 1. **HNF-4α is a positive regulator of human *apoM* gene expression.** *A,* human hepatoma HepG2 cells were transiently transfected with increasing amounts (2.0 and 3.0 μg) of sh-HNF-4α or sh-control producing vectors. 48 h later, total RNA was extracted, and *apoM*, *HNF-4α*, and *apoC-III* mRNA levels were analyzed by RT-PCR. The mRNA levels of the *GAPDH* gene were determined for normalization purposes. The relative mRNA levels of the *apoM*, *HNF-4α*, and *apoC-III* genes were quantified and are shown as a *histograph*. Each value represents the average from three independent experiments. *B,* HepG2 cells were infected with a control adenovirus expressing GFP (Ad-GFP) or with a recombinant adenovirus expressing HNF-4α (Ad-HNF-4) at an m.o.i. of 10. 24 h later, total RNA was extracted, and the mRNA levels of *apoM*, *HNF-4α*, and *apoC-III* were analyzed by RT-PCR. The mRNA levels of the *GAPDH* gene were determined for normalization purposes. The relative mRNA levels of the *apoM*, *HNF-4α*, and *apoC-III* genes were quantified and are shown as a *histograph*. Each value represents the average from three independent experiments. *C,* HepG2 cells were transiently transfected with 3.0 μg of sh-HNF-4α- or sh-control-producing vector, and the intracellular protein levels of apoM, HNF-4α, and actin (loading control) as well as of the secreted apoM were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. *D,* HepG2 cells were infected for 24 h with Ad-GFP or Ad-HNF-4 at an m.o.i. of 10, and the intracellular protein levels of apoM, HNF-4α, and actin (loading control) as well as of the secreted apoM were determined by immunoblotting. The experiment was performed three times, and representative images are presented. **, $p < 0.01$; ***, $p < 0.001$.

Talianidis (Biomedical Sciences Research Center Alexander Fleming, Vari, Greece).

Cell Culture, Transient Transfection, and Reporter Assays—Human hepatoma HepG2 cells and human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. For the treatment of cells with 9-*cis*-retinoic acid, fibrates, 22(*R*)-hydroxycholesterol, T0901317, and T_3, 10% FBS was replaced by 5% charcoal-stripped serum. Transient transfections were performed using the $Ca_3(PO_4)_2$ co-precipitation method. Transient transfections of cells with the shRNA producing vectors were performed with the Lipofectamine™ 2000 reagent according to the manufacturer's instructions. Luciferase assays were performed using the luciferase assay kit from Promega Corp. according to the manufacturer's instructions. Normalization for transfection efficiency was performed by β-galactosidase assays.

Adenovirus Generation and Infection—Recombinant adenoviruses were produced and amplified using the AdEasy adenoviral system (45). Briefly, the human HNF-4α and RXRα cDNA were first cloned in the pAdTrack-CMV vector and then recombined with the pAdEasy1 vector to produce Ad-GFP-HNF-4α or Ad-GFP-RXRα. Empty virus expressing only GFP served as control (Ad-GFP). All viruses were amplified in 911 cells, and viral titers were estimated by a fluorescence forming assay (46). HepG2 cells were infected at an m.o.i. of 10 by adding virus stocks directly to the cell culture medium.

Reverse Transcription-PCR (RT-PCR)—Total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions. One μg of this RNA was reverse-transcribed with random hexamers, and the cDNAs produced were used for PCR amplification with the primers shown in supplemental Table 2. For the normalization of the samples, the cDNA of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was also amplified by PCR. Gene expression levels are expressed relative to *GAPDH* mRNA levels. The quantification of the results was performed by measuring the intensity of the bands using the Tinascan version 2 software of Raytest (Straubenhardt, Germany).

Immunoblot Analysis—For the purification of protein extracts, cells were washed with ice-cold PBS, collected by centrifugation at 5000 rpm for 5 min at 4 °C, and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitors. Lysates were allowed to rotate at 4 °C for 30 min and purified by centrifugation at 13,000 rpm for 5 min at 4 °C. Protein concentration was measured using the Bio-Rad DC protein assay kit, and equal amounts were loaded on SDS, 10.5% (w/v) polyacrylamide gels followed by electrotransfer onto nitrocellulose membranes. Proteins were visualized by probing the membrane with appropriate monoclonal or polyclonal antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence. To normalize the variations for protein amounts, membranes were stripped and re-probed with an antibody to β-actin.

Chromatin Immunoprecipitation (ChIP) Assays—Chromatin immunoprecipitation was performed as described previously (47) using chromatin from HepG2 cells and antibodies against HNF-4α, RXRα, RARα, PPARα, LXRα, and TRβ1. Immunoprecipitated chromatin was analyzed by PCR using primers shown in supplemental Table 3. PCR products were analyzed by agarose gel electrophoresis.

DNA Affinity Precipitation (DNAP)—For the purification of nuclear extracts, HepG2 or transfected HEK293T cells were washed with ice-cold PBS, collected by centrifugation at 4000 rpm for 10 min at 4 °C, and resuspended in a hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.2 mM EDTA) supplemented with protease inhibitors. After a 10-min

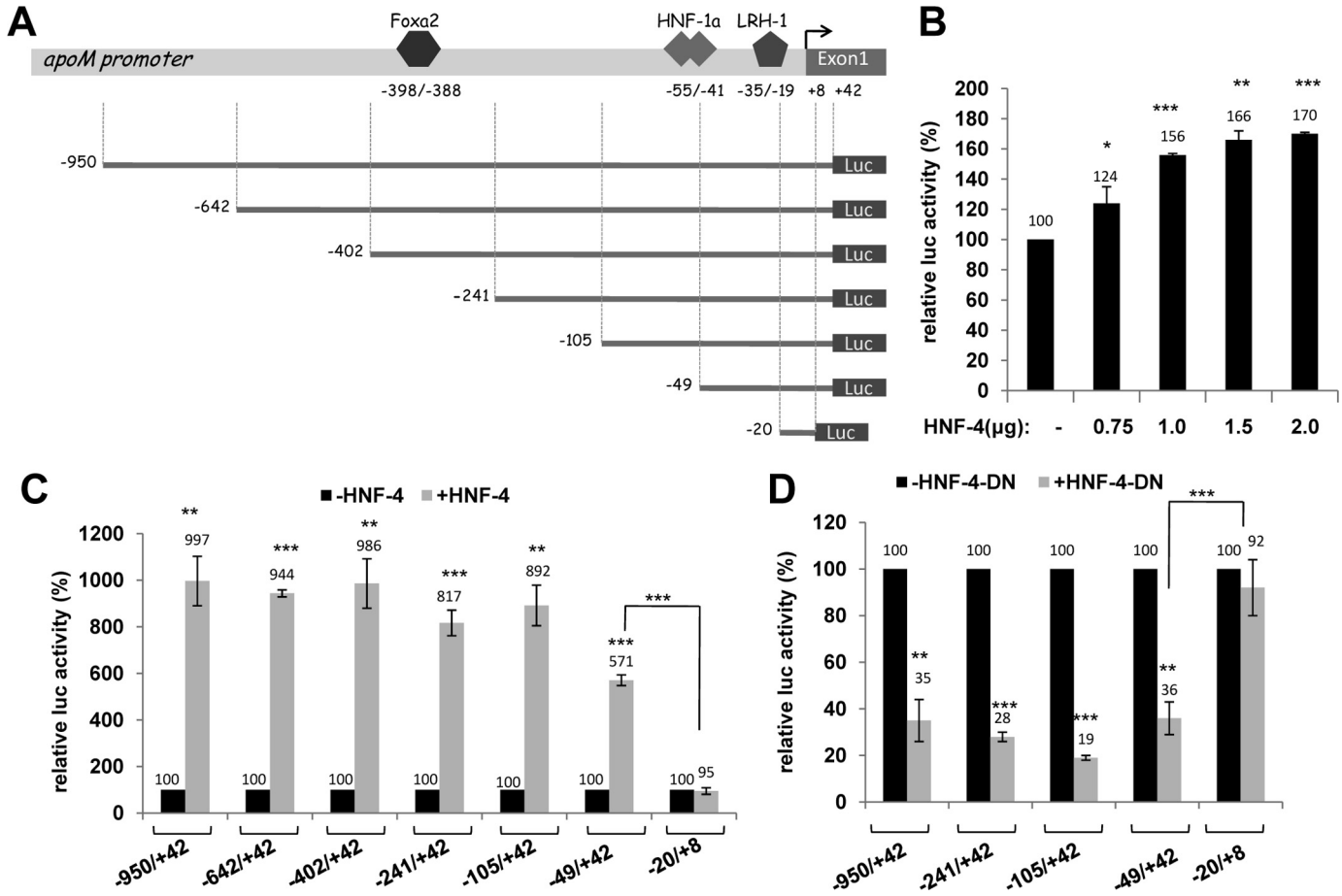

FIGURE 2. **Transactivation of the human *apoM* promoter by HNF-4α requires the proximal −49/−20 region.** *A,* schematic representation of the 5′-deletion derivatives of the (−950/+42) human *apoM* promoter fragments that were cloned upstream of the luciferase (*Luc*) reporter gene and used in the transactivation experiments of *C* and *D*. The position of previously described regulatory elements and factors is also shown. *B,* HepG2 cells were transiently transfected with the (−950/+42) human *apoM*-luc reporter plasmid (1.0 μg) along with increasing concentrations (0.75, 1.0, 1.5, and 2.0 μg) of an HNF-4α expression vector. An expression vector for β-galactosidase (1.0 μg) was included in each sample for normalization purposes. *C,* HEK293T cells were transiently transfected with the luciferase reporter plasmids indicated at the *bottom* of the *graph* (1.0 μg) along with an HNF-4α expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). *D,* HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the *bottom* of the *graph* (1.0 μg) along with the HNF-4α-DN expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). *B–D,* luciferase activity was normalized to β-galactosidase activity and presented with *histographs*. Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

incubation on ice and homogenization with a 27-gauge syringe, nuclei were collected by centrifugation at 6000 rpm for 15 min at 4 °C and resuspended in a low salt buffer (20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, and 0.2 mM EDTA) and a high salt buffer (20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, and 0.2 mM EDTA) supplemented with protease inhibitors followed by rotation on a rotating platform for 30 min at 4 °C. Nuclear extracts were purified by centrifugation at 13,000 rpm for 40 min at 4 °C. For protein-DNA interactions, Dynabeads were washed once with 1× B&W buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 mM EDTA), mixed with 0.58 μM of the biotinylated PCR fragment or oligonucleotide, and incubated at room temperature (25 °C) for 15 min. The oligonucleotide-coupled beads were washed twice with 1× B&W buffer and once with D buffer (20 mM Hepes (pH 7.9), 10% (v/v) glycerol, 40 mM KCl, and 0.5 mM DTT). The protein-DNA binding interactions were allowed to proceed for 30 min on ice in a buffer containing 10% (v/v) glycerol, 20 mM Hepes (pH 7.9), 40 mM KCl, 20 mM $MgCl_2$, 40 mM spermidine, 1 mg/ml BSA, 0.2 mM zinc acetate, 0.5% Nonidet P-40, and 0.5 mM DTT. Each reaction mixture included 30 μg of nuclear extracts, 3 μg of competitor poly(dI/dC), and the biotinylated oligonucleotide-coupled Dynabeads or uncoupled Dynabeads as controls in a total reaction volume of 50 μl. The sequence of the primers utilized for the isolation of the biotinylated PCR promoter fragments or the oligonucleotides used for DNA affinity precipitation assays are shown in supplemental Table 4. Nuclear receptor bound to the oligonucleotides was detected by SDS-PAGE and immunoblotting using the corresponding antibodies.

Statistical Analysis—Results are shown as means ± S.D. Statistical significance was determined using the Student's *t* test. Differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

HNF-4 Is a Positive Regulator of Human apoM Gene Expression in Hepatic Cells—To investigate the role of HNF-4 in *apoM* gene regulation in the liver, RNA interference by shRNAs was employed. For this purpose, an HNF-4 shRNA (sh-HNF-

4)-producing vector was constructed along with a scrambled shRNA (sh-control)-producing vector and used in transient transfection assays in the human hepatoblastoma-derived cell line HepG2. Transient expression of increasing amounts of sh-HNF-4 dose-dependently reduced HNF-4 mRNA levels up to 70% (Fig. 1*A*) and HNF-4 protein levels by ~50% compared with cells expressing sh-control (Fig. 1*C*). Decreased HNF-4 expression in sh-HNF-4-transfected cells was associated with a 50% reduction in *apoM* mRNA levels (Fig. 1*A*) similar to the reduction observed in the mRNA levels of the *apoC-III* gene, a well established target gene of HNF-4 (48–50). Both the intracellular and the secreted levels of apoM protein were also decreased in sh-HNF-4-treated cells compared with sh-control-treated cells (Fig. 1*C*).

In agreement with these findings, overexpression in HepG2 cells of a dominant negative mutant of HNF-4 (HNF-4 DN) that lacks the activation function 2 (AF-2) (51) reduced significantly both *apoM* mRNA levels to 40% of control (supplemental Fig. 1*A*) and protein levels (supplemental Fig. 1*B*). The mRNA levels of the *apoC-III* gene that was used as a positive control showed a comparable reduction in the presence of the HNF-4α DN mutant (supplemental Fig. 1*A*).

The effect of HNF-4 on *apoM* gene expression was next examined using adenovirus-mediated gene transfer. For this purpose, a recombinant adenovirus expressing wild type HNF-4 (Ad-HNF-4) was generated and used to infect HepG2 cells. As a control, a recombinant adenovirus expressing the green fluorescent protein (Ad-GFP) was used. Ad-HNF-4 infection in HepG2 cells led to a 2.4-fold increase in HNF-4 mRNA levels (Fig. 1*B*) and protein levels (Fig. 1*D*) compared with control Ad-GFP-infected cells. Importantly, HNF-4 overexpression was associated with a 1.9-fold increase in *apoM* mRNA levels (Fig. 1*B*) and a significant increase in both intracellular and the secreted apoM protein levels (Fig. 1*D*). In summary, the combined findings of Fig. 1 and supplemental Fig. 1 indicate that HNF-4 is an important regulator of *apoM* gene expression in hepatic cells.

Transactivation of the Human apoM Promoter by HNF-4 Requires the Proximal −49/−20 Region—To investigate further the mechanism of *apoM* gene regulation by HNF-4, a 1-kb genomic fragment bearing the human *apoM* promoter from nucleotide −950 to +42 was amplified by PCR using human genomic DNA as a template and cloned upstream of the luciferase reporter gene (Fig. 2*A*). Transactivation assays in HepG2 cells showed that HNF-4 transactivated the human *apoM* promoter in a dose-dependent manner (Fig. 2*B*).

To identify the minimal regulatory region of the *apoM* promoter that is required for the HNF-4-mediated transactivation, a series of luciferase reporter plasmids containing consecutive 5′ deletions of the *apoM* promoter were constructed (−642/+42, −402/+42, −241/+42, −105/+42, −49/+42, and −20/+8) as shown in Fig. 2*A*, and their transcriptional activity in the presence of HNF-4 was analyzed by transient transfection assays in human embryonic kidney HEK293T cells that do not express endogenously HNF-4 or other hepatocyte-specific nuclear factors. As shown in Fig. 2*C*, HNF-4 overexpression in HEK293T cells was associated with a 10-fold increase in the −950/+42 *apoM* promoter activity. Using the deletion mutants of the *apoM* promoter, it was shown that the proximal *apoM* promoter between nucleotides −49 and +42 was the shortest *apoM* promoter fragment that was responsive to HNF-4 overexpression (5.7-fold, see "Discussion") because further deletion to nucleotide −20 completely abolished the HNF-4-mediated transactivation (Fig. 2*C*). In agreement with these findings, overexpression of the dominant negative form of HNF-4 in HepG2 cells was associated with the reduction of the activity of all *apoM* promoter fragments tested (to 20–35% relative to the control) except for the −20/+8 *apoM* promoter that remained unaffected (Fig. 2*D*). Taken together, the findings of Fig. 2 indicated that the proximal *apoM* promoter region between nucleotides −49 and −20 is required for transactivation by the orphan nuclear receptor HNF-4 in hepatic cells.

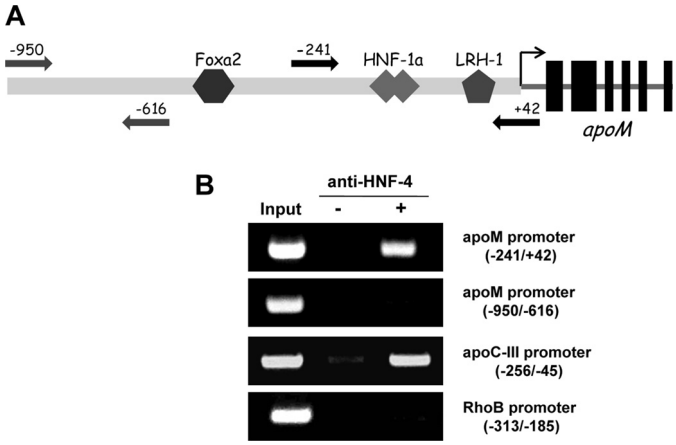

FIGURE 3. **Chromatin immunoprecipitation assays establishing the recruitment of HNF-4α to the proximal human *apoM* promoter in HepG2 cells.** *A*, schematic representation of the human *apoM* promoter region, with *arrows* showing the location of the oligonucleotide primer sets (distal region, −950/−616; proximal region, −241/+42), that were utilized in the chromatin immunoprecipitation assays. *B*, HepG2 cells were subjected to chromatin immunoprecipitation in the absence (*2nd lane*) or presence (*3rd lane*) of an anti-HNF-4 antibody using primers corresponding to the proximal or the distal region of the *apoM* promoter, the proximal region (−256/−45) of the *apoC-III* promoter harboring a DR1 HRE (positive control), or the proximal region (−313/−185) of the *RHOB* promoter (unrelated region, negative control). Nonimmunoprecipitated chromatin was included as a positive control (*1st lane, input*). The experiment was performed three times, and representative images are presented.

HNF-4 Regulates apoM Gene Expression via a Hormone-response Element Located in the −33 to −21 Region of the apoM Promoter—Binding of HNF-4 to the human *apoM* promoter *in vivo* was established using chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig. 3*B*, an antibody against endogenous HNF-4 could efficiently immunoprecipitate the proximal −241/+42 *apoM* promoter but not the distal −950/−616 region of the *apoM* promoter. In control reactions, HNF-4 was found to be associated with the proximal promoter of the human apolipoprotein C-III gene (region −256/−45) but not with the unrelated proximal promoter of the small GTPase *RHOB* gene (region −313/−185) (52) confirming the specificity of the chromatin immunoprecipitation experiment.

Binding of HNF-4 to the proximal *apoM* promoter *in vitro* was established using the DNAP assay. For this purpose, two biotinylated overlapping PCR fragments covering the proximal *apoM* promoter region between nucleotides −241 and +42 were generated (−241/−81 and −105/+42) (Fig. 4*A*). As

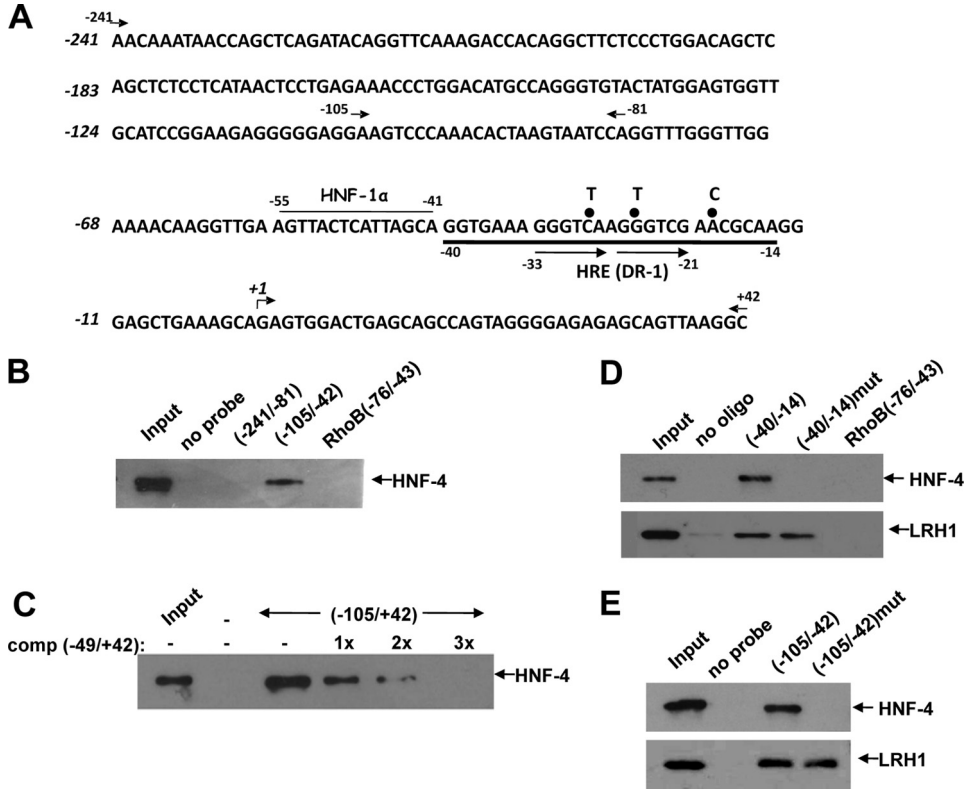

FIGURE 4. **HNF-4α binds to an HRE located in the −33/−21 region of the proximal human *apoM* promoter.** *A,* sequence of the proximal human *apoM* promoter region spanning nucleotides −241 to +42, showing the location of the previously characterized regulatory element that binds HNF-1α as well as the location of the DR1 HRE. The primer sets used for the amplification of the biotinylated promoter fragments −241/−81 and −105/+42 are indicated by *arrows*. Nucleotide substitutions in the two half-repeats of the HRE of the *apoM* promoter are indicated with the *black dots*. *B,* DNA-affinity precipitation using HepG2 nuclear extracts and biotinylated PCR fragments corresponding to the −241/−81 or the −105/+42 region of the human *apoM* promoter or no probe. A biotinylated oligonucleotide corresponding to the −76/−43 region of the human *RHOB* promoter that contains a CAAT box was used as a negative control. Oligonucleotide-bound HNF-4 was detected by Western blotting using a polyclonal anti-HNF-4 antibody. *C,* DNA-affinity precipitation using HepG2 nuclear extracts and a biotinylated PCR fragment corresponding to the wild type −105/+42 region of the human *apoM* promoter in the presence of increasing amounts (1–3-fold molar excess) of a competitor, nonbiotinylated, PCR fragment corresponding to the −49/+42 region of the *apoM* promoter. Oligonucleotide-bound HNF-4 was detected by Western blotting using a polyclonal anti-HNF-4 antibody. *D,* DNA-affinity precipitation experiments using HepG2 nuclear extracts and biotinylated oligonucleotides corresponding to the wild type −40/−14 region of the human *apoM* promoter, the −40/−14 region bearing mutations in the HRE (*mut*), the −76/−43 region of the human *RHOB* promoter, or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-4 or LRH-1 was detected by Western blotting using a polyclonal anti-HNF-4 or anti-LRH-1 antibody, respectively. *E,* DNA-affinity precipitation using HepG2 nuclear extracts and biotinylated PCR fragments corresponding to the wild type or the mutated −105/+42 region of the human *apoM* promoter. Oligonucleotide-bound HNF-4 or LRH-1 was detected by Western blotting using polyclonal anti-HNF-4 or anti-LRH-1 antibodies. All experiments in *B–E* were performed at least three times, and representative images are presented.

shown in Fig. 4*B*, endogenous HNF-4 present in nuclear extracts from HepG2 cells bound to the −105/+42 biotinylated promoter fragment (*4th lane*) but not to the −241/−81 biotinylated promoter fragment (*3rd lane*). In control experiments it was shown that HNF-4 did not bind to the streptavidin Dynabeads (Fig. 4*B, 2nd lane, no probe*) or to a double-stranded biotinylated oligonucleotide corresponding to the −76/−43 region of the human *RHOB* promoter that contains a previously characterized CAAT box (Fig. 4*B*, *5th lane*) (52). In competition DNAP assays, it was shown that binding of HNF-4 to the −105/+42 biotinylated promoter fragment could be competed out by increasing amounts of a nonbiotinylated PCR promoter fragment corresponding to the −49/+42 region of the *apoM* promoter (Fig. 4*C*). This finding, taken together with the transactivation data of Fig. 2, strongly suggested that the HNF-4-binding site is located in the region defined by nucleotides −49 and −20.

The sequence of the proximal human *apoM* promoter contains a putative DR1 (direct repeat with one nucleotide spacing) HRE spanning nucleotides −33 to −21 (Fig. 4*A*). As DR-1 elements are preferred binding sites for HNF-4 (53), we investigated whether HNF-4 binds to the *apoM* promoter via this DR-1 HRE. For this purpose, DNAP experiments were performed using endogenous HNF-4 from HepG2 nuclear extracts and a double-stranded biotinylated oligonucleotide corresponding to the −40/−14 region of the *apoM* promoter that includes the putative HRE. As shown in Fig. 4*D* (*top*), HNF-4 bound efficiently to the −40/−14 biotinylated oligonucleotide probe (*3rd lane*). In line with a previous study (36), the −40/−14 apoM-biotinylated probe bound the liver receptor homolog 1 (LRH-1) (Fig. 4*D, bottom, 3rd lane*). LRH-1 recognizes a 5′-CAAGG-3′ motif present in the −29/−25 region of the *apoM* promoter, and LRH-1 binding as well as LRH-1-mediated transactivation were abolished by a C/T substitution at the first position of this motif (36). To characterize this HRE further, a biotinylated oligonucleotide probe was used bearing three nucleotide substitutions in the two half-repeats and the flanking region. As shown in Fig. 4*D* (*top, 4th lane*), these mutations completely abolished the binding of HNF-4 to this oligonucleotide, whereas they had no effect on binding of LRH-1 (Fig. 4, *bottom, 4th lane*) (see "Discussion").

To exclude the presence of additional HNF-4-binding sites in the proximal *apoM* promoter, the same HRE mutations were introduced into the −105/+42 *apoM* promoter fragment (−105/+42mut). As shown in Fig. 4*E* (*top, 4th lane*), no binding of HNF-4 to the mutated −105/+42 *apoM* promoter fragment was observed. As expected, the mutations had no effect on the binding of LRH-1 to this promoter fragment (Fig. 4*E, bottom, 4th lane*).

To investigate the functional importance of the proximal HRE for the HNF-4-mediated transactivation of the *apoM* promoter, the same mutations in the HRE that abolished the binding of HNF-4 (Fig. 4) were introduced into the (−950/+42)hapoM luciferase reporter plasmid (Fig. 5*A*). As shown in Fig. 5*B*,

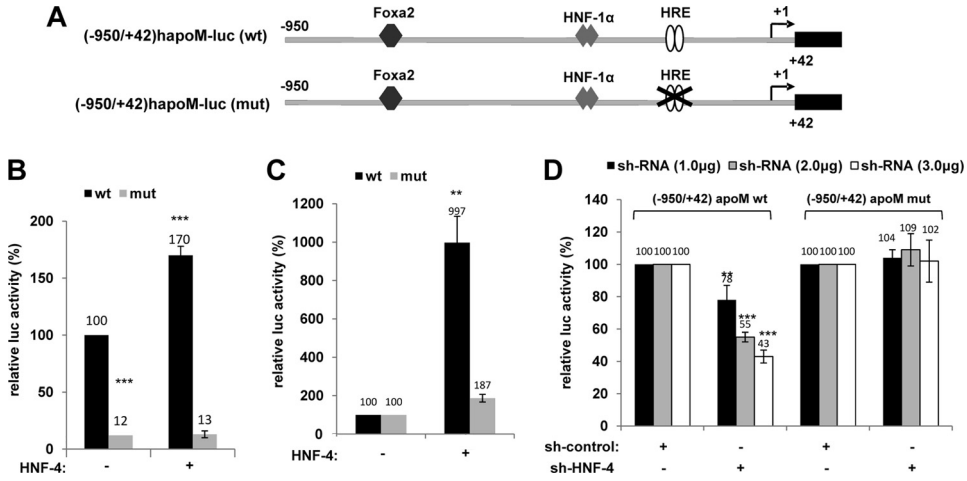

FIGURE 5. **Mutations in the proximal HRE reduce basal *apoM* promoter activity and abolish HNF-4α-mediated transactivation.** *A,* schematic representation of the wild type (−950/+42)human (*h*) *apoM*-luc promoter construct and the corresponding construct bearing the mutations in the HRE that are shown in Fig. 4*A* ((−950/+42)human *apoM*-luc (mut)). *B,* HepG2 cells were transiently transfected with the WT or mutated (−950/+42)human *apoM*-luc reporter plasmid (1.0 μg) along with the HNF-4α expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). *C,* HEK293T cells were transiently transfected with the WT or mutated (−950/+42) human *apoM*-luc reporter plasmid (1.0 μg) along with the HNF-4α expression vector (1.0 μg) and a β-galactosidase expression vector (1.0 μg). *D,* HepG2 cells were transiently transfected with the WT or mutated (−950/+42)human *apoM*-luc reporter plasmid (1.0 μg) along with increasing concentrations of the sh-control- or sh-HNF-4α-producing vector (1.0, 2.0, and 3.0 μg) and a β-galactosidase expression vector (1.0 μg). *B–D,* luciferase activity was normalized to β-galactosidase activity and presented with *histographs.* Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. **, $p < 0.01$; ***, $p < 0.001$.

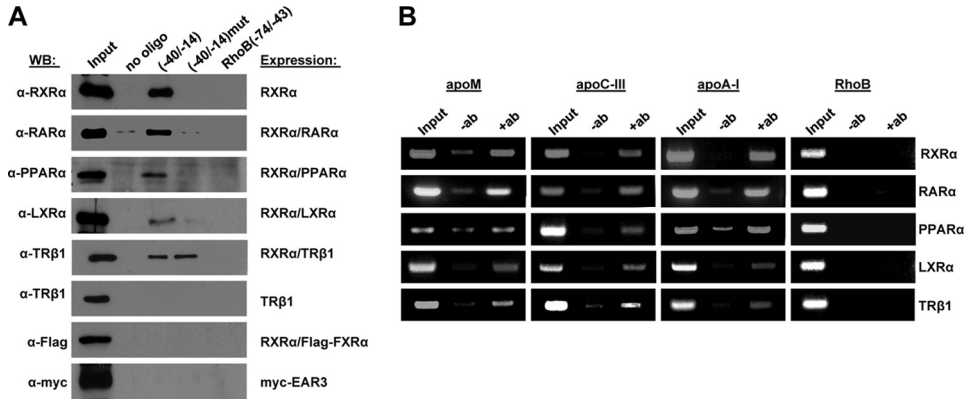

FIGURE 6. **Proximal HRE on the *apoM* promoter binds ligand-dependent nuclear receptors (homo- and heterodimers of RXRα).** *A,* DNA-affinity precipitation experiments using nuclear extracts from HEK293T cells transiently transfected with expression vectors for RXRα and its heterodimer partners or Ear-3 as indicated on the *right* and biotinylated oligonucleotides corresponding to the wild type −40/−14 region of the human *apoM* promoter, the mutated −40/−14 *apoM* promoter, or the −76/−43 region of the human *RHOB* promoter. Oligonucleotide-bound nuclear receptors were detected by Western blotting (*WB*) using the corresponding antibodies shown on the *left*. All experiments were performed three times, and representative images are shown. *B,* HepG2 cells were subjected to chromatin immunoprecipitation in the absence (−*ab*) or the presence (+*ab*) of an anti-RXR, RAR, PPAR, LXR, or TRβ1 antibody as indicated on the *right*. Promoter occupancy was assessed by PCR amplification using primers corresponding to the proximal region of the *apoM* promoter (−241/+42), the proximal region of the *apoC-III* promoter (−256/−45), and of the *apoA-I* promoter (−315/+22) as positive controls or the proximal region of the *RHOB* promoter (−313/−185) as negative control. Nonimmunoprecipitated chromatin was also included (*input*).

mutagenesis of this HRE decreased the basal activity of the (−950/+42)*apoM* promoter in HepG2 cells to 12% of the control indicating the importance of this element for *apoM* gene regulation in hepatic cells. In contrast, the HRE mutations did not affect the basal activity of the *apoM* promoter in HEK293T cells that lack endogenous HNF-4 (Fig. 5*C*). Furthermore, the HRE mutations abolished the HNF-4-mediated transactivation of the *apoM* promoter in both HepG2 (Fig. 5*B*) and HEK293T (Fig. 5*C*) cells. Moreover, expression of increasing amounts of shRNA targeting HNF-4 in HepG2 cells dose-dependently reduced the activity of the wild type promoter to 43% of control, although it did not affect the activity of the mutant promoter (Fig. 5*D*). In agreement with the findings of Fig. 4, the HRE mutations did not abolish the transactivation of the *apoM* promoter by LRH-1 in HEK293T cells (supplemental Fig. 2*A*) or HepG2 cells (data not shown). The combined findings of Figs. 4 and 5 and supplemental Fig. 2 indicated that HNF-4 directly regulates *apoM* gene expression via a novel HRE located in the −33 to −21 region.

Novel HRE on the Proximal apoM Promoter Binds Ligand-dependent Nuclear Receptors—Previous studies have shown that hormone-response elements present in the promoters of various apolipoprotein genes such as *apoC-III*, *apoA-I*, *apoC-II*, *apoA-IV*, or *apoA-II* could be shared by many hormone nuclear receptors, including HNF-4 and homodimers or heterodimers of RXRα (54, 55). Initially, the association of RXRα homodimers and its heterodimers with RARα, PPARα, LXRα, TRβ, FXR, as well as of the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor I (COUP-TFI or Ear-3) with the novel HRE in the proximal *apoM* promoter was examined by DNAP assays. As shown in Fig. 6*A*, RXRα homodimers (*1st lane*) and its heterodimers with RARα (*2nd lane*), PPARα (*3rd lane*), LXRα (*4th lane*), and TRβ1 (*5th lane*) bound to the wild type −40/−14 oligonucleotide. Binding of all heterodimers except RXRα/TRβ was abolished by mutations in the HRE (−40/−14 mut). No binding to the *apoM* HRE was detected by TRβ1 homodimers (Fig. 6*A*, *6th lane*), RXRα/FXRα heterodimers (*7th lane*), or Ear-3 (*8th lane*). Similar to HNF-4, RXRα homodimers and RXRα/RARα heterodimers bound to a larger *apoM* promoter fragment (−105/−42), but this binding was severely affected by the mutations in the HRE (supplemental Fig. 3). In contrast, binding of RXRα/TRβ1 heterodimers to the above *apoM* promoter fragment was not affected by the HRE mutations (supplemental Fig. 3) in agreement with the findings of Fig. 6*A*.

The recruitment of the RXRα homodimers and heterodimers to the *apoM* promoter *in vivo* was investigated by chromatin immunoprecipitation assays in HepG2 cells using antibodies against the corresponding nuclear receptors. These experiments (Fig. 6*B*) showed that nuclear receptors RXRα, RARα, PPARα, LXRα, and TRβ are all recruited to the proximal *apoM* promoter region −241/+42 albeit with different affinities in agreement with the findings from DNAP assays of Fig. 6*A* (strong binding of RXRα and RARα and less efficient binding of PPARα, LXRα, and TRβ). The specificity of the above nuclear receptors for the *apoM* HRE was established using primers that amplified the *apoC-III* and *apoA-I* promoters (positive controls) as well as the *RHOB* promoter (negative control). Altogether, the findings of Fig. 6 indicated that in addition to HNF-4, homodimers of RXRα and heterodimers of RXRα with other ligand-dependent nuclear receptors can occupy the same HRE in the proximal *apoM* promoter with different affinities.

Regulation of apoM Gene Expression by 9-cis-Retinoic Acid—To assess the functional relevance of the association of RXRα homo- and heterodimers with the novel HRE of the proximal *apoM* promoter, nuclear receptor signaling was stimulated in HepG2 cells by the administration of the corresponding ligands, and their effect on *apoM* gene expression was evaluated using RT-PCR. As shown in Fig. 7*A*, treatment of HepG2 cells with 9-*cis*-RA for different times (2–24 h) resulted in a biphasic mode of *apoM* gene regulation with an early activation phase (at 2–4 h), which was followed by a repression phase (at 6–24 h). To investigate the mechanism of this biphasic regulation of *apoM* gene transcription by 9-*cis*-RA, we determined the mRNA levels of RXRα, HNF-4, and HNF-1, all of which have been shown previously to be regulated by RXR (56–58). HNF-4 and HNF-1 exhibited a similar response to retinoic acid treatment; their mRNA levels were increased at 2–6 h of treatment and returned to basal levels at 8–24 h of treatment (Fig. 7*A*). In addition, a time-dependent down-regulation of the RXR mRNA levels was observed in the presence of 9-*cis*-RA resulting in 30% repression relative to control at 8 h of treatment (Fig. 7*A*). A strong reduction was also observed in the protein levels of RXRα in 9-*cis*-RA-treated HepG2 cells (supplemental Fig. 4) Surprisingly, when apoM protein levels were monitored, a significant increase was observed at 2 h of 9-*cis*-RA treatment that persisted for up to 24 h (Fig. 7*B*) possibly due to a stabilizing effect of 9-*cis*-RA on apoM protein given the fact that 9-*cis*-RA is a ligand for apoM (59).

To investigate further the role of RXRα in the regulation of *apoM* gene expression in hepatic cells, a recombinant adenovirus expressing RXRα (Ad-RXR) was generated and used to infect HepG2 cells. As shown in Fig. 8, *A* and *B*, infection of HepG2 cells with Ad-RXRα in the presence of its ligand 9-*cis*-RA led to a significant increase in both RXRα and *apoM* mRNA and protein levels (intracellular and secreted) compared with control Ad-GFP-infected cells thus establishing RXRα as a positive regulator of *apoM* gene expression.

Transient overexpression of RXRα in HEK293T cells resulted in a strong transactivation of the wild type (−950/+42)*apoM* promoter in the presence of 9-*cis*-RA (12.6-fold), whereas the mutations in the proximal HRE that abolished binding of RXRα to this element (Fig. 6*A*) reduced this transactivation to 3-fold (Fig. 8*C*).

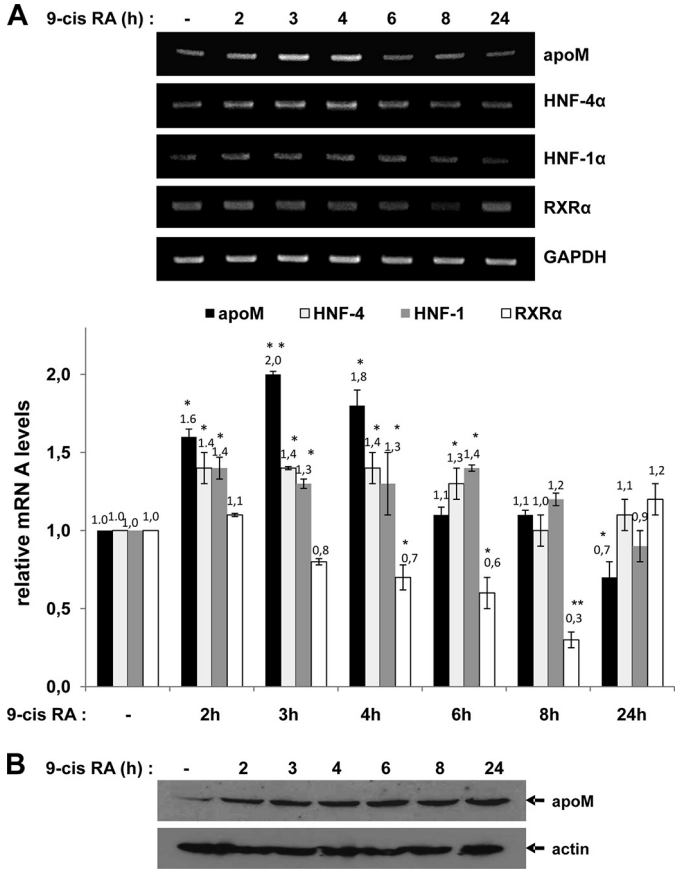

FIGURE 7. **Regulation of *apoM* gene expression by 9-*cis*-retinoic acid.** *A*, HepG2 cells were treated with 9-*cis*-RA (1 μM) for the indicated times or left untreated. Total RNA was extracted, and *apoM*, *HNF-4α*, *HNF-1α*, and *RXRα* mRNA levels were analyzed by RT-PCR, normalized relative to the mRNA levels of the *GAPDH* gene, quantified, and are shown as a *histograph*. Each value represents the average from three independent experiments. *B*, HepG2 cells were treated with 9-*cis*-RA (1 μM) for the indicated times or left untreated, and the protein levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. *, $p < 0.05$; **, $p < 0.01$.

Overexpression of RXRα along with its heterodimeric partner RARα in HEK293T cells was associated with a strong (24-fold) transactivation of the wild type (−950/+42)*apoM* promoter in the presence of 9-*cis*-RA but not that of the promoter bearing mutations in the HRE (Fig. 8*C*). Using the *apoM* promoter deletion constructs, it was established that, similarly to HNF-4, deletion of the promoter region between nucleotides −49 and −20 abolished RXRα- and RXRα/RARα-dependent transactivation of the *apoM* promoter (Fig. 8*D*).

Induction of apoM Gene Expression by Fibrates, Oxysterols, and T_3—To assess the functional contribution of the other RXRα heterodimers (RXRα/PPARα, RXRα/TRβ, and RXRα/LXRα) in the regulation of the *apoM* gene, HepG2 cells were treated with the corresponding ligands for 3 and 24 h, and the expression levels of apoM were monitored by RT-PCR assays and immunoblotting. As shown in Fig. 9*A*, treatment of HepG2 cells with fibrates, 22(OH)C, and T_3 (ligands of PPARα, LXRα, and TRβ) caused a 5.1-, 5.5-, and 3.1-fold increase in *apoM* mRNA levels, respectively, compared with untreated cells. ApoM protein levels were also significantly increased after treatment of cells with the ligands for 3 h (Fig. 9*B*). Induction of

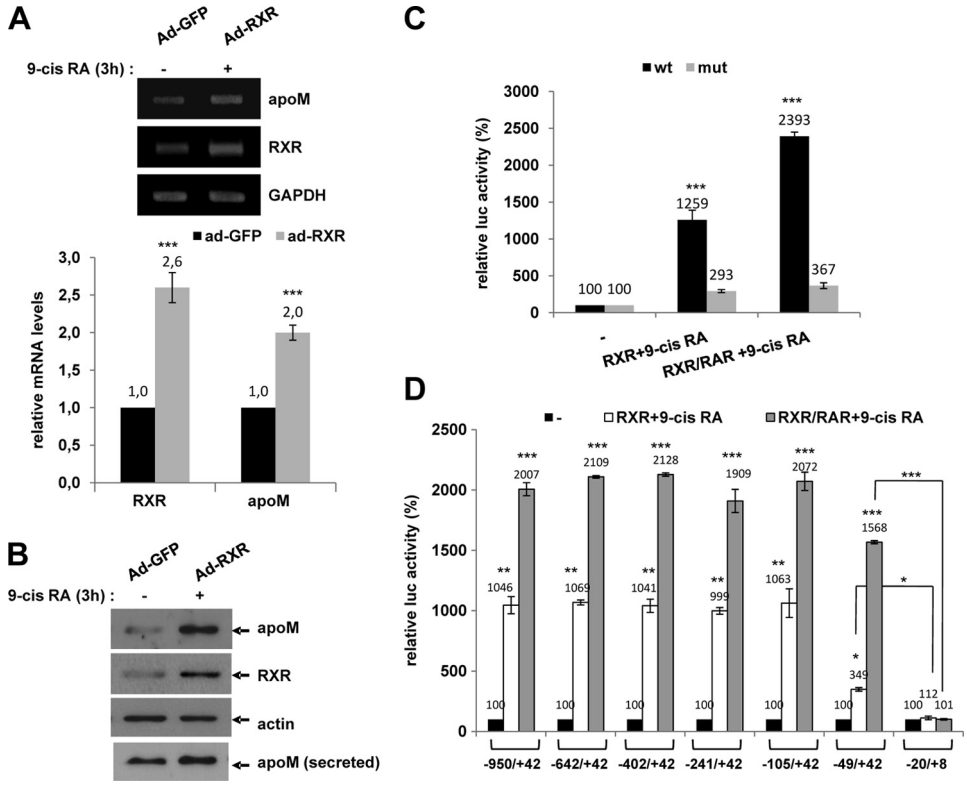

FIGURE 8. **Induction of *apoM* gene transcription by retinoic acid receptors RXR and RAR.** *A,* HepG2 cells were infected with a control adenovirus expressing GFP (*Ad-GFP*) or with a recombinant adenovirus expressing RXRα (*Ad-RXR*) at an m.o.i. of 10 for 24 h and treated with 9-*cis*-RA (1 μM) for 3 h. Total RNA was extracted, and the mRNA levels of *apoM*, *RXR*α, and *GAPDH* genes were analyzed by RT-PCR. The normalized relative mRNA levels of the apoM and RXRα genes were quantified and are shown as a *histograph*. Each value represents the average from three independent experiments. *B,* HepG2 cells were infected for 24 h with Ad-GFP or Ad-RXR at an m.o.i. of 10 and treated with 9-*cis*-RA (1 μM) for 3 h, and the protein levels of intracellular apoM, RXR, and actin (loading control) and of the secreted apoM were determined by immunoblotting. The experiment was performed three times, and representative images are presented. *C,* HEK293T cells were transiently transfected with the WT or mutated (−950/+42)human *apoM*-luc reporter plasmid (1.0 μg) along with expression vectors for RXRα or both RXRα and RARα (1.0 μg each). The β-galactosidase expression plasmid (1.0 μg) was included in each sample for normalization of transfection variability. Following transfection, cells were treated with 9-*cis*-RA (1 μM) for 24 h. Luciferase activity was normalized to β-galactosidase activity and presented with a *histograph*. Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. *D,* HEK293T cells were transiently transfected with the indicated *apoM* promoter deletion plasmids (1.0 μg) along with expression vectors for RXRα (1.0 μg) or both RXRα and RARα (1.0 μg each) along with a β-galactosidase expressing plasmid (1.0 μg). Following transfection, cells were treated with 9-*cis*-RA (1 μM) for 24 h. Luciferase activity was normalized to β-galactosidase activity and presented with a *histograph*. Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

apoM mRNA and protein levels by these ligands persisted for 24 h of treatment (Fig. 9, *C* and *D*). Significant increase in the protein levels of the secreted apoM was also observed in the media of HepG2 cells that had been treated with 9-*cis*-RA, fibrates, and 22(OH)C for 24 h (supplemental Fig. 5).

Overexpression of the RXRα along with PPARα, LXRα, or TRβ1 in HEK293T cells that had been treated with the corresponding ligands was associated with a strong transactivation of the wild type (−950/+42)*apoM* promoter (28.4-, 46.5-, and 38.9-fold, respectively), whereas no significant effect was observed on the activity of the mutated *apoM* promoter (Fig. 9*E*). Strong transactivation by the RXR homo- and heterodimers as well as by HNF-4 and LRH-1 was observed using the mouse *apoM* promoter (−761/−7), which contains the same HRE that is 100% homologous with the human (supplemental Fig. 6, *A* and *B*). No transactivation could be observed by RXRα/FXRα heterodimers in the presence of chenodeoxycholic acid in accordance with the DNA binding data of Fig. 6*A* (data not shown). Finally, deletion analysis of the *apoM* promoter showed that the RXR/PPAR, RXR/LXR, and RXR/TRβ heterodimers in the presence of their ligands transactivated strongly all *apoM* promoter fragments tested except the (−20/+8) reporter plasmid that lacks the proximal HRE (Fig. 9*F*), thus establishing the contribution of the proximal HRE in the stimulation of *apoM* gene expression by fibrates, oxysterols, and T_3.

DISCUSSION

The impact of apoM on HDL metabolism has been studied mainly in mouse models. These studies indicated that apoM might affect the quality rather than the quantity of HDL and that apoM overexpression reduces the development of atherosclerosis in an atherosclerosis-prone setting (23, 24) suggesting that exploring new ways of *apoM* gene up-regulation may provide new therapeutic tools for the treatment of coronary artery disease.

In this study, we show that the apolipoprotein M gene is under the control of hormone nuclear receptors that have been shown to play pivotal roles in lipid and glucose metabolism such as HNF-4 and the receptors for retinoic acid, thyroid hormone, fibrates, and oxysterols (54, 55, 60). Among these nuclear receptors, HNF-4 seems to play a major role in the regulation of *apoM* gene expression in the liver. This is in agreement with a previous cDNA microarray analysis of HNF-4α-induced genes in human hepatoma cells that showed a 4.5-fold increase in apoM expression levels by overexpressed HNF-4 (44). HNF-1 knock-out mice are characterized by the complete absence of apoM in plasma and abnormal HDL profiles (24). Previous studies had shown that the *HNF-1* promoter contains a binding site for HNF-4 and that the two factors participate in a transcriptional network operating in hepatic cells that control the transcription of many liver-specific genes, including genes involved in lipoprotein metabolism (61–63). The importance of HNF-4 and HNF-1 in lipid homeostasis is also supported by a recent genome-wide association study that showed a statistically significant association between polymorphisms in the two genes and abnormalities in the HDL and LDL levels (43).

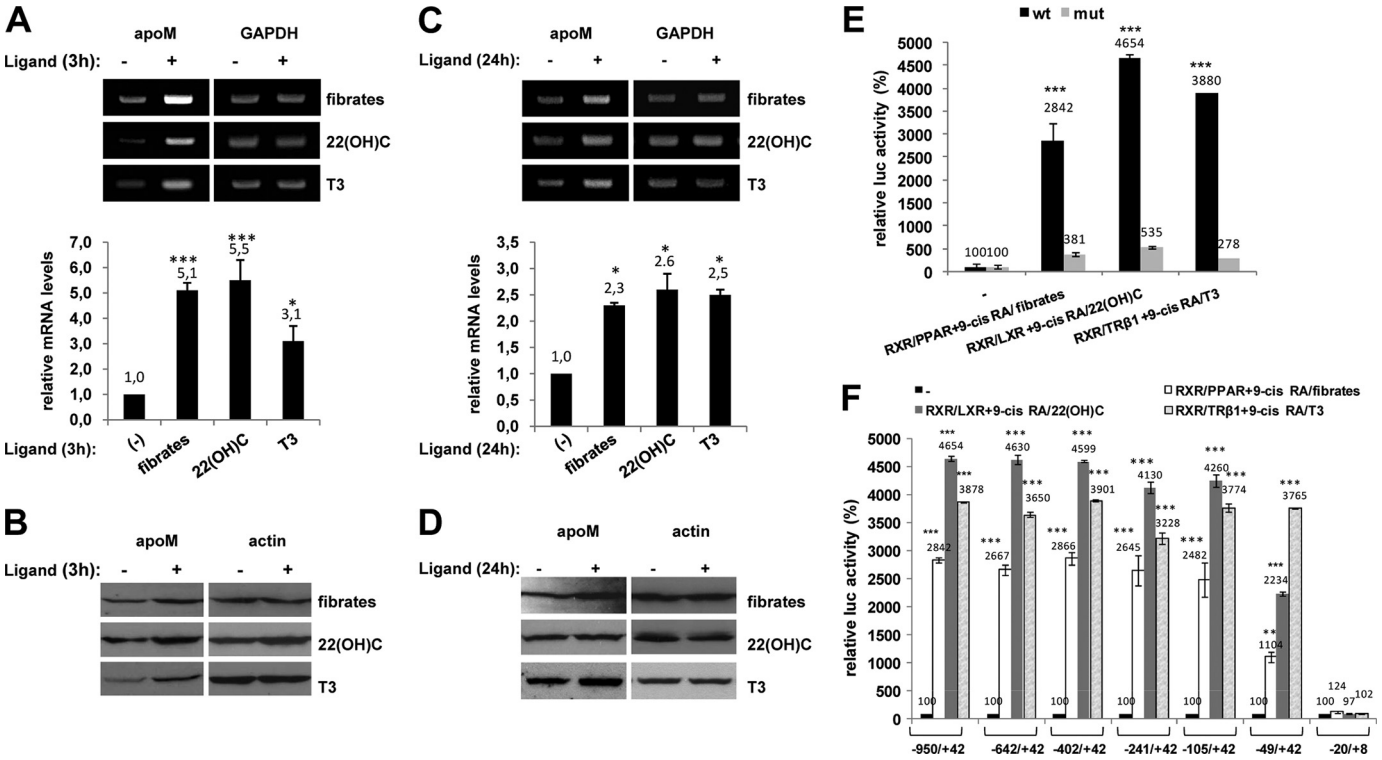

FIGURE 9. **Induction of *apoM* gene expression by fibrates, oxysterols, and T_3.** *A* and *C,* HepG2 cells were treated with fibrates (250 μM), 22(OH)C (1 μM), or T_3 (1 μM) for 3 h (*A*) or 24 h (*C*). Total RNA was extracted, and *apoM* mRNA levels were analyzed by RT-PCR. The normalized mRNA levels of the *apoM* gene were quantified and are shown as a *histograph*. Each value represents the average from three independent experiments. *B* and *D,* HepG2 cells were treated with fibrates (250 μM), 22(OH)C (1 μM) or T_3 (1 μM) for 3 h (*B*) or 24 h (*D*), and the expression levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. Each experiment was performed three times, and representative images are presented. *E,* HEK293T cells were transiently transfected with the WT or mutated (−950/+42)human *apoM*-luc reporter plasmid (1.0 μg) along with expression vectors for RXRα, PPARα, LXRα, and TRβ1 (1.0 μg each) at the combinations indicated and a β-galactosidase expressing plasmid (1.0 μg). Following transfection, cells were treated with the appropriate ligands for 24 h (9-*cis*-RA (1 μM), fibrates (100 μM), 22(OH)C (1 μM), or T_3 (1 μM)). Luciferase activity was normalized to β-galactosidase activity and presented with a *histograph*. Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. *F,* HEK293T cells were transiently transfected with the indicated *apoM* promoter luciferase reporter plasmids (1.0 μg) along with expression vector for RXRα, PPARα, LXRα, or TRβ1 (1.0 μg each) at the combinations indicated along with a β-galactosidase expressing plasmid (1.0 μg). Following transfection, cells were treated with the appropriate ligands as in *E* for 24 h. Luciferase activity was normalized to β-galactosidase activity and presented with a *histograph*. Each value represents the average (±S.D.) from at least three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

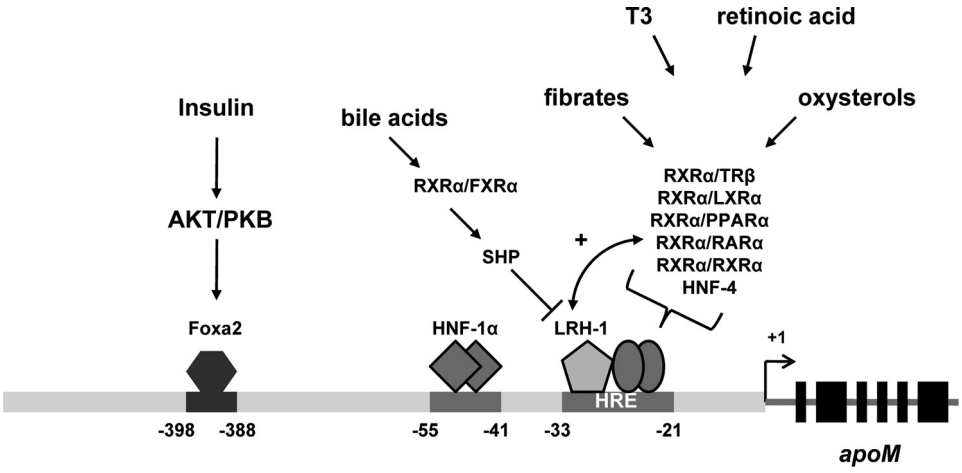

FIGURE 10. **Summary of regulatory elements and transcription factors that participate in the control of human *apoM* gene transcription.** See text for details.

A second key finding of this study is that *apoM* gene expression is subject to regulation by oxysterols that are the natural endogenous ligands of the LXRs. Previous studies had provided contradictory results regarding the effect of LXR ligands on *apoM* gene regulation. In one study, Zhang *et al.* (64) showed that oral administration of the synthetic LXR agonist T0901317 in mice was associated with a reduction in basal *apoM* mRNA levels in the liver. In another study, Calayir *et al.* (65) showed that T00901317 down-regulated *apoM* gene expression in mouse liver, but it up-regulated *apoM* gene expression in mouse intestinal cells. Similar up-regulation was observed when the natural LXR ligand 22(OH)C was used (65). These contradictory findings should be evaluated in light of a previous study showing that T0901317 is a dual LXR/FXR agonist that activates FXR more efficiently than its natural ligand, the bile acid chenodeoxycholic acid (66). Furthermore, Venteclef *et al.* (36) showed recently that bile acids suppress *apoM* gene expression in

hepatic cells via an LRH-1 (liver receptor homologue-1)-binding site in the proximal *apoM* promoter and that this repression required SHP. SHP is an inhibitory nuclear receptor activated by FXRs that interacts physically with many nuclear receptors, including LRH-1, and interferes with their transactivation potential (67). The LRH-1 site reported by Venteclef *et al.* (36) is located inside the proximal *apoM* HRE that was characterized in this study. Interestingly, the C/T substitution in the first base of the LRH-1-binding site that was shown by Venteclef *et al.* (36) to abolish binding of LRH-1 and to inhibit the LRH-1-mediated transactivation of the *apoM* promoter was not equally effective in our study. This controversy could possibly be accounted for by the two additional substitutions that we introduced to the *apoM* HRE, which possibly restored LRH-1 binding to this element and the LRH-1-mediated transactivation as shown in Fig. 4, *D* and *E,* and supplemental Fig. 2*A*.

Based on the above findings, we are tempted to speculate that the negative effect of T0901317 on *apoM* gene expression in liver cells reported previously (64, 65) could be due to the activation of the FXR/SHP pathway that inhibits LRH-1 in hepatic cells. In agreement with this hypothesis, we found that treatment of HepG2 cells with 1 μM T0901317 for 3 h caused a 2-fold increase in SHP mRNA levels. In contrast, the SHP mRNA levels were not affected by 22(OH)C (supplemental Fig. 7*A*). Furthermore, T0901317 treatment or SHP overexpression inhibited the LRH-1-mediated transactivation of the −105/+42 *apoM* promoter bearing a mutation in the LXR element in HepG2 cells suggesting that in hepatic cells T0901317 suppresses *apoM* gene transcription via an SHP/LRH-1 pathway (supplemental Fig. 7, *C* and *D*). In contrast to HepG2 cells, T0901317 failed to inhibit *apoM* gene transcription in intestinal Caco-2 cells where LRH-1 is expressed at low levels or in Caco-2 cells overexpressing LRH-1 suggesting that the T0901317/SHP pathway is not operating in these cells. As a result, T0901317 induced *apoM* gene expression in Caco-2 cells (supplemental Fig. 7*B*). Although the proximal *apoM* HRE resembles a DR-1 element (direct repeat with 1 nucleotide spacing), which is not favored by LXRα/RXRα heterodimers, a recent study showed that binding of LXR/RXR to such HREs is not prohibited (68).

Fig. 10 summarizes our current understanding of the regulation of *apoM* gene expression in the liver. Although it is possible that other key players are missing from this picture, strong evidence indicates that liver *apoM* gene expression is controlled by the interplay between liver-enriched factors such as HNF-1, HNF-4, LRH-1, and FOXA2/HNF-3β with ligand-dependent nuclear receptors such as homo- and heterodimers of RXR. Thus, *apoM* is a novel target for ligands shown previously to have a beneficial effect on HDL levels (oxysterols and fibrates). Synergistic interactions between the above factors could be required for optimal *apoM* gene expression. In support of this hypothesis, we showed that HNF-4 transactivates the *apoM* promoter synergistically with LRH-1 (supplemental Fig. 2, *B* and *C*) and HNF-1 (data not shown).

HDL is an important atheroprotective molecule, and many different HDL-based therapies of diabetes, obesity, metabolic syndrome, and coronary artery disease are currently under development. Having established the importance of apoM for HDL physiology and cholesterol homeostasis, it is anticipated that the detailed understanding of how this gene is regulated in health and disease will provide novel therapeutic and diagnostic tools for the above diseases that affect a large proportion of the population in Western countries.

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REGULATION OF HUMAN APOLIPOPROTEIN M GENE EXPRESSION BY ORPHAN AND LIGAND-DEPENDENT NUCLEAR RECEPTORS

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Supplementary Figures

Suppl. Figure 1. Hepatocyte nuclear factor 4α (HNF-4α) is a positive regulator of human apoM gene expression. (A) HepG2 cells were transiently transfected with an expression vector for HNF-4α DN (10μg) and 48h later, total RNA was extracted and apoM and apoC-III mRNA levels were analyzed by RT-PCR. The normalized (relative to GAPDH) mRNA levels of the apoM and apoC-III genes and are shown as a histograph. Each value represents the average from three independent experiments. (B) HepG2 cells were transiently transfected with 10μg of the HNF-4α DN expressing vector and the protein levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. Key: **p<0.01

Suppl. Fig. 2. HNF-4 and LRH-1 synergistically transactivate the human apoM promoter. (A) HEK293T cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with an LRH-1 expression vector (1.0μg) and a β-galactosidase expression plasmid (1.0μg). Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. (B) HEK293T cells were transiently transfected with the wt (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with the expression vectors

for HNF-4α (1.0μg) or LRH-1 (1.0μg) or both and a β-galactosidase expression plasmid (1.0μg). (C) HepG2 cells were transiently transfected with the wt (-950/+42)human apoM-luc reporter plasmid (1.0μg) along with a sh-control or sh-HNF-4α producing vector (1.0μg) and a β-galactosidase expression vector (1.0μg).

In Panels A-C, luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

Suppl. Figure 3. Mutations in the proximal HRE of the human apoM promoter does not affect binding of TRβ1 homodimers. DNA-affinity precipitation experiments using nuclear extracts from HEK293Tcells transiently transfected with expression vectors for RXRα, RARα or TRβ as indicated on the right and biotinylated PCR fragments corresponding to the wild type or the mutated -105/+42 region of the human apoM promoter. Oligonucleotide-bound nuclear receptors were detected by Western blotting using the corresponding antibodies. The experiment was performed at least three times and representative images are presented.

Suppl. Figure 4. Retinoic acid decreases the protein levels of RXRα in HepG2 cells. HepG2 cells were treated with 9-cis RA (1μM) for 8h (+) or left untreated (-) and the protein levels of HNF-4α, HNF-1α, RXRα and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times and representative images are presented.

Suppl. Figure 5. Retinoic acid, fibrates and oxysterols increase the levels of secreted apoM in HepG2 cells. HepG2 cells were treated with 9-cis RA (1μM),

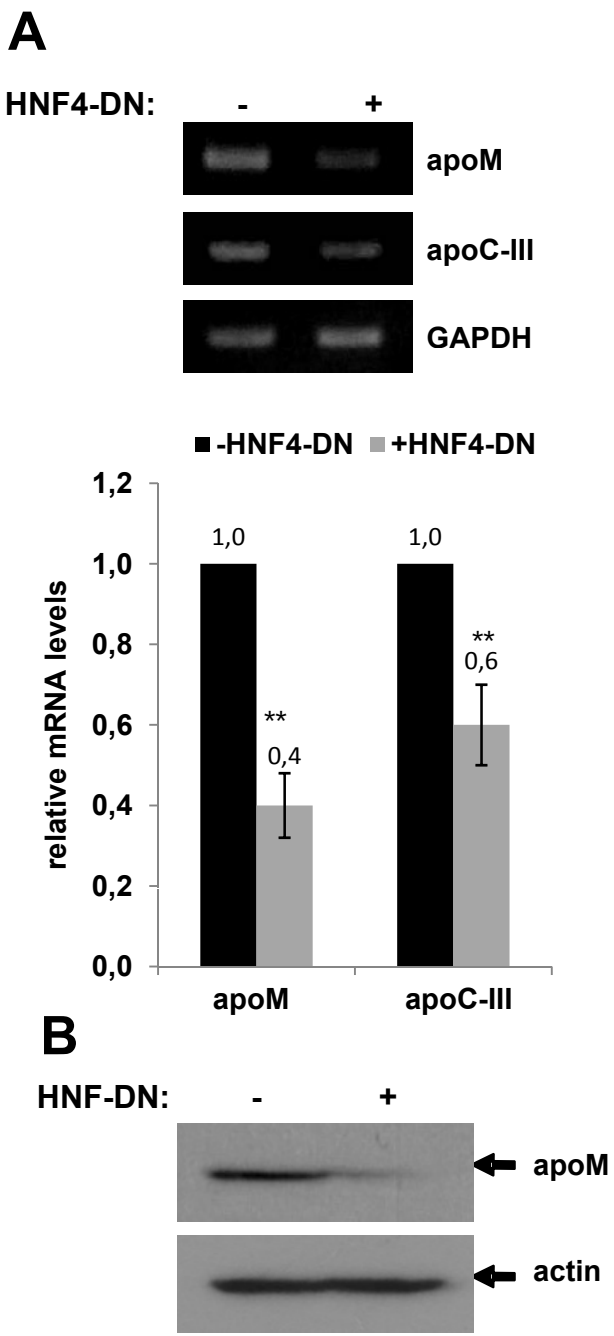
fenofibrates (250 μM) or 22(OH)C (1 μM) for 24h (+) or left untreated (-). Equal amounts of culture medium was subjected to SDS-PAGE and immunoblotting using an anti-apoM polyclonal antbody. The experiment was performed three times and a representative image is presented.

Suppl. Figure 6. HNF-4 and RXR homodimers and heterodimers activate mouse apoM promoter activity. Alignment of the human and mouse apoM proximal promoter regions. Vertical lines indicate residues that are identical and light and dark gray boxes the HNF-1α response element and the HRE respectively. The sequence identity between human and mouse apoM is 100% in the HRE and approximately 93% in the HNF-1α site. (B) HEK293T cells were transiently transfected with the (-761/-7)mouse apoM-luc reporter plasmid (1.0μg) along with expression vectors for HNF-4α, LRH-1, RXRα, RARα, PPARα, LXRα or TRβ1 (1.0μg each) and treated with the corresponding ligands as indicated. Each sample included a β-galactosidase expression plasmid (1.0μg) used for normalization of transfection variability. Luciferase activity was normalized to β-galactosidase activity and presented with a histogram. Each value represents the average (±SD) from at least three independent experiments performed in duplicate. Key: **p<0.01, ***p<0.001

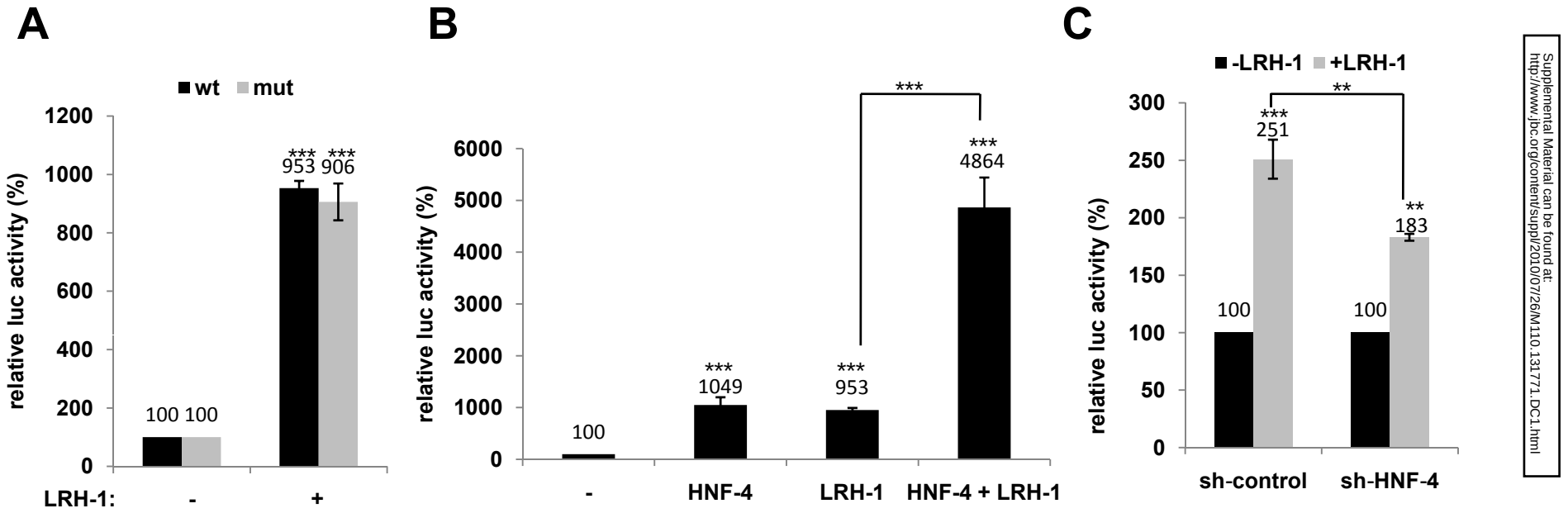
Suppl. Figure 7. Effect of LXR ligands on the expression of apoM gene in hepatic and intestinal cells. (A) HepG2 cells were treated with LXR ligands 10901317 or 22(OH)C (1μM) for 3h or with DMSO (vehicle). Total RNA was extracted and apoM, SHP and GAPDH mRNA levels were analyzed by RT-PCR. Normalized values (relative to GAPDH) represent the average from three independent experiments. (B) Caco-2 cells that had been transiently transfected with an LRH-1 expressing vector or

a control vector (-) were treated with 10901317 (1μM) for 24 h or DMSO. Total RNA was extracted and apoM, ABCA1, SHP and GAPDH mRNA levels were analyzed by RT-PCR. Normalized values (relative to GAPDH) represent the average from three independent experiments. Expression of exogenous LRH-1 in transfected Caco-2 cells was monitored by immunoblotting using an anti-LRH-1 antibody. The experiment was performed three times and representative images are presented. (C) HepG2 cells were transiently transfected with the mutated (-105/+42)human apoM-luc reporter plasmid (1.0μg) along with an expression vector for LRH-1 (1.0 μg) and were either treated with the LXR ligand 10901317 (1μM) for 24h or DMSO. The β-galactosidase expression vector (1.0μg) was included for normalization purposes. (D) HepG2 cells were transiently transfected with the mutated (-105/+42)human apoM-luc reporter plasmid (1.0μg) along with expression vectors for LRH-1 and/or SHP (1.0 μg). The β-galactosidase expression vector (1.0μg) was included for normalization purposes. In Panels C and D, luciferase activity was normalized to β-galactosidase activity and presented with histographs. Each value represents the average (±SD) from at least three independent experiments performed in duplicate

Suppl. Figure 1



Suppl. Figure 2



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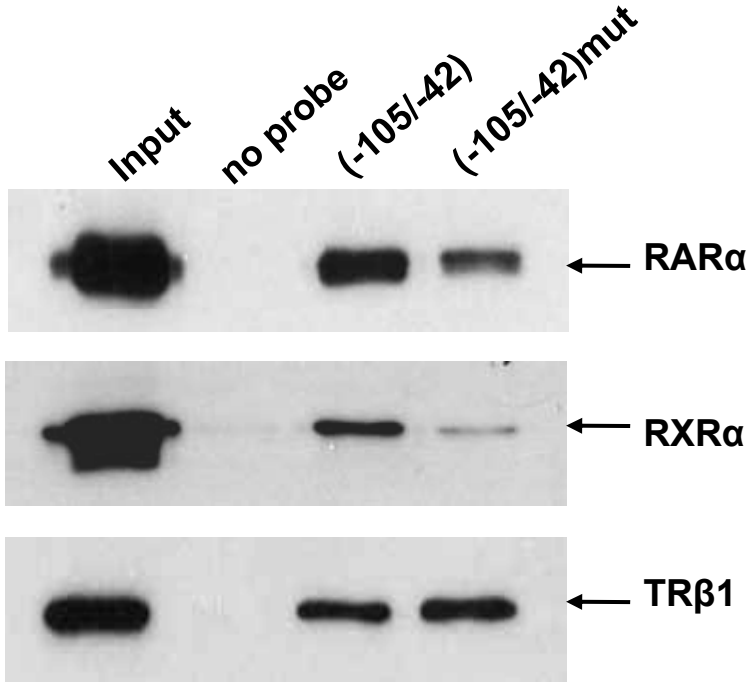

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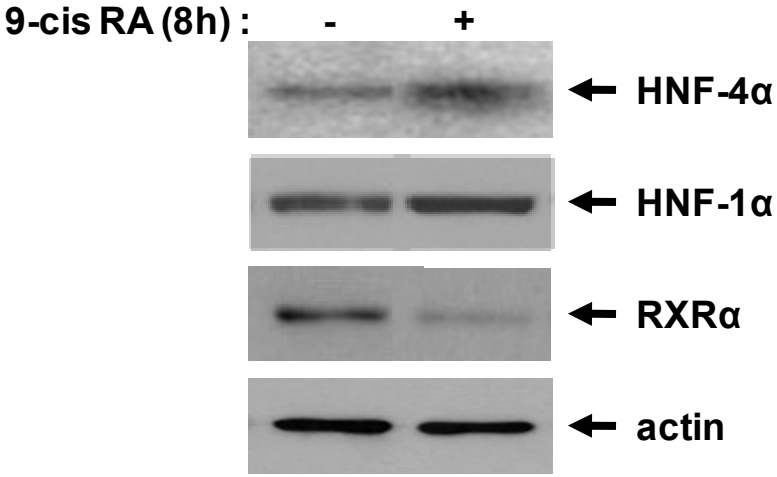

Suppl. Figure 5

A

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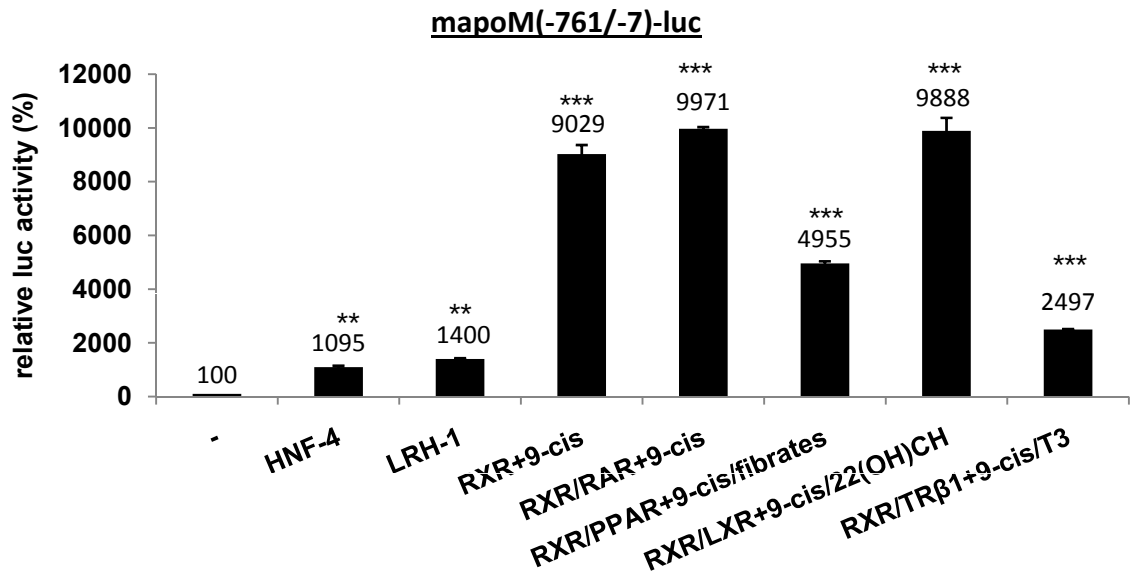
human   -187  GCTCAGCTCTCCTCATAACTCCTGAGAAACCCTGGA-CATGCCAGGGTGTACTATGGAGT
              ||||||||  |||||  |||  |||  |||  |  ||||  ||||||  ||||  ||  |  |  ||||
mouse   -238  GCTCAGCTTTCCTCCTAAGGCCTCAGAGTCACTGGGTCATGCCCGGGTATATTGTAGAGT

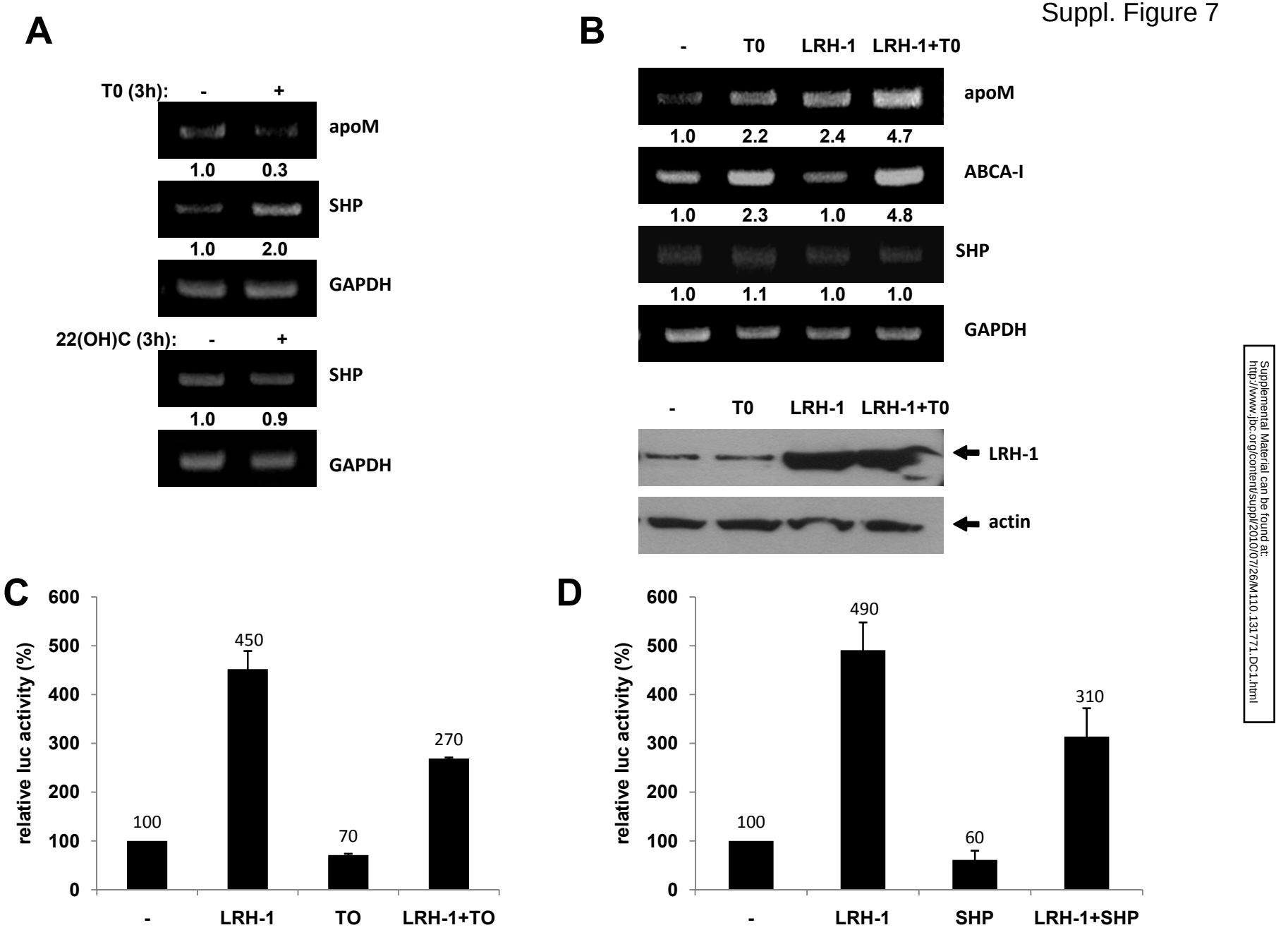
human   -128  GGTTGCATCCGGAAGAGGGGGAGGAAGTCCCAAACACTA-AGT-AATCCAGGTTTGGGTT
              ||||||||||||||||||||||||||||  |||  |  |  |  |||  ||||  |||  |||||||
mouse   -178  GGTTGCATCCGGAAGAGGGGGAGGAAGGCCCCGATGCCATAGTCAATCTAGGCTTGGGTT

                                  HNF-1a                    HRE
human   -70   GGAAAACAAGGTTGAAGTTACTCATTAGCAGGTGAAAGGGTCAAGGGTCGA
              ||||||||||||||||||||  ||||||||||  ||||||||||||||||||
mouse         
              -118  GGAAAACAAGGTTGAAGTTACTTATTAGCAGGTTAAAGGGTCAAGGGTCGA
```

B



Suppl. Figure 7



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Supplementary Tables 1-4

Supplementary Table 1.
Oligonucleotides used for cloning

name	sequence
hapoM-950F	5' -CGGGGTACCGGTGGTGTTTTGTTTGTTTGGAGAC- 3’
hapoM-642F	5’-CGGGGTACCTTGGACACATTCTGCCTGACACCAA- 3’
hapoM-402F	5’-CGGGGTACCCGACTTCTTCAAGATCAGGGGCTATG- 3’
hapoM-241F	5’-CGGGGTACCAACAAATAACCAGCTCAGATACAGG- 3’
hapoM-105F	5’-CGGGGTACCGAAGTCCCAAACACTAAGTAATCCA- 3’
hapoM-49F	5’-CGGGGTACCTCATTAGCAGGTGAAAGGGTCAAGG- 3’
hapoM-20F	5’-CAACGCAAGGGAGCTGAAAGCAGAGTGGAGAATTC A- 3’
hapoM+42R	5’ -CCCAAGCTTGCCTTAACTGCTCTCTCCCCTACTG- 3’
hapoM+8R	5’-AGCTT GAATTCTCCACTCTGCTTTCAGCTCCCTTGCGTTGGTAC- 3’
mapoM-761F	5’ -CGGGGTACCCTGAATAAACGTGAGCCCATTTGTC- 3’
mapoM-7R	5’ -CCCAAGCTTACCTCCGCCTTAACTGTTCTCTGAT- 3'
hapoM-44Fmut	5’-AGCAGGTGAAAGGGT***T***AAG***T***GTCGA***C***CGCAAGGGAGCTGAA-3’
hapoM-4Rmut	5’-TTCAGCTCCCTTGCG***G***TCGAC***A***CTT***A***ACCCTTTCACCTGCT-3’
HNF-4 shRNA sense	5’-GATCCCC*GGCAGTGCGTGGTGGACAA*TTCAAGAGA*TTGTCCACCACGCACT GCC*TTTTTA- 3’
HNF-4 shRNA antisense	5’ -AGCTTAAAAA*GGCAGTGCGTGGTGGACAA*TCTCTTGAA*TTGTCCACCACGC ACTGCC*GGG- 3’
scrambled shRNA sense	5’ -GATCCCC*TTCTCCGAACGTGTCACGT*TTCAAGAGA*ACGTGACACGTTCGGA GAA*TTTTTA- 3’
scrambled shRNA antisense	5’ -AGCTTAAAAA*TTCTCCGAACGTGTCACGT*TCTCTTGAA*ACGTGACACGTTCG GAGAA*GGG- 3’

*Kpn*I (GGTACC), *Hind*III (AAGCTT) and *Eco*RI (GAATTC) sites are underlined
Nucleotide substitutions in the primers used for site-directed mutagenesis are in bold and italic
si-RNA sequence targeting HNF-4 or si-control (scrambled) sequence is italic and underlined

Supplementary Table 2.
Primers used in RT-PCR analysis

name	sequence
hapoM-F	5’ -TATCCTTAACTCCATCTACCAGTGC- 3’
hapoM-R	5’ -AGTTACAGGTCAGTTATTGGACAGC- 3’
hapoC-III-F	5’ -AGGAGTCCCAGGTGGCCCAGCAG- 3’
hapoC-III-R	5’ -CACGGCTGAAGTTGGTCTGACCTCA- 3'
HNF-4α-F	5’ -CGAGCAGATCCAGTTCATCA- 3’
HNF-4α-R	5’ -CCAGCGGCTTGCTAGATAAC- 3’
RXRα-F	5’ -GTTGAACTCGCCTCTTTTGC- 3’
RXRα-R	5’ -GACACTTTCTTCCCCACCAA- 3’
HNF-1α-F	5’ -TCATCGAGACCTTCATCTCCAC- 3’
HNF-1α-R	5’ -ATGAACAGGCTTTGCTCCTAGC- 3’
SHP-F	5’ -GGAGTCCTTCTGGAGCCTGGAGCTT- 3’
SHP-R	5’ -AATGTGGGGTGTGGCTGAGTGAAGA- 3’
ABCA1-F	5' - GAAGCCACAAAAACATTGCTGCAT- 3'
ABCA1-R	5' - CCTCATACCAGTTGAGAGACTTGAT - 3'
GAPDH-F	5’ -ACCACAGTCCATGCCATCAC- 3’
GAPDH-R	5’ -TCCACCACCCTGTTGCTGTA- 3’

Supplementary Table 3.
Primers used in ChIP assays

name	sequence
hapoM-241F	5’ -AACAAATAACCAGCTCAGATACAGG- 3’
hapoM+42R	5’ -CCCAAGCTTGCCTTAACTGCTCTCTCCCCTACTG- 3’
hapoM-950F	5’ -CGGGGTACCGGTGGTGTTTTGTTTGTTTGGAGAC- 3’
hapoM-616R	5’ -TGTTGGTGTCAGGCAGAATGTGTCCA- 3’
hapoC-III-256F	5’ -CAG GCCCACCCCCAGTTCCTGAGCTCA- 3’
hapoC-III-45R	5’ -CCTGTTTTATATCATCTCCAGGGCAGCAGGC- 3’
hapoA-I-315F	5’ -AGAGTGACCGGGGCAGGCAGCAGGAC- 3’
hapoA-I+22R	5’ -GGCAACTGCCCACACACTCCCATGGAGG- 3’
hRhoB-313F	5’ -TCTGAATGGGAGTCGCCAACGC- 3’
hRhoB-185R	5’ -AGTCCGCGCTGCTGCTGC- 3’

Supplementary Table 4.
Oligonucleotides used in DNAP assays

name	sequence
hapoM-241F-Bio	5’ -Bio-CGGGGTACCAACAAATAACCAGCTCAGATACAGG- 3’
hapoM-105F-Bio	5’ -Bio-CGGGGTACCGAAGTCCCAAACACTAAGTAATCCA- 3’
hapoM-81R	5’ -TGGATTACTTAGTGTTTGGGACTTC- 3’
hapoM+42R	5’ -CCCAAGCTTGCCTTAACTGCTCTCTCCCCTACTG- 3’
hapoM-40F-Bio	5’-Bio-GGTGAAAGGGTCAAGGGTCGAACGCAA- 3’
hapoM-40F-Bio-mut	5’ -Bio-GGTGAAAGGGT*T*AAG*T*GTCGA*C*CGCAA- 3’
hRhoB-76F-Bio	5’ -Bio-GCCGGCTGGTTTCCCATTGGACGGCTATATTAAG- 3’