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Analysis of Gene Regulatory Functions of the Human Acyl-CoA-Binding-Protein in Lipid Metabolism

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Summary

The human acyl-CoA binding protein (ACBP) is an ubiquitary expressed multifunctional protein. ACBP is localized in the cytosol, nucleus, endoplasmatic reticulum and Golgi apparatus. As an intracellular pool former and carrier of acyl-CoAs, ACBP profoundly influences fatty acid metabolism. ACBP transports acyl-CoAs to the nucleus and interacts with the transcription factor hepatocyte nuclear factor 4 alpha (HNF-4α). The gene encoding ACBP is up-regulated by glucose and lipogenic factors and is down-regulated under fasting conditions. Here, the up- and down-regulation of the ACBP gene was simulated in liver HepG2 cells by an overexpression and RNAi approach to study the gene regulatory function of ACBP.

In order to identify ACBP target genes, genome-wide transcript profiling under siRNA-mediated ACBP knockdown in human liver HepG2 cells was performed. Based on a single sided permutation T-test ($p<0.05$) 256 down-regulated and 198 up-regulated transcripts with a minimal fold change of 1.32 (log 0.5) were identified. TaqMan-based qRT-PCR of 10 selected genes confirmed high accuracy of the array data. Gene annotation enrichment analysis revealed ACBP-mediated down-regulation of 18 genes encoding key enzymes in glycerolipid (i.e. mitochondrial glycerol-3-phosphate acyltransferase), cholesterol (i.e. HMG-CoA synthase 1 and HMG-CoA reductase) and fatty acid (i.e. fatty acid synthase) metabolism. Integration of these genes in common pathways suggested a decrease of lipid biosynthesis under ACBP knockdown. Accordingly, saturated (16:0) and monosaturated (16:1, 18:1) fatty acids were significantly reduced to 75% in ACBP-depleted cells.

In order to assess the gene regulatory function of ACBP in more detail, reporter gene analysis was performed. Using this approach, ACBP repressed the HNF-4α-induced activity of a 617bp HMGCS1 promoter fragment by about 75% in HepG2 as well as in non-endodermal HeLa cells devoid of HNF-4α. Interestingly, reporter assays without co-transfection of HNF-4α revealed an ACBP-induced reduction of HMGCS1 promoter activity by about 60-80% in both cell lines. Activities of 417bp and 317bp HMGCS1 promoter fragments were 2-4-fold decreased by ACBP.

Concordantly, HMGCS1-mRNA and -protein levels were diminished to about 40% and 30% in ACBP-expressing HeLa cells, respectively. Additionally, ACBP diminished promoter activity and transcript levels of the cholesterogenic HMGCR.

Taken together, by using the RNAi approach in combination with genome-wide expression profiling in HepG2 cells, evidence for the function of human ACBP in lipid metabolism at the level of gene expression was obtained. This effect seems to be translated to the cellular level of certain fatty acids. An overexpression approach suggested an essential function for human ACBP as a transcriptional regulator for HMG-CoA synthase 1 and HMG-CoA reductase, encoding the rate-limiting enzymes of cholesterol synthesis. The 454 identified ACBP-regulated genes represent a first reference of the human ACBP-*regulon*.

Zusammenfassung

Das humane Acyl-CoA-Bindungsprotein (ACBP) ist ein ubiquitär exprimiertes und multifunktionelles Protein. ACBP ist im Cytosol, im Zellkern, am Endoplasmatischen Retikulum sowie am Golgi-Apparat lokalisiert. ACBP dient zur Speicherung von zellulären Acyl-CoAs und ist mit dieser Funktion maßgeblich am Fettstoffwechsel beteiligt. ACBP transportiert Acyl-CoAs in den Zellkern und interagiert mit dem Transkriptionsfaktor Hepatozyten Nukleärer Faktor 4 alpha (HNF-4α). Durch Glukose und lipogene Faktoren wird das ACBP-Gen in seiner Transkription gesteigert, sowie unter Fasten reprimiert. Diese physiologischen Bedingungen sollen in der vorliegenden Arbeit in Zellkulturexperimenten nachgestellt werden, um die mögliche Funktion des ACBPs in der Genexpression zu untersuchen.

Zur Identifizierung von ACBP-Zielgenen wurden genomweit die Transkriptprofile, nach RNAi-vermittelter ACBP-Depletion in humanen HepG2-Zellen, mittels DNA-Array erfasst. Auf der Basis eines einseitigen Permutations T-Testes ($p<0.05$) konnten 256 herunter- und 198 hoch-regulierte Transkripte, bei einer minimalen Veränderung um das 1.32-fache, erfaßt werden. 10 ausgesuchte Transkripte wurden durch quantitative RT-PCR nach dem TaqMan-Prinzip verifiziert. Die Ergebnisse bestätigen die hohe Genauigkeit der Array-Daten. Eine Gen-Annotations-Anreicherungs-Analyse sowie eine funktionelle Allokation der ACBP-sensitiven Gene führten zur Identifizierung von 18 reprimierten Genen. Diese kodieren Schlüsselenzyme der Glyzerolipid- (z.B. mitochondrielle Glyzerol -3-Phosphat Acyltransferase), der Cholesterol- (z.B. HMG-CoA Synthase 1, HMG-CoA Reduktase) und Fettsäure- (z.B. Fettsäure-Synthase) -Synthese. Auf Metabolitebene führte die ACBP-Depletion in HepG2-Zellen zu einer signifikanten Erniedrigung der gesättigten C16:0, der einfach ungesättigten C16:1 und der C18:1 Fettsäuren um 25%.

Zur weiteren Untersuchung der genregulatorischen Funktion des ACBPs wurden Reporter-Gen-Analysen in HepG2-Zellen und HeLa-Zellen, die kein HNF-4α exprimieren, durchgeführt. In beiden Zelllinien konnte gezeigt werden, dass die ACBP-Überexpression die HNF-4α induzierte Aktivität eines 617bp HMGCS1

Promotorfragmentes um 75% vermindert. Interessanterweise ist diese Unterdrückung nicht von der Überexpression des HNF-4α abhängig. Die Aktivität von zwei kürzeren HMGCS1 Promotorfragmenten wird durch ACBP-Expression ebenfalls um das 2-4-fache reduziert. Darüber hinaus führt die ACBP-Überexpression in HeLa-Zellen zur Erniedrigung der HMGCS1 mRNA- bzw. Protein-Spiegel um 40% bzw. 30%. Die HMGCR wird ebenfalls auf Promotor- und mRNA Ebene unter ACBP-Überexpression reprimiert.

In dieser Arbeit wurde erstmals gezeigt, dass ACBP zentrale Gene des Lipid- und Cholesterinstoffwechsels auf Ebene der Genexpression reguliert und dass dieser Effekt teilweise auf Metabolitebene abbildbar ist. Durch ACBP-Überexpression konnte die wesentliche Bedeutung des humanen ACBPs als transkriptioneller Regulator der HMG-CoA synthase 1 und der HMG-CoA Reduktase zusätzlich bestätigt werden. Mit der Identifizierung von 454 ACBP-sensitiven Genen ist es schließlich gelungen eine erste Version des humanen ACBP-*Regulons* zu erstellen.

General Introduction

Nutrigenomics is a new field in nutritional science focusing on the molecular relationship between diet and the human genome. Post-genomic technologies are used to assess gene expression level (transcriptomics), protein level (proteomics) as well as metabolites (metabolomics) as a function of different nutrients. With these approaches, beneficial and/ or detrimental effects of food components on health and disease were studied to a certain extent during the last years (1-4). In this context, the multifunctional human acyl-CoA binding protein (ACBP) was identified as a candidate gene for psychiatric disorders and the metabolic syndrome. ACBP controls the intracellular concentration of fatty acids contributing to the development of insulin resistance. The gene encoding ACBP is up-regulated by glucose and lipogenic factors and down-regulated under fasting conditions. In the following, the up- and down-regulation of the ACBP gene was simulated in liver HepG2 cells by an overexpression and RNAi approach in order to study the gene regulatory function of ACBP.

Mogensen et al. (1987) have firstly described the existence of ACBP (5). After the purification of this small 10 kDa protein from bovine liver a vast amount of work was done describing its cellular function. However, the exact role of ACBP is still not fully understood. Several synonyms are actually used for ACBP, indicating the functional versatility of this ubiquitary expressed and highly conserved protein. Firstly, ACBP was described as diazepam binding inhibitor (DBI) or endozepine (EP) according to its ability to displace the anxiolytic drug diazepam or valium from the benzodiazepine recognition site of gamma-aminobutyric acid (GABA) receptor in rat brain (6). Thereinafter, a protein from porcine intestine was isolated that specifically stimulated cholecystokinin release and thus pancreatic enzyme secretion. Accordingly, Herzig et al. (1996) named it cholecystokinin-releasing peptide (CCK-RP). This peptide however was identical to the porcine DBI (7). The common name ACBP came up as binding studies verified sequestering of acyl-CoAs with very high affinity (K_D=1-5nM) (8).

ACBP belongs to a group of lipid binding proteins harboring also fatty acid binding protein (FABP) and sterol carrier protein (SCP). ACBP exerts high affinity towards

long-chain fatty acid coenzyme A esters (acyl-CoA) and low affinity towards the shorter acetyl- and butyryl-CoAs. Free fatty acids and cholesterol are not bound by ACBP (8-10). The high affinity to sequester acyl-CoAs depends on various structural features of ACBP (Fig. 1). Nuclear magnetic resonance spectroscopy displayed 4 α-helices in *up-down-down-up* orientation (11-14). Studies with ACBP/ palmitoyl-CoA complexes showed subdivision of the ligand binding sites into one for the adenine ring, one for the 3'-phosphate and one for the palmitoyl part of the ligand. The adenine moiety is bound into a hydrophobic pocket formed by the aromatic ring of tyrosine at position 31. The 3'-phosphate group interacts strongly with the protein through hydrogen bounds belonging to tyrosin at position 28 and lysine at position 32 and 54, making up 40% of the total binding energy. The orientation of the ligand is profoundly determined by pantetheine and cysteamine residues. Beside ACBP, mouse endozepine-like peptide (ELP) (15), membrane-associated (MA)-DBI (16) and DBI-related sequence 1 (DRS-1) (17) also possess the acyl-CoA binding domain. Recently, a crystallization study revealed a novel dimeric mode of ligand binding for human ACBP. Thus, the acyl-CoA moiety is positioned across two ACBP molecules what may enable them to flip-out (18).

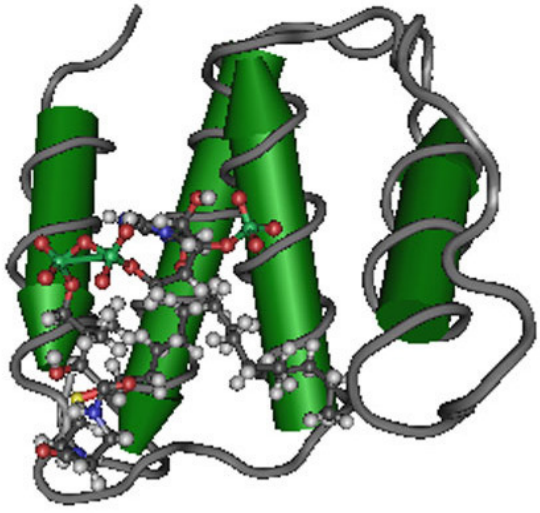

Fig. 1 3D model of bovine ACBP in complex with palmitoyl-CoA

The model is based on NMR structure established by Kragelund et al. (2003) (11) and was adopted from NCBI *Structure* database.

The ACBP gene is located on chromosome 2q12-q21, whereas an inoperable ACBP-related pseudogene is localized on chromosome 6 (19). Computational promoter analysis classified ACBP as a typical housekeeping gene (20). So far, three transcripts encoding for polypeptides of 86, 88 and 104 amino acids were identified (21,22). The three ACBP isoforms differ within their N-terminal amino acid residues and arise from alternative usage of the first exon. Most functional work however was done on the ACBP of 86 amino acid residues. ACBP is ubiquitary expressed with highest abundance in tissues of elevated energy requirement as in steroid-producing cells of adrenal cortex or testis and in epithelial cells responsible for secretion or water transport (23). At the cellular level, microscopy revealed distribution of ACBP in the cytosol, nucleus (24), endoplasmatic reticulum (ER) and Golgi (25).

The broad abundance of ACBP from yeast to mammals may indicate essentiality of this small protein. Concordantly, ACBP depletion in the parasite *Trypanosoma brucei* was lethal (26). Less severely, Acb1p (the yeast ACBP homologue) exhausted yeast were growth retarded and showed perturbed membrane assembly, organization and structure (27,28). Experimental cell culture data were rather contradictory (29-31). The loss of ACBP in mouse was not lethal but hair lipid profile and hair appearance were altered, with sparse, matted hair with greasy appearance (32).

In mammals, ACBP regulates highly specific processes depending mainly on cell and tissue type (Fig. 2). Interestingly, ACBP might control glucose homeostasis. The occurrence in glucagon-producing A cells of the endocrine pancreas suggested a paracrine interaction of ACBP with insulin secretion. Indeed, *in vitro* perfusion studies from rat pancreas (33) and isolated rat islets (34) have shown a moderate inhibitory effect of ACBP on glucose-induced insulin secretion. Inconsistently, *in vivo* administration of ACBP could not reconstitute the *in vitro* conditions (35). Thus, the impact of ACBP on insulin secretion is still under discussion. ACBP expression in brain is functionally linked with behavioral disorders. Binding of ACBP and its processing products triakontatetraneuropeptide (TTN) and octadecaneuropetide (ODN) to GABA-receptor as well as the synthesis of neuroactive steroids act neuromodulatory (36). The administration of these

peptides initiated pro-conflict behavior in rats (37,38). In humans, ACBP level were increased in patients with severe anxiety and epilepsy (39). Data concerning Alzheimer disease are conflicting (40,41). Thus, ACBP was regarded as a candidate gene for anxiety disorders. A single nucleotide polymorphism (SNP) at position 88 leading to an amino acid change (valine to methionine) was associated with anxiety mediated panic attacks (42). Moreover, external stimuli such as nicotine (43) and alcohol (44) induced ACBP-mRNA expression in mouse brain after long-term administration and thus might promote behavioral changes. In addition, hippocampus-dependent learning was impaired in ACBP transgenic mice (45).

In steroidogenic cells and tissues, ACBP and its proteolytic peptides are involved in cholesterol metabolism since their administration stimulated mitochondrial cholesterol uptake and subsequent steroid hormone synthesis (46,47). The interaction of ACBP with peripheral benzodiazepine receptor (PBR) or 18 kDa translocator protein is decisive for this function (48). ACBP is also involved in cell proliferation (49), bacterial defense (50), apoptosis (30,51) and inflammation (52,53). Beside these highly specific functions, its role in fatty acid metabolism was predominantly in focus of former work. The induction of medium-chain fatty acid synthesis by ACBP (5) established the importance of this protein in general lipid metabolism.

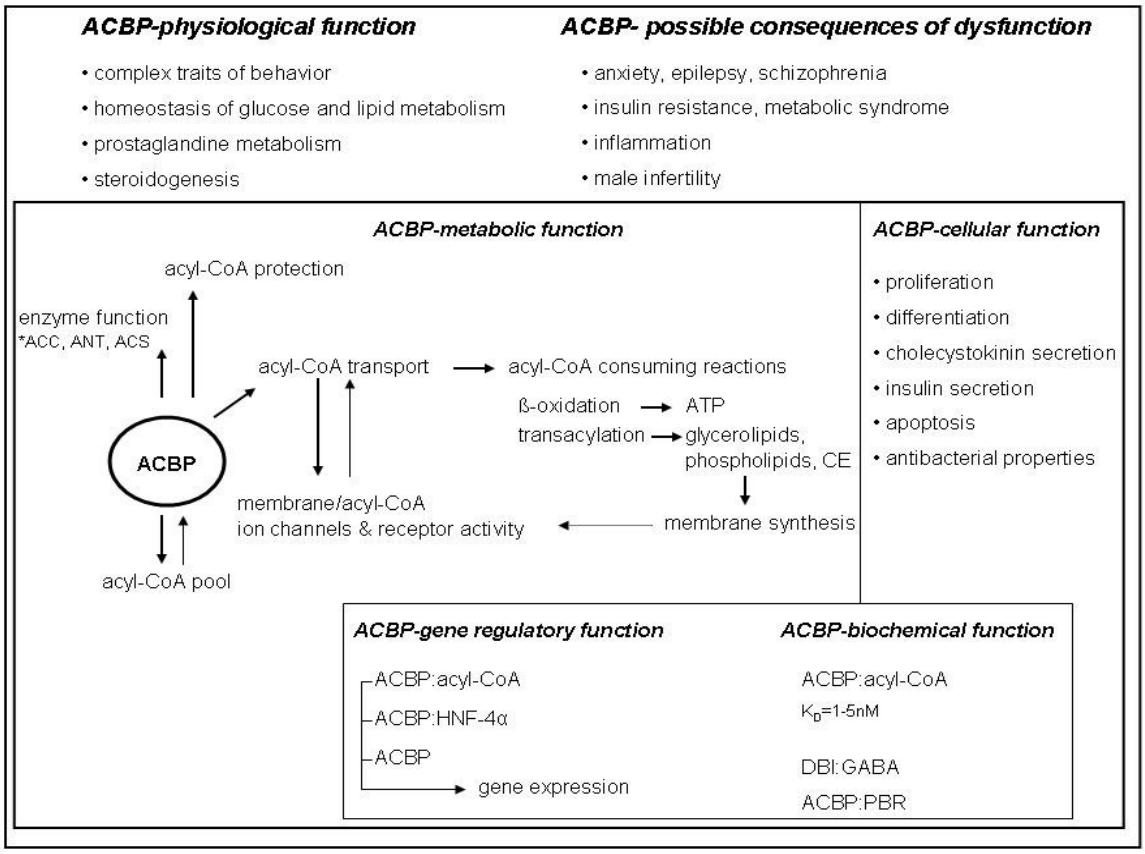

Fig. 2 Functions of ACBP at different levels of biological complexity

ACBP binds and transports acyl-CoAs towards metabolizing enzyme systems for energy production but also for the synthesis of complex lipids. Enzymes, ion channels and signal transduction pathways are also protected against acyl-CoAs detergenic effects. Because ACBP is also nuclear localized it might directly or indirectly affect gene expression. ACBP exerts highly specialized functions dependent on cell and tissue type. At physiological level, ACBP fulfils versatile functions contributing to certain pathophysiological conditions.
ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; ANT, adenine nucleotide translocase; CE, cholesteryl ester; GABA, gamma-aminobutyric acid; HNF-4α, hepatocyte nuclear factor 4α; PBR, peripheral benzodiazepine receptor

Compelling evidence that ACBP participates in acyl-CoA transport and pool formation was obtained from various *in vivo* and *in vitro* studies. *In vivo* studies in yeast expressing bovine or yeast ACBP (54,55) showed higher levels of acyl-CoAs. Moreover, overexpression of Acb1p changed acyl-CoA composition profoundly with an accumulation of C16:0-CoA and a reduction of C18:0- and C18:1-CoA (55). Concordantly, acyl-CoA composition in Acb1p knockout yeast was shifted towards C18:0-CoA enrichment and C26:0-CoA reduction (27). Similar to yeast, total acyl-CoA pool did rise in ACBP transgenic mice and rats. Data according acyl-CoA composition are rather conflicting (56,57). Accumulation

seems however to be specific to the membrane/ microsomal pool (56). The changes observed in acyl-CoA composition and length in yeast as well as in rodents further strengthen the role of ACBP in termination of fatty acid synthesis and transport of the former towards the elongation system. *In vitro*, the presence of ACBP attenuated the inhibitory effect of long-chain acyl-CoAs on long-chain acyl-CoA carboxylase (ACC) and adenine nucleotide translocase (ANT). Both enzymes are rate-limiting in energy metabolism. Additionally, ACBP protects acyl-CoAs against hydrolyses by microsomal acyl-CoA hydrolase and stimulates mitochondrial long-chain acyl-CoA synthetase (58,59). This might additionally contribute to the enlargement of the acyl-CoA pool in various overexpression approaches.

Binding and transport of acyl-CoAs by ACBP essentially affects the biological function and utilization of acyl-CoA esters. Especially *in vitro* data support the ability of ACBP to transfer acyl-CoAs between membranes. This affects the activity of membrane-bound enzymes and leads to the desorption and donation of acyl-CoAs (60). Mitochondrial carnitine palmitoyltransferase (CPT) I enzyme activity correlated with the concentration of ACBP-bound acyl-CoA and was therefore proposed to interact directly with the ACBP/ acyl-CoA-complex (61). Moreover, ACBP donates acyl-CoAs to various transacylation systems, thereby also changing enzyme activity. This was described for the acyl-CoA:lysophospholipid acyltransferase (LAT) (62) or acyl-CoA:cholesterol acyltransferase (63).

Acyl-CoAs are important intermediates for lipid metabolism as well as for signaling pathways. The availability of acyl-CoAs is also dependent on the nutritional status of a cell. ACBP protein levels are elevated under the administration of a high fat diet and diminished under a fasting regime (64). Although acyl-CoAs rise under both conditions, ACBP levels behave opposing. Thus, under a high fat diet ACBP redistributes acyl-CoAs away from the mitochondrial membrane towards the ER for triacylglyceride synthesis, whereas under fasting conditions ACBP levels decline in order to increase acyl-CoA availability for ß-oxidation. Hence, ACBP expression is regulated by lipo-oxidative peroxisome proliferator-activated receptor (PPAR) (65) as well as lipogenic sterol regulatory element-binding protein (SREBP) (66,67). Both transcription factors are key-regulators of genes involved in

lipid metabolism. Dual regulation of the ACBP gene through PPAR and SREBP establishes further ACBPs role as acyl-CoA donor and carrier (67,68). The expression of ACBP is also modulated by hormones, such as androgens (69,70) or insulin (71).

Since ACBP is nuclear localized (24) and was shown to interact with hepatocyte nuclear factor (HNF)-4α (72), it is assumed that ACBP might also possess a gene regulatory function. Moreover, the nuclear transport and abundance of acyl-CoAs might impact the activity of various transcription regulators such PPAR, HNF-4α or the thyroid hormone receptor (TR) (73,74). In Acb1p knockout yeast, transcript profiling revealed a putative gene regulatory function of ACBP. The aim of the current work was to gain insight into ACBPs gene regulatory function in human cells. As first approach, RNAi-mediated ACBP knockdown was employed in liver HepG2 cells in order to identify and characterize ACBP target genes.

Chapter I

C. Vock, K. Biedasek, I. Boomgaarden, A. Heins, I. Nitz, F. Döring*:* "ACBP knockdown leads to down-regulation of genes encoding rate-limiting enzymes in cholesterol and fatty acid metabolism". *submitted in Physiological Genomics*

In our second approach we analyzed the putative gene regulatory function of ACBP in the cholesterol biosynthesis pathway. Thus, by applying ACBP overexpression in liver HepG2 and cervical HeLa cells, we investigated the role of ACBP in the transcriptional regulation of HMG-CoA synthase 1 and HMG-CoA reductase genes. Both genes encode rate-limiting enzymes of cholesterol synthesis.

Chapter II

C. Vock, F.Döring, I. Nitz*:* "Regulation of the rate-limiting cholesterogenic enzymes HMG-CoA synthase and HMG-CoA reductase by human ACBP".
Cell Physiol Biochem. 2008;22(5-6):515-24

General Discussion

ACBP was initially classified as a typical housekeeping gene (20). Later studies revealed, that ACBP is regulated by nutritional status (64), hormones (71,75) and lipogenic transcription factors (65,67). Since ACBP controls the intracellular fate of fatty acids and acyl-CoAs, it was described as a candidate gene for diabetes type 2 (76). ACBPs nuclear localization (24) and its role in the nuclear delivery of acyl-CoAs gave rise to the assumption that ACBP might also possess a gene regulatory function. Supportively, ACBP interacts with HNF-4α (72), a nuclear factor controlling genes of glucose and fatty acid metabolism. The present work aimed to unravel the gene regulatory function of ACBP and to identify putative ACBP target genes.

In order to identify ACBP target genes, a combined approach of ACBP knockdown by RNA interference and gene expression profiling by microarray technology was employed in liver HepG2 cells (*Chapter I*). The reliability for siRNA mediated gene knockdown for gene function analysis was approved by De Souza et al. (77), since transcript profiles after siRNA mediated PPARα depletion in hepatocytes were highly comparable to those of PPARα knockout mice. Transfection of an ACBP specific siRNA efficiently reduced cellular ACBP protein levels to 20% in liver HepG2 cells. The degree of knockdown was comparable to other studies (78-80) and was not accompanied by pleitrophic effects (cytotoxicity and/ or apoptosis). Till today the essentiality of ACBP is questionable. Target disruption of ACBP in the parasite *Trypanosoma brucei* is lethal (26) and yeast exhausted in Acb1p (the ACBP homologue) are growth-retarded (27). A mutation within the ACBP locus in mice resulted in sparse hair, male infertility, failure to thrive, hydrocephaly and anemia (81). Results from *in vivo* cell culture experiments seem to be conflicting. We alike others did not find impaired viability of ACBP-exhausted cells. In contrast, Faergeman et al. (82) observed severe cell death after ACBP depletion. Knockdown efficiency might partly explain divergent results obtained from cell culture experiments. In this study a 80% reduction of the ACBP protein was achieved. Other studies reported a complete depletion of ACBP.

In this study, a homogenous cell population cultured under standardized conditions was used in order to identify ACBP target genes. 454 differential regulated genes with a minimal fold change of 1.32 were identified under ACBP knockdown. The selected threshold of 1.32 indicates rather subtle changes of transcript abundance but may facilitate the identification of physiological networks. In comparison, pharmaceutical agents lead to drastic effects on the transcriptome because of high specificity and affinity on biological targets (83). Hence, data from the current study might be more comparable to effects achieved by nutritional interventions and thus physiological conditions. Nevertheless, fold changes in transcript levels assessed by microarray were verified by qRT-PCR.

According to ACBPs function in fatty acid and acyl-CoA metabolism, gene annotation enrichment analysis revealed 22 ACBP-regulated genes allocating to cellular lipid metabolism. The vast amount of these genes was transcriptionally repressed and could be classified to fatty acid, glycerolipid and cholesterol synthesis. Thus, depletion of the lipid binding protein ACBP promotes transcriptional repression of genes encoding rate-limiting lipogenic enzymes. ACBP knockdown leads to a down-regulation of fatty acid synthase (FASN), ELOVL family member 6 (ELOVL6) and peroxisomal trans 2-enoyl CoA reductase (PECR). FASN is the rate-limiting enzyme of fatty acid synthesis since it possesses all enzymatic activities required for each cycle of fatty acid chain synthesis towards palmitic acid. ELOVL and PECR are both involved in fatty acid elongation. In accordance to gene expression data, the levels of saturated fatty acid C16:0 and the monounsaturated fatty acids C16:1 and C18:1 were reduced under ACBP knockdown. It should be mentioned, that the translation of expression profiles into metabolites is not always assured (84).

Consistent with disturbed phospholipid metabolism observed in Acb1p knockout yeast (85), mitochondrial glycerol-3-phosphate acyltransferase (GPAM) was down-regulated in our study. GPAM encodes the acylation of glycerol-3-phosphate to lysophosphatic acid. Moreover, cellular depletion of ACBP repressed four cholesterogenic genes, including HMG-CoA synthase (HMGCS1) and HMG-CoA reductase (HMGCR), encoding the rate-limiting enzymes of cholesterol synthesis. Additionally, the more down-stream acting isopentenyl-diphosphate delta

isomerase 1 (IDI1) and lanosterol synthase (LSS) were transcriptionally repressed. Therefore, only certain genes of the cholesterol biosynthesis pathway are differential expressed under ACBP knockdown. This phenomenon was also observed by others (84). Former studies already showed the implementation of ACBP in cholesterol metabolism for steroidogenic tissues (47,86). A putative gene regulatory role for ACBP in cholesterol synthesis was not yet described. Accordingly, the role of ACBP in the transcriptional control of HMGCS1 and HMGCR was investigated in a more comprehensive way (*Chapter II*). Expression of ACBP strongly suppressed HMGCS1 and HMGCR promoter activity in HepG2 and cervical HeLa cells. Moreover, HMGCS1 and HMGCR transcript levels were repressed in HeLa cells. As HMGCS1 protein was only moderately reduced by ACBP expression, cellular cholesterol levels were not significantly changed under the experimental set-up used. This might also reflect complex regulation of cholesterol biosynthesis not only at the transcriptional level but also through negative feedback control or by post-transcriptional mechanisms (87).

ACBP overexpression leads to a decreased expression of HMGCS1 and HMGCR but cellular cholesterol levels were not affected in our experimental set-up using cervical HeLa cells. In steroidogenic tissues, altered cellular ACBP level may affect steroid hormone synthesis by substrate deficiency. Modified ACBP level may also influence the steroidogenesis via the PBR signaling pathway (88). Additionally, changed ACBP level might impact the hydrophobic environment of the mitochondrial membrane and hence cholesterol uptake (89). Infertility of ACBP knockout mice *in vivo* support the profound function of ACBP in steroidogenesis (32).

In general, attenuated cholesterol build-up might impair membrane synthesis and structure. Recent studies have already shown fragmented vacuoles in yeast lacking the cholesterol equivalent ergosterol (90). Supportively, aberrant membrane structures and vesicle accumulation were observed in Acb1p knockout yeast (28). Gene expression profiling in these yeast cells showed a repression of the cytochrome P450 lanosterol 14a-demethylase which participates in the ergosterol biosynthesis pathway (85). In accordance, an altered fatty acid composition under ACBP knockdown was found in this study (Chapter I). Similar

results were also reported by others (82). In transgenic animals plasma (57) and tissue (56) cholesterol levels were not changed and likewise membrane disturbances were not observed in a yeast ACBP overexpression approach (54). Plasma membranes are crucial for the exchange of nutrients, metabolites and signal transduction. The lipid raft hypothesis (91) further strengthens the role of cholesterol for membrane organization and vesicular processes. Lipid rafts are special sphingolipid- and cholesterol-rich microdomains containing a variety of signaling and transport proteins as well as signaling lipids. Cholesterol has been proposed to be the basic element for raft formation (92). Thus, ACBP depletion might also have an impact on transduction of signals or cellular nutrient uptake.

The obtained data (*Chapter I & II*) implicate a key role for human ACBP in cholesterol synthesis at the level of gene expression. Unexpectedly, HMGCS1 and HMGCR were transcriptional repressed under ACBP knockdown and overexpression of ACBP. Thus, a transcription factor function of ACBP does not seem very presumable, although the protein is nuclear localized (24). This was supported by chromatin immunoprecipitation since binding of ACBP towards genomic sites of the HMGCS1 promoter was not observed (*Chapter II*). In general, an orchestra of transcription factors act together in controlling transcription, not only by direct DNA binding but also by co-factor recruitment acting as co-activators or co-repressors (reviewed in (93)). Physical interaction of ACBP with the transcription factor HNF-4α was shown to transactivate an apolipoprotein B (ApoB) promoter construct. Additionally, HNF-4α activates a wide variety of genes, including those involved in cholesterol, fatty acid and glucose metabolism (94). However, ACBP suppressed the promoter activity of HMGCS1 and HMGCR in endodermal HeLa cells devoid of HNF-4α (95). Based on these findings, we propose a co-factor function of ACBP in gene expression. Beside HNF-4, other transcription factor may interact with ACBP. Sterol regulatory element-binding protein (SREBP)-2 activates cholesterogenic genes under sterol-deprivation and decreases expression to basal levels in the presence of cholesterol (87). Hence, SREBP-2 is the principle regulator of cholesterol synthesis and might also interact with ACBP. This has to be approved in ongoing studies but in initial experiments co-transfection of SREBP-2 in HeLa cells could not abolish ACBPs repressive effect on basal HMGCS1 promoter activity.

Synergistic and/ or allosteric transcriptional control could be mechanisms to explain similar effects on gene expression by ACBP overexpression and ACBP knockdown. Depending on co-factor recruitment an activator could switch to become a repressor (96). Regarding ACBPs proposed co-factor function, its expression level may influence the recruitment of transcription factors, which may increase or decrease gene transcription. Post-translational modification of ACBP may also effect expression of target genes. It has been shown, that stress-induced phosphorylation of ACBP leads to trafficking of the protein from the nucleus to the cytosol (97). A shift in cellular fatty acid and/ or fatty acid derivatives could also be implicated in transcriptional suppression of cholesterol biosynthesis since these metabolites are involved in signal transduction and gene expression (59). In this regard, ACBP influences the balance between the levels of fatty acids and acyl-CoA esters (*Chapter I*). Total fatty acid content behaves proportional to cellular ACBP levels, whereas acyl-CoAs levels, especially of stearate-CoA, increased both under ACBP depletion and overexpression (27,56).

As shown recently, transcriptional changes in Acb1p depleted yeast were not complementable by a yeast mutant unable to bind acyl-CoA esters (85). The ACBP/ acyl-CoA complex or the ability of ACBP to donate acyl-CoAs towards the nucleus might modulate gene expression. Acyl-CoAs agonize and antagonize several nuclear factors (74,98). For example, acyl-CoAs activate HNF-4α under concurrent inhibition of PPARα transcriptional activity (99). Also thyroid hormone receptor (TR) interacts with acyl-CoA esters (73). Especially, acyl-CoA chain length and degree of saturation influence the preference of transcription factors for ligand binding (100). Fatty acid level and composition were modified in various experimental setups (*Chapter I) (28,56).* PPARα, retinoid X receptor (RXR)-α or liver X receptor (LXR)-α are directly activated by fatty acids whereas SREBP1c and nuclear factor κB (NF-κB) are regulated by indirect mechanisms (reviewed by (74)). The cross-talk between various nuclear factors including also transcription factor displacement from the promoter might be an additional regulatory determinant (101).

The proposed gene regulatory mechanisms of ACBP are summarized in Fig. 3. In sum, many studies describe opposing results on target gene expression after co-factor depletion and overexpression (102,103) whereas others did not (104). The

rationale for transcriptional suppression of HMGCS1 and HMGCR after ACBP depletion and overexpression is not clear but could be explained by a co-factor function of ACBP.

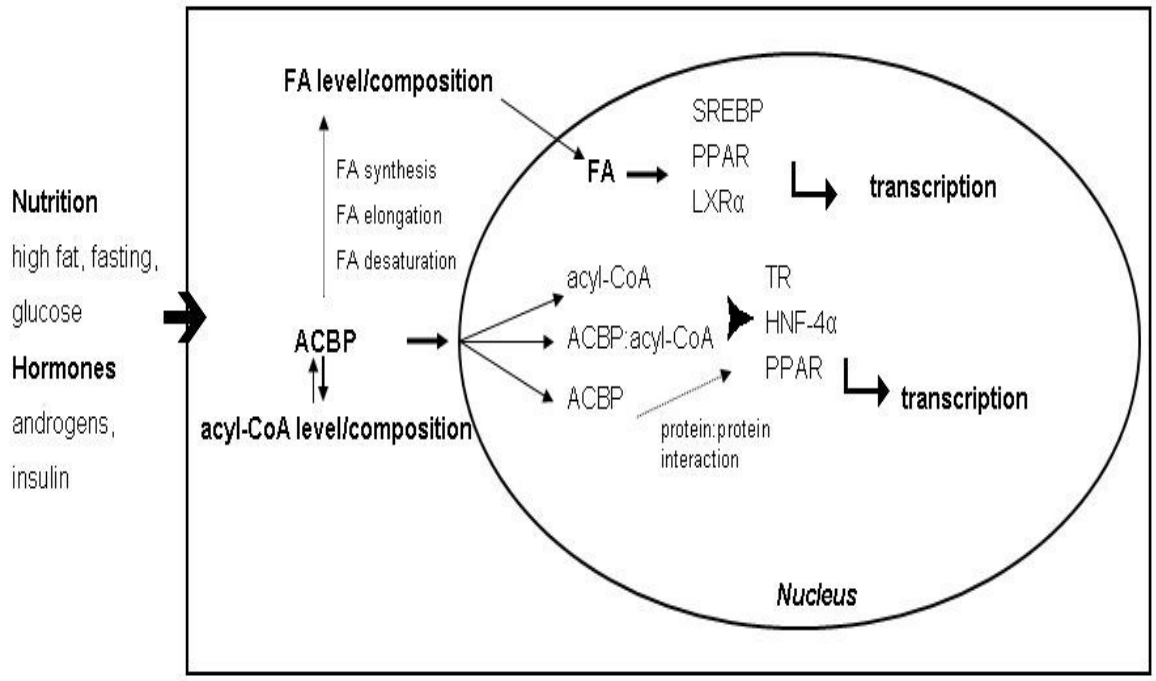

Fig. 3 Putative gene regulatory mechanisms of human ACBP
The cellular ACBP levels are modulated by nutritional regimes and hormones. This could affect the gene regulatory function of ACBP. Different modes of gene regulation by ACBP are possible. In an indirect way, ACBP level might modify the availability of specific hydrophobic-ligands for nuclear factors (e.g. FA & acyl-CoA). In a more direct manner, ACBP might act via protein-protein interaction as transcriptional co-factor.

In this study, an isolated cell culture model was used in order to unravel the gene regulatory function of ACBP. It should be mentioned, that cell cultures are artificial systems and obtained results have to be verified in more complex setups. On the other hand, cell culture systems are more feasible to study basic gene function under highly standardized conditions. Cell culture studies are also usable for future approaches, in order to anticipate the impact of special dietary factors (e.g. different fatty acids) on the gene regulatory function of ACBP. The 454 identified ACBP regulated genes represent a first reference of the human ACBP *regulon*.

This will substantially facilitate the understanding of ACBPs cellular function also in regard to its role as a disease-related candidate gene.

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Submitted Manuscripts

Chapter I

ACBP knockdown leads to down-regulation of genes encoding rate-limiting enzymes in cholesterol and fatty acid metabolism

Chapter II

Regulation of the rate-limiting cholesterogenic enzymes HMG-CoA synthase and HMG-CoA reductase by human ACBP

ACBP knockdown leads to down-regulation of genes encoding rate-limiting enzymes in cholesterol and fatty acid metabolism

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Running head: ACBP knockdown and gene expression

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Abstract

The human Acyl-CoA binding protein (ACBP) is a structural and functional highly conserved protein. As an intracellular pool former and carrier of acyl-CoAs, ACBP influences the overall lipid metabolism. Its nuclear abundance and physical interaction with hepatocyte nuclear factor 4α suggested a gene regulatory function of ACBP. In order to identify ACBP target genes we performed genome-wide transcript profiling under siRNA-mediated ACBP knockdown in human liver HepG2 cells. Based on a single sided permutation T-test ($p<0.05$) we identified 256 down-regulated and 198 up-regulated transcripts with a minimal fold change of 1.32 (log 0.5). TaqMan-based qRT-PCR of 10 selected genes confirmed high accuracy of the array data. Gene annotation enrichment analysis revealed ACBP-mediated down-regulation of 18 genes encoding key enzymes in glycerolipid (i.e. mitochondrial glycerol-3-phosphate acyltransferase), cholesterol (i.e. HMG-CoA synthase and HMG-CoA reductase) and fatty acid (i.e. fatty acid synthase) metabolism. Integration of these genes in common pathways suggested a decrease of lipid biosynthesis under ACBP knockdown. Accordingly, saturated (16:0) and monosaturated (16:1, 18:1) fatty acids were significantly reduced to 75% in ACBP-depleted cells. Taken together, we obtained evidence that ACBP functions in lipid metabolism at the level of gene expression. This effect seems to be translated into certain metabolites. The identified 454 ACBP regulated genes present a first reference for further studies to define the ACBP regulon in mammalian cells.

Keywords: RNAi, ACBP, HepG2 cells, fatty acids, cholesterol metabolism

Introduction

The human Acyl-CoA binding protein (ACBP) is a highly conserved protein, which can be found in nearly all eukaryotes. It is a typical housekeeping gene, although it's expression varies between different cells and tissues (33). ACBP possesses a number of important physiological and biochemical functions, such as inhibition of diazepam binding to benzodiazepine binding site of gamma-aminobutyric acid-ergic (GABA)-receptor (21, 22), regulation of glucose-induced insulin secretion from pancreatic beta-cells (3, 6), release of cholecystokinin (CCK) in intestine (26), stimulation of steroidogenesis through peripheral-type benzodiazepine receptor (PBR) (2, 4, 42) and modulation of cell proliferation (18). ACBP seems also to be part of the calcium-dependent proteolytic system through the activation of calpains (37). In this respect ACBP seems to be involved in pro-apoptotic processes and cell death (52).

ACBPs participation in acyl-CoA metabolism is well established and supported by its ability to bind and sequester long chain acyl-CoAs (LCFACoA) with high affinity. This suggests that ACBP functions as intracellular carrier and pool former of LCFACoA. Thereby, ACBP protects membranes and transport functions as well as enzymes against LCFACoAs detergenic and inhibitory effects (30, 34, 47, 55). Furthermore, ACBP mediates intermembrane transport of acyl-CoAs by displacing them from membranes and donating them to utilizing enzymes, i.e. ß-oxidation in mitochondria, microsomal glycerolipid synthesis and cholesteryl ester acylation (7, 44, 45). Yeast studies applying Acb1p (the yeast homologue of ACBP) overexpression or knockout, substantiate the proteins function in overall lipid metabolism. Especially knockout of ACBP evoked profound changes in membrane structure, assembly and organization (17). Loss of ACBP in mice causes defective cutaneous fatty acid metabolism (31).

ACBP is localized throughout the cytosol and at special cell organelles as Golgi apparatus or endoplasmic reticulum (23), in nucleus and in the perinuclear area (43). Except for a distinct nuclear localization during adipocyte differentiation (24), a functional relevance for ACBPs nuclear abundance is not known. A direct impact of ACBP on gene regulation was shown by Petrescu et al. (2003), which reported physical and functional interaction with the hepatocyte nuclear factor (HNF)-4α (43). Expression profiling in Acb1p yeast knockout strain revealed transcriptional changes

which were however not complementable by an Acp1p mutant unable to bind acyl-CoA esters. This supports finally the idea of ligand-mediated impact of ACBP on gene expression (16).

Here, a genome-wide expression profiling approach was applied in HepG2 cells to identify human ACBP target genes and their functional allocation. Therefore we performed siRNA mediated knockdown of ACBP and the resulting changes in gene expression were assessed with Affymetrix microarray technology. Our study shows, that ACBP-depletion in HepG2 cells reduces the transcript levels of central rate-limiting enzymes of cholesterol, fatty acid as well as glycerolipid synthesis. Accordingly, the fatty acid content of ACBP-depleted cells was also reduced

Material & Methods

Cell culture

The human hepatocellular carcinoma cell line HepG2 was cultured in RPMI-Medium + 1% Glutamax (Invitrogen, Carlsbad, USA) containing 10% fetal calf serum in a 5% CO_2/95% air atmosphere at 37°C. Media was replaced every two days and cells were passaged at subconfluency with 0.25% Trypsin in PBS to maintain exponential growth.

siRNA design

Two ACBP specific stability enhanced twenty-one-nucleotide short interfering RNAs (siRNAs), were purchased from Dharmacon Research (Lafayette, USA). siRNA1 (5'-GAAAAAATACGGGATATGA -3'), located in exon 4 at 246-264 bp relative to ATG start codon 1A, was designed according to Reynolds design criteria (46) and in consideration of RNA secondary structure. To assess secondary structure of ACBP mRNA (NM_001079862) we used the Mfold web server (57). siRNA2 (5'-GGAAGATGCCATGAAAGCT -3') located in exon 4 at 201-219 bp relative to ATG start codon was according to Faergeman et al. (15). Sequence information about ACBP was taken from GenBank (NCBI, http://www.ncbi.nlm.nih.gov). To avoid off-targeting effects of the designed siRNAs, sequence comparisons of the ACBP protein family and ACBP domain containing proteins were done using web based alignment tools at NCBI and EBI (http://www.ebi.ac.uk). Control siRNAs targeted to Lamin A/C, Cyclophilin B and a stabilized scrambled siRNA were also obtained from Dharmacon Research Inc.

Transient Transfection

For transfection experiments $1.1x10^5$ cells were seeded in 24-well plates (Sarstedt, Newton, USA). Cells were transfected at 30-40% confluency in a final siRNA concentration of 100nmol/L with Dharmafect4 transfection reagent (Dharmacon Research, Lafayette, USA) according to the manufacturer's instructions. 24 h post-transfection medium was changed. Cells treated with transfection reagent were used as negative control (mock control).

Transfection efficiency was initially monitored with fluorescein-labeled siRNA (Qiagen, Hilden, Germany) by microscopy.

Real-time RT-PCR analysis

Real-time reverse transcriptase PCR (RT-PCR) was carried out on total RNA from HepG2 cells transfected with ACBP specific or scrambled siRNA after 24 h, 36 h and 48 h on an ABI PRISM 7000 Sequence Detection System instrument (PE Applied Biosystems, Foster City, CA). For RNA isolation RNeasy Kit (Qiagen, Hilden, Germany), for cDNA preparation iScript cDNA synthesis kit (BioRad Laboratories, Hercules, USA) were used. cDNA was diluted 1:5 with RNase-free H_2O before PCR analysis. Differential regulated target genes identified by the expression profiling approach were verified accordingly. Each real-time RT-PCR reaction was done in triplicate according to manufacturer's instructions. Gene expression assays [ACBP (Hs01554584_m1), 3-hydroxy-3-methylglutaryl-CoA synthase (Hs_00266810_m1), 3-hydroxy-3-methylglutaryl-CoA reductase (Hs_00168352_m1), isopentenyl-diphosphate delta isomerase 1 (Hs_01057440_m1), lanosterol synthase (Hs_01057440_m1), acyl-CoA:cholesterol acyltransferase 2 (Hs_00186048_m1), fatty acid synthase (Hs_00188012_m1), mitochondrial glycerol-3-phosphate acyltransferase (Hs_00326039_m1), peroxisomal trans 2-enoyl CoA reductase (Hs_00218365_m1), early growth response 1 (Hs_00152928_m), 3-hydrox-3-methylglutaryl-CoA lyase (Hs_00609306_m1)] were obtained from ABI. Quantities of target mRNA were determined via standard curves using hypoxanthine phosphoribosyl-transferase 1 (HPRT1) as endogenous control. Two negative controls (-reverse transcriptase/-RNA template) were included to detect possible contaminations.

Cytotoxicity and apoptosis assays

Cytotoxicity was determined 72 h after transfection of HepG2 cells using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to manufacturer's instructions with regard of modified lysis time of 30 min. Luminescence was measured in a GloMax™ microplate luminometer (Promega, Mannheim, Germany) as a signal proportional to the amount of ATP present in metabolically active cells.

Caspase 3 and/ or caspase 7 activity was determined 72 h post-transfection using the Caspase-Glo™ 3/7 Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions with prolonged lysis of 30 min. Camptothecin (Sigma Aldrich, Saint Louis, USA) was used as a positive control for apoptosis induction. 16

h before caspase 3/7 measurement cells were incubated with 5µmol/L Camptothecin. All experiments were performed at least in triplicate.

Gas chromatography

For fatty acid analysis, $4x10^5$ HepG2 cells were plated on 6-well dishes. Transfection of ACBP specific and scrambled control siRNA was performed as described above. Accordingly, 72 h post-transfection cells were trypsinized, resuspended in PBS and stored at -80°C. For GC-analysis cells were extracted in dichloromethane:methanol (2:1). Methyl margaric acid (C17:0; 250 µg) was added as internal standard. After incubation for 15 min at 37°C, lipid phase was extracted using a $CaCl_2$ (0.04%)-solution in HPLC H_2O and dichloromethane/methanol. For preparation of fatty acid methyl esters (FAME) solvents were evaporated under nitrogen, remixed with 1.5 ml methanolic NaOH (0.5 M), and incubated at 105°C for 30 min. After adding 2 ml boron trifluoride, samples were incubated another 35 min at 105°C. 5 ml saturated NaCl-solution and 2 x 1 ml isooctane were added. The upper phase was used for gas chromatography analysis, performed on Hewlett-Peckard 6890 gas chromatograph equipped with a J&W 122-2362 DB-23 capillary column (Agilent, Palo Alto, USA) with hydrogen carrier flow rate at 40 ml/min, and air at 450 ml/min. Injection split was 1:20. The following temperature program was used: first temperature was set at 80°C, 25°C/min to 180°C, hold for 2 min, 2°C/min to 182°C, hold for 16 min, 6°C/min to 197°C, hold for 10 min, 40°C/min to 240°C, hold for 2 min, at least 250°C for 10 min. FAMEs were detected by a flame ionization detector. Sample peaks were compared with retention times of known standards for specific fatty acid identification.

Microarray experiment

Microarray experiments were performed on the human Genome U133 Plus 2.0 Genechip array (Affymetrix, Santa Clara, USA). For the experiments $4x10^5$ HepG2 cells were seeded in 6-well plates and transfected after 24 h with 100 nmol/L ACBP-specific siRNA1 or scrambled siRNA as control for 72 h. Total RNA was prepared with RNeasy Kit (Qiagen, Hilden, Germany). RNA amount and integrity was assessed spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) and by using the Bioanalyzer (Agilent, Palo Alto, USA). Experiments were done in triplicate and each approach consisted 3 pooled wells.

Processing and statistical analysis was done by the KFB-Center of Excellence for Fluorescent Bioanalysis (University of Regensburg, Germany) based on Affymetrix

protocols (Affymetrix, Santa Clara, USA). The samples were hybridized to the human genome array which contains ~38,500 full-length human genes and EST clusters. Digitized chip image data were processed using Affymetrix GeneChip Analysis Suite software version 5.0 (MAS5.0). Comparison of expression profiles from the scrambled siRNA control (set as baseline) and the ACBP specific siRNA1 transfected sample (designated as the experiment) was done with Genomatix ChipInspector software (Munich, Germany), which includes a single sided permutation T-test. For determination of differentially regulated genes the fold change was set 1.32 and false discovery rate (FDR) rests with 1%. Beside that, only significant detected transcripts after MAS5.0, as a single-rank call algorithm, were taken for further analysis (32).

Computational analysis

Gene Ontology (GO) functional classification was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (www.david.abcc.ncifcrf.gov/home.jsp) (9). Classification according biological processes and molecular function was performed on GO term level 2. Statistical significant GO terms with a *p*-value lower than 0.05, based on Benjamini-Hochberg correction for multiple testing, were presented. We utilized the text-mining system Genomatix *BiblioSphere Pathway Editio*n (BSPE) (www.genomatix.de) to identify the putative functional connection between the 454 regulated target genes. BSPE is based on co-citation of gene names and synonyms (48). The lowest co-citation filter (GFG level G0) based on abstract level was applied. Subsequent sub-analysis was applied by filtering differential regulated genes according to the GO-term of *cellular lipid metabolic process*. Promoter sequences of selected target genes were adopted from Genomatix *Gene2Promoter*. Promoter length was adjusted to 600 bp, 500 bp upstream and 100 bp downstream of transcriptional start site (TSS). Promoter sequences were subjected to *in silico* promoter analysis using Genomatix *MatInspector* to search for common transcription factor binding sites (TFBS). TFBS were represented by position weight matrices from the Genomatix Family library (Version 7.0) (5, 29).

Statistical analysis

Statistical analysis was performed with GraphPad Prism4 (Graphpad software, Inc., San Diego, USA) using 2-tailed Student´s paired t-test. All values were expressed as mean ± SD. Significant differences were considered for P values less than 0.05.

Results

siRNA-mediated knockdown of ACBP expression in HepG2 cells

For ACBP knockdown we have designed two specific siRNA sequences and analyzed their silencing effect in HepG2 cells. A non-silencing scrambled siRNA served as a negative control. Based on a fluorescence-labeled siRNA, transfection efficiency was between 50-70% (data not shown). Using 100 nmol/L ACBP-siRNA1 or -siRNA2, a reduction of ACBP-mRNA levels to 63% and 73% was observed 24 h after transfection, respectively (Fig. 1A). 36 h post-transfection, a reduction of ACBP-mRNA to 39% (siRNA1) and 48% (siRNA2) could be obtained. No further silencing effect was achieved by increasing transfection time to 48 h where ACBP-mRNA levels were decreased to 48% (siRNA1) or 54% (siRNA2). At protein level, both ACBP-siRNAs caused an approximately 80% reduction of ACBP 72 h post-transfection in HepG2 cells (Fig. 1B). Thus, we were able to establish siRNA-mediated knockdown of ACBP at mRNA and protein level in HepG2 cells.

Cytotoxic or pro-apoptotic events are not evoked by ACBP knockdown

Since ACBP is described as a cell death promoting protein (53) we determined whether ACBP knockdown causes cytotoxicity and/or apoptosis in HepG2 cells. For this purpose, 72 h after transfection of ACBP-siRNAs or control siRNAs, cell viability and apoptosis were determined using ATP and caspase 3/7 measurements. As shown in Fig. 2A, transfection of scrambled siRNA, lamin A/C-siRNA, or cyclophilin B-siRNA is not accompanied by cytotoxic effects. Importantly, the cell viability is not pertubated by ACBP knockdown. For apoptotic measurements, the well known pro-apoptotic agent camptothecin serves as a positive control. As shown in Fig. 2B, camptothecin raised caspase 3/7 activity to about 2042% compared to mock cells. ACBP-siRNA1 did not increase caspase 3/7 activity in comparison to scrambled- or cyclophilin B-siRNA. In contrast, lamin A/C-siRNA and ACBP-siRNA2 increase the caspase 3/7 activity significantly. Therefore, we used the non-cytotoxic and non-apoptotic ACBP-siRNA1 for ongoing experiments.

Transcript profiling under ACBP knockdown showed 454 differential regulated genes in HepG2 cells

A profound depletion of ACBP protein to 20% compared to scrambled control siRNA was observed 72 h after siRNA transfection of HepG2 cells. This experimental set-up was used in order to identify putative ACBP-regulated genes. We performed microarray analyses applying human Genome U133 Plus 2.0 Genechip arrays (Affymetrix) in three independent experiments and compared the mRNA profile of control cells (scrambled siRNA) with ACBP-depleted cells. Based on a single sided permutation T-test and "present" designated probe sets with a minimal fold change of 1.32 (log 0.5) we identified 454 transcripts which were consistently regulated under ACBP-depletion. 256 transcripts were down- and 198 were up-regulated. As a mandatory control of our whole set-up, we found a down-regulation of the ACBP-mRNA by a factor of 2.35±0.19 (mean±SD). As a further control, 10 transcripts were selected for verification by TaqMan-based qRT-PCR. As shown in Table 2, we verified all selected genes. The consistency between array and qRT-PCR data is mentionable. For example, array data revealed a down-regulation of the PECR-gene with a fold change of -1.53±0.01 (mean±SD) whereas qRT-PCR shows a factor of -1.49±0.09. Taken together, we identified 454 ACBP-regulated genes with high accuracy and assurance by using an RNAi-approach and whole genome expression profiling in HepG2 cells.

Gene annotation enrichment analysis revealed that ACBP-regulated genes are involved in fatty acid, glycerolipid and cholesterol metabolism

To allocate the corresponding genes of regulated transcripts with regard to *biological processes* and *molecular function* the functional annotation tool DAVID was applied. We used Gene Ontology (GO) classification of level 2 which maintains good term coverage and provides meaningful term specificity (9). Table 1 shows the number of ACBP-regulated genes which are significantly overrepresented in certain GO-terms. For example, 169 genes allocate to the term *regulation of biological processes,* 156 genes assign to *regulation of cellular processes* and 102 allocate to *cellular component organization and biogenesis*. According to *molecular function* half of the ACBP-regulated genes allocate to *protein binding*. As a second approach to allocate identified genes we used the text-mining system BibliospherePathwayEdition (BSPE) which allows a more comprehensive sub-analysis. Since ACBP has a designated

function in fatty acid and steroid metabolism, we subsequently filtered 223 ACBP-regulated genes according to the GO-classification *cellular metabolic process* and *cellular lipid metabolic process*. Based on this, we identified 16 down-regulated and 4 up-regulated genes (Tab. 2) allocated to *cellular lipid metabolic process.* Independent from GO-based gene selection we identified the 3-hydroxy-3-methylglutaryl-Coenzyme A lyase (HMGCL; +1.33) and the early growth response 1 gene (EGR1; -1.67) as regulated by ACBP. Thus, we subjected 22 ACBP regulated to further sub-analysis. A consistent down-regulation of four genes involved in cholesterol biosynthesis was observed: 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (HMGCS1; -1.60), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR; -1.36), isopentenyl-diphosphate delta isomerase 1 (IDI1; -1.32) and lanosterol synthase (LSS; -1.37). Acyl-CoA:cholesterol acyltransferase 2 (ACAT2; +1.35) was up-regulated. Eight differential expressed genes were involved in *fatty acid metabolic processes*, including acyl-CoA oxidase (ACOX1; -1.35), CD74 molecule (CD74; +1.39); cytochrome P450, family 4, subfamily F, polypeptide 3 (CYP4F3; +1.32); ELOVL family member 6 (ELOVL6; -1.34); liver fatty acid binding protein (L-FABP 1; -1.39), fatty acid synthase (FASN; -1.40), peroxisomal trans 2-enoyl CoA reductase (PECR; -1.53) and protein kinase, AMP-activated, beta 2 non-catalytic subunit (PRKAB2; -1.49). Three regulated genes encoded enzymes involved in *membrane lipid metabolic processes*: mitochondrial glycerol-3-phosphate acyltransferase (GPAM; -1.45); inositol (myo)-1(or 4)-monophosphatase 1 (IMPA1; -1.49) and ST3 beta-galactoside alpha-2,3-sialyltransferase 6 (ST3GAL6; -1.39).

ACBP knockdown reduces the fatty acid content in HepG2 cells

ACBP-regulated genes involved in lipid metabolism (Tab. 2, n=22) were allocated into common metabolic pathways based on literature and database searches. As shown in Fig. 3, 10 regulated genes encode key enzymes which could be integrated into fatty acid, glycerolipid and cholesterol metabolism. As described, the regulation of these genes was verified by qRT-PCR (Tab. 2). A close inspection of the depicted metabolism suggested a down-regulation of lipid biosynthetic pathways. Consequently, we determined fatty acid content in HepG2 cells after ACBP knockdown. As shown in Fig. 4, saturated (16:0) and monosaturated (16:1, 18:1) fatty acid were significantly reduced to nearly 75% in ACBP-depleted cells. Thus, the

ACBP-siRNA mediated down-regulation of the lipid metabolism at mRNA-level seems to be translated into the relevant metabolites.

In silico promoter analysis of ACBP target genes

We performed *in silico* promoter analysis to identify common regulatory motifs in the 20 ACBP regulated genes allocating the GO-term cellular *lipid metabolic process* (Tab. 2). Promoter sequences were extracted and analyzed according common transcription factor binding sites (TFBS). Comparative analysis resulted in the significant identification of TFs belonging to EGR/nerve growth factor induced protein C & related factor (EGRF) (p=4.33E-6), zinc factor binding protein factors (ZFBP) (p=8.75E-4) and GC-Box factors SP1/GC (SP1) (p=1.2 E-3) family. Based on the observed transcriptional repression of EGR1 we concentrated on EGR-family. Sub-analysis allocated putative binding sites for EGR1 in 16 of total 20 differential expressed genes: ACOX1, CMAS, ELOVL6, FASN, GPAM, HMGCR, HMGCS1, IDI1, IMPA1, LSS, PECR, PNPLA3, PRKAB2, RDH11, SBF2, ST3GAL6.

Four identified target genes seem not be regulated by EGR1, possess however putative TFBS for other EGR-family members. CD74 might be regulated by Wilms tumor 1 (WT1) and nerve growth factor-induced protein C (NGFIC). WT 1 and EGR2 seem to bind to CYP4F3 promoter, whereas EGR2 possesses a target site in FABP1-promoter and collagen krox protein (CKROX) in ACAT2-promoter.

Discussion

The ubiquitous expressed multifunctional protein ACBP is a well known intracellular transporter of acyl-CoAs thereby determining profoundly their intracellular fate. Through binding and directing acyl-CoAs towards nucleus and nuclear factors ACBP may influence gene expression (25). Supportively, ACBP is located in the nucleus (12) and interacts with HNF-4α (43). Here we aimed to unravel the impact of human ACBP in gene expression by applying RNAi-mediated silencing of ACBP in HepG2 cells in combination with microarray gene chip technology. We achieved a down-regulation of the ACBP protein of about 80%. Similar reductions were also reported in other siRNA-mediated knockdown studies (13, 51, 56). Our microarray approach revealed high accuracy because the measured transcript levels by microarray were very consistent to qRT-PCR verification data. In comparison to complex tissues available form ACBP overexpressing (27, 41) and knockout mice (31), our approach employed a homogenous cell population cultured under highly standardized conditions. Therefore, the identified 454 ACBP regulated transcripts in liver HepG2 cells is an appropriate collection to unravel the cellular function of ACBP in gene expression.

The broad abundance of the multifunctional ACBP in the eukaryotic kingdom could be a hint for its essential function. Indeed, target disruption of ACBP in the parasite *Trypanosoma brucei* leads to lethality (38). In ACBP-depleted yeast a retarded cell growth was described (49). A mutation within the ACBP locus in mice resulted in sparse hair, male infertility, failure to thrive, hydrocephaly and anemia (40). Furthermore, Faergeman et al. (15) showed growth arrest, cell detachment and apoptosis-induction in ACBP-depleted HepG2, HeLa and Chang cells. In contrast but in accordance to our study in HepG2 cells, ACBP knockdown in murine 3T3-L1 fibroblasts (35) and HeLa cells (52) did not point out impairment of cell viability. The differences in the degree of ACBP knockdown may partly explain conflicting results in cell culture experiments. We obtained a 80% reduction of the ACBP protein, whereas other studies showed almost complete ACBP depletion. However, to investigate the putative gene regulatory function of ACBP it seems to be desirable to avoid negative pleiotrophic effects such as cytotoxicity and/ or apoptosis by ACBP knockdown. This was achieved in our study. Also expression data excluded the ACBP mediated induction of apoptosis-related genes since those were not over-estimated according

to GO classification of *biological processes*. Thus, siRNA-mediated depletion of ACBP in HepG2 cells is a suitable tool to study its gene regulatory function. Recent studies revealed even a function of ACBP in promoting and transmitting apoptosis at the level of protein-protein interaction. In this respect, suppression of ACBP ameliorated BH3 interacting domain death agonist (Bid)-induced mitochondrial damage and therefore prevented cell death (52). Solstad et al. (54) uncovered ACBP recently as phosphorylation target in the initial phase of apoptosis.

Metabolic as well as transcriptional profiling data in ACBP-depleted cells and organisms are summarized in Tab. 3. Comprehensive studies were raised in yeast and comprised mainly parameters of lipid composition (14, 17, 49) and some gene expression data (16). Human cell culture studies focused on ACBPs role in adipocyte differentiation (35) and apoptosis induction (52). In this study, we identified 454 genes to be differential expressed in ACBP-depleted HepG2 cells. Based on gene annotation enrichment analysis using DAVID and BSPE 20 ACBP-regulated genes were allocated towards *cellular lipid metabolic processes*. Remarkably, the vast amount of these lipogenic genes were transcriptionally repressed in ACBP-depleted HepG2 cells, whereas 6 of these down-regulated genes were classified to fatty acid metabolic processes. In accordance, we observed a significant reduced content of the fatty acids C16:0, C16:1 and C18:1n9 in ACBP exhausted HepG2 cells. Studies in yeast (49) and mice (27) confirmed the role of ACBP in fatty acid and acyl-CoA metabolism. Acb1p knockout yeast strain showed reduced total fatty acid content, especially of the very long chain FA C26:0 (17).

The genes encoding FASN, ELOVL6 and PECR were transcriptional repressed in ACBP-exhausted HepG2 cells. This may explain the reduced fatty acid content in ACBP depleted cells, since all three genes encode key enzymes of fatty acid synthesis and elongation. FASN is a multifunctional protein, which has all enzymatic activities required for each cycle of fatty acid synthesis towards palmitic acid. ELOVL6 encodes for a protein catalyzing the elongation of palmitic acid towards long chain fatty acid. ELOVL is predicted to be important for tissue fatty acid composition and is also involved in obesity-induced insulin resistance (36, 39). PECR functions in fatty acid elongation, possesses high affinity to fatty acids with chain lengths up to 16 carbons and shows marked expression in liver and kidney (8). Other genes that were

down-regulated following ACBP-depletion were FABP1 and ACOX1. FABP1 encodes for a liver-specific lipid binding protein, sequestering hydrophobic molecules including fatty acids, acyl-CoAs and cholesterol (20). ACOX1 encodes for a peroxisomal fatty-acyl-CoA oxidase involved in ß-oxidation of long and very long chain fatty acid. Interestingly, ACOX1 was documented to interact with ACBP in a yeast-two-hybrid map of *Drosophila melanogaster* (19). Taken together, the obtained data suggest transcriptional repression of general fatty biosynthesis and elongation. This is supported by fatty acid profiles assessed in our ACBP knockdown HepG2 cells as well in Acb1p knockout yeast (49).

The GPAM gene was also repressed in ACBP knockdown cells. GPAM encodes a key enzyme in phospholipid synthesis catalyzing the acylation of glycerol-3-phosphate to lysophosphatic acid. This suggested a pertubated function of phospholipid metabolism under ACBP knockdown. Supportively, the relative phospholipid content and its composition was shown to be reduced in Acb1p exhausted yeast (16). Another interesting finding of our study is that ACBP-depletion changes expression of five genes allocating to *steroid metabolic processes.* Four of these cholesterogenic genes were commonly repressed. These included the two rate-limiting enzymes HMGCS1 and HMGCR as well as the more down-stream acting IDI1 and LSS. ACAT2 is involved in cholesterol esterification and was up-regulated. The fact, that not all genes of cholesterol metabolism were differential regulated was also observed by others (10). Former studies in steroidogenic cells and tissues uncovered the involvement of ACBP in mitochondrial cholesterol up-take and conversion towards pregnenolone via the interaction with the peripheral-type benzodiazepine receptor (PBR) (2, 4). In consideration of current data, depletion of ACBP may lead to low cholesterol level thereby interfering also with steroid hormone synthesis. Low cholesterol synthesis might also contribute to disturbed membrane assembly and organization (17). Recent studies have already shown fragmented vacuoles in yeast lacking the cholesterol equivalent ergosterol (28). Moreover, a disturbed vesicular trafficking was observed in Acb1p-exhausted yeast (14, 17). Gene expression profiling in this yeast cells showed a repression the cytochrome P450 lanosterol 14a-demethylase which participates in the ergosterol biosynthesis pathway (16). Together, ACBP may have a conserved gene regulatory function in cholesterol/ ergosterol biosynthesis.

A number of transcription factors are known to substantially regulate key genes of the lipid metabolism. In the context of our study, the sterol regulatory element-binding proteins 1a and 2 (SREBP) are of special interest (1, 11, 50) because they regulate most of the genes depicted in Fig. 3. Thus, an interaction between ACBP and SREBP is considerable but has to be tested in future experiments by using promoter-reporter assays, for example. *In silico* promoter analysis of the identified 20 ACBP target genes allocating to *cellular lipid metabolic processes* revealed that 16 genes possess a putative binding site for EGR1. Interestingly, the EGR1 gene itself was transcriptional repressed in ACBP exhausted HepG2 cells. The precise role of EGR1 in ACBP-mediated suppression of target genes remains open but seems to be a promising approach to unravel the gene regulatory function of ACBP.

In conclusion, genome-wide expression profiling in HepG2 cells under ACBP knockdown revealed a gene regulatory function of human ACBP in cholesterol and fatty acid biosynthesis. This effect seems to be translated into relevant metabolites. Moreover, the identified 454 ACBP regulated transcripts may present a first reference for further studies to define the ACBP regulon in mammalian cells.

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Figures

Fig.1. **Repression of ACBP transcript- (A) and protein- (B) levels after siRNA transfection.**

HepG2 cells were transfected with 100 nmol/L of ACBP specific siRNA1, siRNA2 or scrambled siRNA as negative control. A) For qRT-PCR RNA was isolated after 24, 36 and 48 h of transfection. Expression data are relative to HPRT1 as housekeeping gene and presented as mean ± SD (*p<0.05; n=2-4). B) Samples for western blot analyses were generated 72 h post-transfection. Beta-actin served as loading control. A representative immunoblot is depicted.

Fig.2. **Effects of ACBP knock-down on cell viability (A) and induction of apoptosis (B)**

HepG2 cells were transfected with 100nmol/L of ACBP specific siRNAs1, siRNA2 or control siRNAs against lamin A/C, cyclophilin B and scrambled siRNA for 72 h. Cytotoxicity and induction of apoptosis were determined by chemiluminescent measurement. Camptothecin (5µmol/L) was incubated for 16 h as a pro-apoptotic agent. Mock control cells were only incubated with transfection reagent. Data are represented relative to scrambled siRNA as mean ± SD (*p <0.001; n=4-5).

Fig.3. **Catalyzed reactions of regulated target genes allocated to *cellular lipid metabolism* and the involvement of human ACBP**

Human ACBP in involved in overall acyl-CoA metabolism. RNAi-mediated ACBP depletion resulted in down-regulation of rate-limiting enzymes of fatty acid, glycerolipid as well as cholesterol metabolism. ACBPs impact on gene regulation is assumed based on its nuclear localization. Attention was particularly given to regulated genes connected to acyl-CoA metabolism.

G3P, glycerol-3-phosphate; LA, lysophosphatic acid; CE, cholesterol ester

Fig.4. **Effect of ACBP depletion in HepG2 cells on fatty acid content**

HepG2 cells were transfected with 100nmol/L ACBP specific siRNA1 and scrambled control siRNA. Fatty acid content was assessed 72 h post-transfection by gas chromatography as described in Material & Methods. Data are presented as mean ± SD (n=3; *p<0.05).

Tables

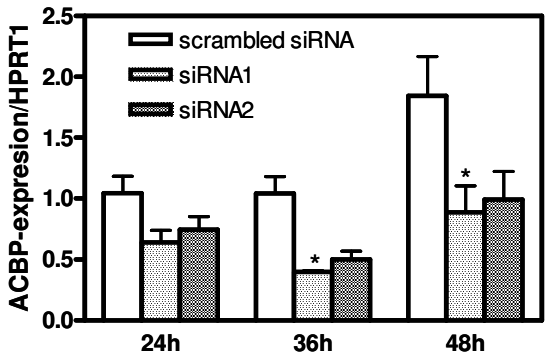
Table 1. **Gene Ontology classification of significant regulated genes in ACBP-depleted HepG2 cells according *biological processes and molecular function***

Table 2. **ACBP regulated genes allocating to the GO term *cellular lipid metabolic process* and verification by qRT-PCR**

Table 3. **Metabolic and transcriptional profiling studies conducted in ACBP-depleted cells and organisms**

Figure 1

A


ACBP-expresion/HPRT1
2.5
2.0
1.5
1.0
0.5
0.0
scrambled siRNA
siRNA1
siRNA2
*
*
24h
36h
48h

B

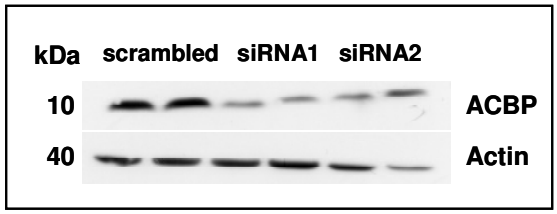
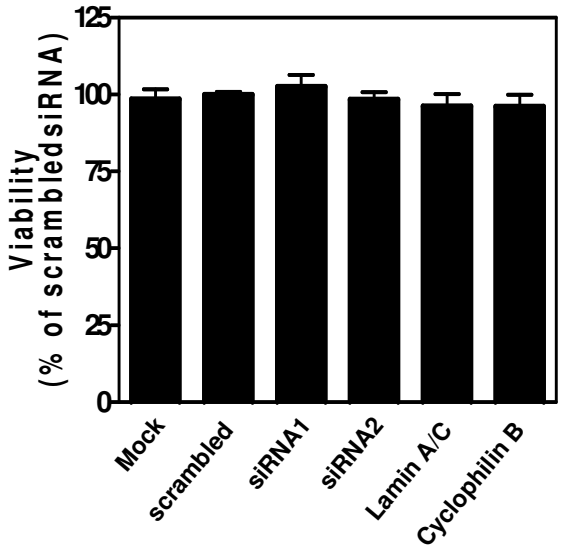

kDa
scrambled
siRNA1
siRNA2
10
40
ACBP
Actin

Figure2

A


Viability
(% of scrambled siRNA)
125
100
75
50
25
0
Mock
scrambled
siRNA1
siRNA2
Lamin A/C
Cyclophilin B

B

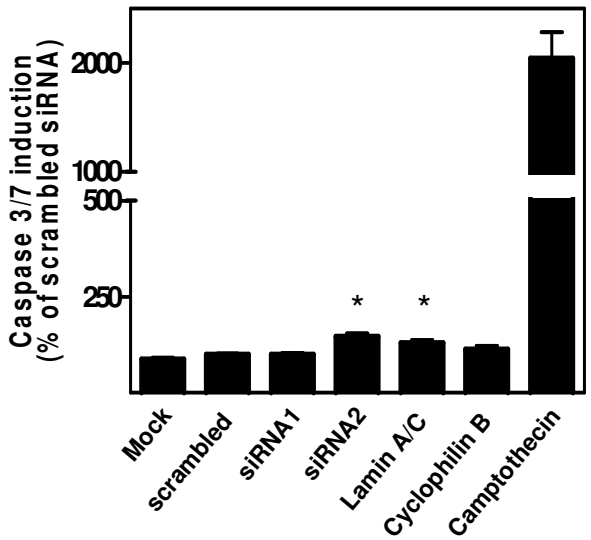

Caspase 3/7 induction
(% of scrambled siRNA)
2000
1000
500
250
*
*
Mock
scrambled
siRNA1
siRNA2
Lamin A/C
Cyclophilin B
Camptothecin

Figure 3

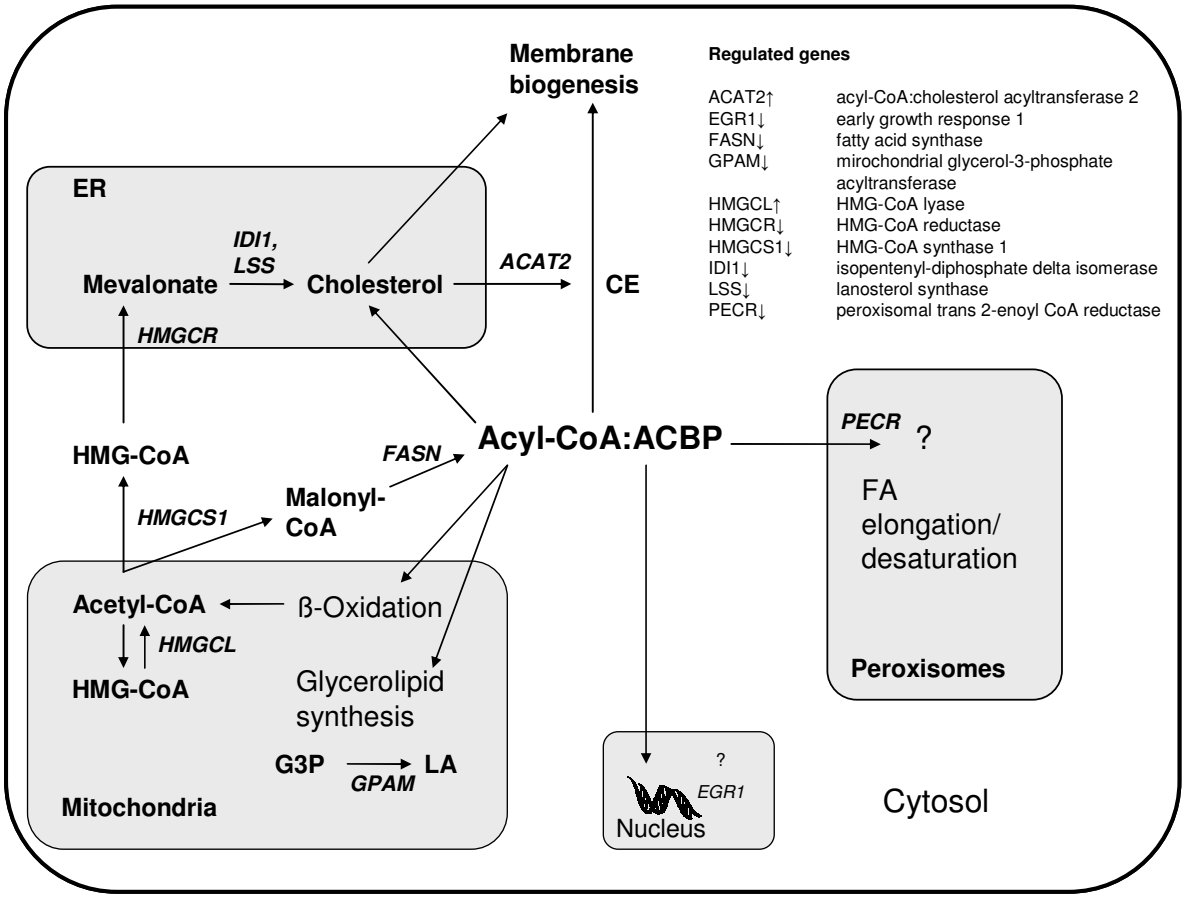

Membrane biogenesis
Regulated genes
ACAT2↑ acyl-CoA:cholesterol acyltransferase 2
EGR1↓ early growth response 1
FASN↓ fatty acid synthase
GPAM↓ mirochondrial glycerol-3-phosphate acyltransferase
HMGCL↑ HMG-CoA lyase
HMGCR↓ HMG-CoA reductase
HMGCS1↓ HMG-CoA synthase 1
IDI1↓ isopentenyl-diphosphate delta isomerase
LSS↓ lanosterol synthase
PECR↓ peroxisomal trans 2-enoyl CoA reductase
ER
IDI1, LSS
Mevalonate
Cholesterol
ACAT2
CE
HMGCR
HMG-CoA
FASN
Acyl-CoA:ACBP
PECR
?
FA elongation/ desaturation
Peroxisomes
HMGCS1
Malonyl-CoA
Acetyl-CoA
ß-Oxidation
HMGCL
HMG-CoA
Glycerolipid synthesis
G3P
LA
GPAM
Mitochondria
?
EGR1
Nucleus
Cytosol

Figure 4

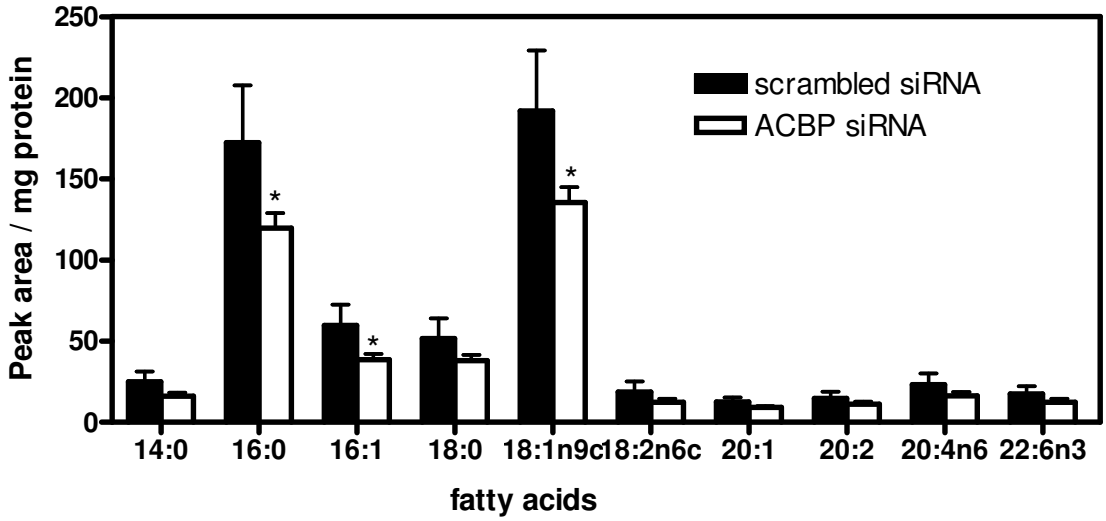

250
200
150
100
50
0
Peak area / mg protein
scrambled siRNA
ACBP siRNA
*
*
*
14:0
16:0
16:1
18:0
18:1n9c
18:2n6c
20:1
20:2
20:4n6
22:6n3
fatty acids

Table 1

	Gene count	*p*-value	Benjamini[1]
GO term biological process			
Anatomical structure development	92	2.5E-6	4.1E-4
Regulation of biological process	169	4.6E-6	3.8E-4
Multicellular organismal development	97	5.2E-5	2.8E-4
Cell cycle	47	1.2E-5	4.7E-4
Cell development	59	1.6E-5	5.1E-4
Regulation of cellular processes	156	2.3E-5	6.2E-4
Cellular developmental process	77	4.2E-5	9.8E-4
Cellular component organization and biogenesis	102	1.6E-4	3.2E-3
Death	41	2.0E-4	3.6E-3
Anatomical structure morphogenesis	49	8.0E-4	1.3E-2
Cell cycle process	36	8.0E-4	1.2E-2
Regulation of molecular function	27	3.4E-3	4.5E-2
GO term molecular function			
Protein binding	256	2.2E-13	2.5E-11
Transcription cofactor activity	19	9.7E-4	5.4E-2
Transmembrane transport	43	1.3E-3	4.7E-2

[1] p-value after Benjamini-Hochberg correction for multiple comparison

Table 2

Gene ID	Gene symbol	Gene description	Fold change	
			microarray	Real-time RT-PCR
8435	ACAT2[a]	Acyl-CoA:cholesterol acyltransferase 2	+1.35±0.08	+1.31±0.30
51	ACOX1[b, i]	acyl-Coenzyme A oxidase 1, palmitoyl	-1.35±0.09	
972	CD74[b, f]	CD74 molecule, major histocompatibility complex, class II invariant chain	+1.41±0.05	
55907	CMAS[f, g]	cytidine monophosphate N-acetylneuraminic acid synthetase	-1.33±0.12	
4051	CYP4F3[b]	cytochrome P450, family 4, subfamily F, polypeptide 3	+1.32±0.08	
1958	EGR1[k]	early growth response 1	-1.67±0.33	-1.62±0.09
79071	ELOVL6[b, f]	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	-1.34±0.02	
2168	FABP1[b]	fatty acid binding protein 1, liver	-1.39±0.03	
2194	FASN[b, f]	fatty acid synthase	-1.40±0.01	-1.31±0.21
57678	GPAM[c, f]	glycerol-3-phosphate acyltransferase, mitochondrial	-1.45±0.13	-1.37±0.13
3155	HMGCL[k]	3-hydroxy-3-methylglutaryl-Coenzyme A lyase	+1.33±0.03	+1.27±0.51
3156	HMGCR[a, e, f]	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-1.36±0.09	-1.39±0.25
3157	HMGCS1[a, e,f]	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-1.60±0.10	-1.56±0.14
3422	IDI1[a, e, f]	isopentenyl-diphosphate delta isomerase 1	-1.32±0.07	-1.23±0.20
3612	IMPA1[c, f]	inositol(myo)-1(or 4)-monophosphatase 1	-1.50±0.16	
4047	LSS[a, f]	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	-1.37±0.11	-1.11±0.33
55825	PECR[b, f]	peroxisomal trans-2-enoyl-CoA reductase	-1.53±0.01	-1.49±0.09
80339	PNPLA3[d, i, j]	patatin-like phospholipase domain containing 3	-1.36±0.21	
5565	PRKAB2[b, f]	protein kinase, AMP-activated, beta 2 non-catalytic subunit	-1.48±0.08	
51109	RDH11[e]	retinol dehydrogenase 11 (all-trans/9-cis/11-cis)	-1.35±0.03	
81846	SBF2[h]	SET binding factor 2	+1.33±0.14	
10402	ST3GAL6[c]	ST3 beta-galactoside alpha-2,3-sialyltransferase 6	-1.39±0.08	

Gene expression profiling was performed 72 h after ACBP siRNA1 transfection in HepG2 cells. Data compromise 3 independent experiments. Selected target genes were verified with real-time RT-PCR. Data are present mean fold changes ± SD. GeneID is based on NCBI GenBank database.

Sub-analysis of GO-term *cellular lipid metabolic process*

[a] steroid metabolic process; [b] fatty acid metabolic process; [c] Membrane metabolic process; [d] neutral lipid metabolic process;

[e] isoprenoid metabolic process; [f] lipid biosynthetic process;

[g] lipopolysaccharide metabolic process; [h] lipid modification; [i] cellular lipid catabolic process ; [j] glycerolipid metabolic process,

[k] literature-based allocation

Table 3

Cell type/organism	Essentiality	Transcriptional and/or metabolic effects
Yeast		
Schjerling et al. 1996	*no*	growth rate↓; C18:0-CoA↑; Δ9-desaturase (OLE-1) expression↑; FA-composition ↔, FA-content ↓
Gaigg et al. 2001	*no*	C18:0-CoA ↑; phospholipids ↔; C26:0-CoA ↓; sphingolipids ↓; perturbed plasma membrane structure; vesicle accumulation,; Elo1/2/3p expression ↔; OLE-1 expression ↑
Faergeman et al. 2004	*no*	ceramide ↓; altered vacuole morphology & assembly; phospholipid composition altered
Feddersen et al. 2007	*no*	134 differential expressed genes (44↓, 90↓) induction of FA-; phospholipid-; glycolysis-synthesis and stress response related genes; phospholipid composition altered
Cell culture		
this study *HepG2 cells*	*no*	no morphological changes; no apoptosis & cytoxicity; 454 differential expressed genes (256 ↓, 196↑), repression of rate-limiting genes of FA-synthesis, glycerolipid and cholesterol synthesis, FA-content ↓
Boujrad et al. 1993 *MA-10 Leydig cells*	*no*	hormone-stimulated steroid hormone synthesis ↓
Mandrup et al. 1998 *3T3L1 fibroblasts*	*no*	lipid accumulation ↓; adipocyte differentiation ↓; PPARγ, C/EBPα expression ↓; expression of adipogenic genes ↓
Faergeman et al. 2002 *HeLa, HepG2, Chang-cells*	*yes*	growth arrest, detachment of cells, early apoptosis induction, cell death
Shulga et al. 2003 *HeLa*	*no*	prevention of apoptosis, t-Bid-induced activation of mitochondrial μ-calpain ↓
other		
Milne et al. 2001 *Trypanosoma brucei*	*yes*	cell death
Lee et al. 2007 *mouse*	*no*	sebocyte hyperplasia, sparse, matted hair, abnormal hair lipid profile, liver lipids ↔

Transcriptional regulation of HMG-CoA synthase and HMG-CoA reductase genes by human ACBP

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Running title: ACBP regulates cholesterol synthesis

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Abbreviations

ChIP, chromatin immunoprecipitation; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HNF-4α, hepatocyte nuclear factor 4 alpha; LDL, low density lipoprotein; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; SI, sterol isomerase; VDR, vitamin D receptor

Key words: cholesterol, gene regulation, HNF-4α, promoter analysis

Abstract

The acyl-CoA binding protein (ACBP) is an ubiquitary expressed multi-functional protein which regulates basic cellular functions such as fatty acid and steroid metabolism. Since ACBP is described to interact with the transcription factor hepatocyte nuclear factor 4 alpha (HNF-4α), we investigated the role of human ACBP on transcriptional regulation of the putative HNF-4α target gene HMG-CoA synthase 1 (HMGCS1). As shown by promoter-reporter assays ACBP represses the HNF-4α-induced activity of a 617bp HMGCS1 promoter fragment by approximately 80% in HepG2 cells as well as in non-endodermal HeLa cells devoid of HNF-4α. Interestingly, reporter assays without co-transfection of HNF-4α revealed that ACBP reduces the activity of the HMGCS1 promoter by about 60 to 80% in both cell lines. Activities of 417bp and 317bp HMGCS1 promoter fragments were 2.5 to 4 fold decreased by ACBP. Concordantly, the levels of HMGCS1-mRNA and -protein were diminished to 60% and 70% in ACBP-expressing HeLa cells, respectively. Additionally, ACBP reduces the promoter activity and the mRNA levels of the cholesterogenic HMG-CoA reductase (HMGCR). In conclusion, we provide evidence that ACBP is a transcriptional regulator of the HMGCS1 and HMGCR genes encoding rate-limiting enzymes of cholesterol synthesis.

Introduction

Acyl-CoA binding protein (ACBP) is an essential member of lipid binding proteins that was originally isolated as diazepam binding inhibitor (DBI) from rat brain with its ability to displace diazepam from gamma aminobutyric acid receptors [1]. The ubiquitously expressed ACBP shows a high degree of structural and functional conservation [2] and exerts versatile cell and tissue type-specific functions as: regulation of glucose-induced insulin secretion from pancreatic beta cells [3,4], modulation of cell proliferation in testicular Leydig cells [5], modulation of monocyte mediated inflammation [6,7], antibacterial properties [8], release of cholecystokinin from intestine [9] or stimulation of steroidogenesis [10-12].

The abundance of ACBP in energy-consuming cells and tissues, such as adrenal cortex or testis, and its high affinity towards medium- and long chain acyl-CoAs, support ACBPs crucial role in fatty acid metabolism [13-15]. Besides sequestering activated fatty acids, ACBP is particularly involved in the protection and pool formation of acyl-CoAs [16,17] and their donation to cell organelles, membranes and special enzyme systems [16,18-22]. Beside being intermediates and substrates in metabolism acyl-CoAs are regulators and signaling molecules, affecting a number of cellular functions including activity of ion channels, ion pumps or enzymes [reviewed in [23]]. Moreover, they are described to impact signal transduction pathways as well as gene expression [24]. The gene-regulatory function of acyl-CoAs is based on their ability to act as agonistic or even antagonistic ligands for a number of nuclear factors. This was in detail shown for the endodermal derived hepatocyte nuclear factor (HNF)-4 alpha [25], originally regarded as orphan nuclear factor, but also for peroxisome proliferator-activated receptors (PPARs) [26]. In this respect, ACBP impacts the availability and accessibility of acyl-CoAs not only for general metabolism but also for gene regulatory properties. Recently, expression profiling confirmed the importance of acyl-CoA ligand binding for the induction of transcriptional changes in Acb1p-depleted yeast [27]. A putative gene regulatory function of ACBP is moreover implicated, as ACBP directly interacted with HNF-4α, thereby activating the transcription of genes involved in lipid and glucose metabolism [28]. In keeping with this, gene expression analysis revealed numerous genes of fatty acid, phospholipid as well as glycerol synthesis to be up-regulated in Acb1p-depleted yeast [27].

Until today data about the impact of ACBP on cholesterol metabolism are limited to steroidogenic cells and tissues. ACBP and its processing products trikontatetraneuropeptide (TTN) and octadecaneuropeptide (ODN) are described to enhance steroidogenesis by activating the transport of cholesterol towards the inner mitochondrial membrane, a process thought to be mediated by the interaction with peripheral-type benzodiazepine receptor (PBR) [11,29]. In the present study we analyzed the impact of ACBP on two key rate-limiting enzymes, HMG-CoA synthase (HMGCS1) and HMG-CoA reductase (HMGCR), in two different human cell lines by an ACBP overexpression approach.

Material and Methods

Cell culture and transient transfection

Human hepatocellular HepG2 and cervical HeLa cells were purchased from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). HepG2 cells were maintained in RPMI + 1% Glutamax (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS). HeLa cells obtained MEM media (Invitrogen, Carlsbad, USA) containing 10% FCS and 1% non-essential amino acids (PAA, Cölbe, Germany). Cells were grown until preconfluent density in a humified 5% CO2/95% air atmosphere at 37°C. Media was changed every 2 days and cells were passaged with 0.25% trypsin in PBS (HepG2) and 0.05% trypsin/0.02% EDTA solution. Transient transfections were performed with FuGene6 transfection reagent according to the manufacturer's protocol (Roche, Basel, Switzerland). For qRT-PCR and immunodetection HepG2 ($1.5x10^5$) and HeLa ($4x10^4$) cells were plated in 24-well dishes. After 24 h, cells were transfected with 800 ng ACBP-*V5* expression plasmid in 6:1 ratio of FuGene6 reagent (µl) to DNA (µg). For ChIP analysis, $1x10^6$ HeLa cells were seeded on 10 cm dishes (4 dishes per condition). Cells were transfected at 50% confluence with 15 µg ACBP-*V5* expression plasmid in 3:1 ratio of FuGene6 reagent (µl) to DNA (µg) for 24 h.

Plasmids

All plasmids were produced using cloning based on PCR and Gateway technology (Invitrogen, Carlsbad, USA). Preparation of the human HNF-4α expression plasmid is described by Klapper et al. [30]. Human ACBP isoform 1A (NM_001079862, NP_001073331) including stop-codon (ACBP) was cloned into the expression vector pcDEST40. To allow C-terminal fusion with V5 (ACBP-*V5*), site-directed mutagenesis (Stratagene, La Jolla, USA) was performed according to the manufacturer's protocol. Mutagenic primers were as follows (mutated sites underlined): for: 5'-AAAAATACGGGATA<u>G</u>GAAAGGGTGGGCG-3' and rev: 5'-CGCCCACCCTTTC<u>C</u>TATCCCGTATTTTT-3. Promoter fragments -617/-1 bp, -417/-1 bp, -317/-1 bp, and -217/-1 bp of human HMG-CoA synthase 1 (HMGCS1, NM_002130) and promoter fragment -528/-1 bp of human HMG-CoA reductase (HMGCR, NM_000859) were cloned into pSEAP-basic vector (Promega, Mannheim, Germany), containing cassette C of the Gateway vector conversion system. Positions are given

relative to transcription start sites. PCR-primers for cloning of promoter fragments are depicted in Tab.1. All plasmids were approved by sequencing.

SEAP reporter gene assay

Promoter analyses were performed using secretory alkaline phosphatase (SEAP) as reporter gene. HepG2 ($3x10^4$) and HeLa ($5x10^3$) cells were seeded in 96-well plate format and cultured for 24 h. Cells were transfected using FuGene6 transfection reagent (Roche, Basel, Switzerland) after manufacturer's instruction in 6:1 ratio of FuGene6 to DNA with 60 ng of promoter construct, 37.6 ng ACBP/ACBP-*V5* expression plasmid and 2.4 ng of luciferase-containing pGl3 control plasmid (Promega, Madison, USA) as transfection efficiency control. For transfection with human HNF-4α, 50 ng promoter plasmid, 31.5 ng ACBP expression plasmid, 16 ng HNF-4α and 2.0 ng pGl3 were accordingly transfected. The total amount of 100 ng plasmid per well was adjusted with empty pcDEST40 expression vector. Transfection of pSEAP2-basic without promoter fragment was used as negative control for background correction.

After 48 h media was removed and frozen at -20°C until measurement of SEAP activity with Great EscAPe SEAP chemiluminescence detection kit (BD Bioscience Clontech, Palo Alto, USA). Cells were washed with 1x PBS, lysed by the addition of Glo Lysis Buffer (1x) and kept at -20°C (Promega, Madison, USA). Luciferase activity was measured with commercial Bright-Glow Assay Reagent (Promega, Madison, USA) after manufacturer's protocol. Chemiluminescence measurement was performed on 96-well flat-bottom opaque microplate in GloMax™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). Each experiment was performed at least 3 times in triplicate.

Real-time RT-PCR (TaqMan®)

Quantification of HMGCS1 and HMGCR mRNA level was performed with quantitative RT-PCR based on TaqMan® principle. In brief, total RNA was isolated after 12, 24 and 48 h of transfection using RNeasy kit (Qiagen, Hilden, Germany). cDNA was prepared using iScript cDNA synthesis kit (BioRad Laboratories, Hercules, USA). QRT-PCR was done in triplicate in at least three independent cell culture experiments using ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, USA). HMGCS1 and HMGCR gene expression assays were commercially available from PE ABI. Quantification was based on comparative ΔCt method using hypoxanthine phosphoribosyl-tranferase 1 (HPRT1) as endogenous control.

Two negative controls (-RT/-RNA template) were included to detect possible contamination. PCR was performed according to the manufacture's protocol (PE Applied Biosystems, Foster City, USA).

Immunodetection of ACBP and HMGCS1

Transient transfection of HeLa cells was performed as described. After 72 h cells were harvested and lysed in 1x reaction buffer (5 mmol/L colic acid, 0.1% Triton X-100 in PBS, pH7.4) incubated for 20 min at 4°C and subsequently sonicated. Protein concentration was measured with BCA kit (Pierce, Rockford, USA) according manufacturer's instruction. Reaction buffer (1x) was used as lysis buffer for subsequent cholesterol measurement [31].

Whole cell extracts were suspended in NuPage LDS sample buffer (Invitrogen, Carlsbad, USA) and denaturated at 70°C for 10 min. Samples were loaded on 12 % polyacrylamide gel and transferred by electroblotting to a PVDF membrane (Roth, Karlsruhe, Germany). ACBP specific polyclonal antibody (ACBP_total) was raised in rabbit by Eurogentec (Seraing, Belgium) after immunization of specific antigen (^{58}AWNELKGTSKEDAMK^{72}) mapping exon 3 and 4 of ACBP 1A isoform (NP_001073331).

For immunodetection membranes were probed with specific primary antibodies Ab ACBP_total (1:2000), HMG-CoA synthase 1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) or actin goat polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, USA) followed by horseradish peroxidase-coupled secondary antibody (1:10.000). Immunoreactive bands were visualized using chemiluminescent substrate ChemiGlow and quantified with Alpha Ease FC software (Alphaimager HP, AlphaInnotech, San Leandro, USA).

Cholesterol measurement

Cellular cholesterol content was measured by Amplex® Red Cholesterol Assay (Molecular Probes/Invitrogen, Carlsbad, USA). Protein concentration of the samples was measured with BCA kit according manufacturers instructions (Pierce, Rockford, USA). Cellular cholesterol is oxidised to H_2O_2 and detected by fluorescent measurement using 10-acetyl-3, 7-dihydrophenoxazine (Amplex Red reagent) with Infinite F500 plate reader (λ_{Ex}= 535nm; λ_{Em}=590 nm, Tecan Crailsheim, Germany).

Results are expressed as cholesterol concentration (µg/ml) in corresponding samples of 10 µg protein absolute. Each sample was measured in duplicate.

Chromatin immunoprecipitation

ChIP assay was performed using ChIP-IT^{TM} Express enzymatic kit (Active Motif, Carlsbad, USA) after manufacturer's protocol. Protein/DNA complexes were fixed with formaldehyde and chromatin was sheared enzymatically into uniform fragments of 200-1000 bp size. Specific ACBP/DNA complexes were precipitated overnight using 3 µg of ACBP_total primary antibody. To control efficiency of chromatin immunoprecipitation and to exclude non-specific DNA binding, 3 µg of rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, USA) was used in parallel. Chromatin was isolated according manufacturer's instructions and stored at -80°C until PCR analysis.

Chromatin was subjected to PCR analysis to amplify putative HMGCS1 promoter regions subjected to ACBP interaction. Primers for amplification of 207 bp HMGCS1 promoter fragment were as follows: forward 5'-TGGCCCGCATCTCCTCTCAC-3' and reverse 5'-GCTAGGATTTTCCCTCGTG -3. Amplification was carried out under following cycling conditions: 3 min at 94°C, 5 cycles with 30s at 94°C, 30s at 64°C, 30 s at 72°C, 5 cycles with 30s at 94°C, 30s at 62°C, 30 s at 72°C, followed by 30 cycles of 30s at 94°C, 30s at 60°C, 30 s at 72°C and final extension at 72°C for 10 min. As negative control genomic primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used (F: 5'-TCCATGACAACTTTGGTATCGTGG-3', R: 5'-GACGCCTGCTT-CACCACCTTCT-3'. Amplification of chromatin prior to immunoprecipitation was used as an input control. ChIP was performed in two independent experiments comprising independent cell passages. Chromatin of each experiment was precipitated twice with appropriate antibody.

Statistical analysis

Statistical analysis was performed with GraphPad Prism4 (Graphpad software, Inc., San Diego, USA) using 2-tailed Student´s paired t-test. All values were expressed as mean ± SD. Significant differences were considered for P values less than 0.05.

Results

ACBP represses the HNF-4α-induced promoter activity of HMGCS1

ACBP is described to interact with HNF-4α [28], a transcription factor that controls glucose, fatty acid and cholesterol metabolism [32]. Because ACBP is also described as putative regulator of cholesterol metabolism [11,29], we investigated the impact of HNF-4α and ACBP on the promoter activity of HMGCS1. This gene encodes a rate-limiting enzyme of cholesterol synthesis and contains a putative HNF-4α binding site within its promoter region at position -274 to -249. We performed promoter-reporter assays with a functional 617bp segment of the HMGCS1 promoter. This HMGCS1-SEAP construct showed an about 60-fold higher activity in comparison to the negative control plasmid pSEAP-basic (data not shown) and could therefore be classified as moderate promoter. As shown in Fig. 1, HNF-4α led to 2-fold activation of basal HMGCS1 promoter activity in human liver HepG2 cells. Interestingly, co-transfection of a V5-tagged human ACBP cDNA (ACBP-*V5*) together with HNF-4α strongly decreased the HNF-4α effect by a factor of 5 (Fig. 1). In comparison to HepG2 cells which contain endogenous HNF-4α, HeLa cells show no HNF-4α expression [33]. Here, the HNF-4α-induced activity of the HMGCS1 promoter is decreased by ACBP-*V5* to nearly 25% (Fig. 1). Thus, we found evidence in two different cell lines that ACBP represses the HNF-4α-induced promoter activity of the HMGCS1 gene.

ACBP is a negative transcriptional regulator of HMGCS1

Based on our findings we speculate that ACBP may function as a negative transcriptional regulator of HMGCS1. To test this, ACBP-mediated activity of the HMGCS1 promoter was determined without co-transfection of HNF-4α. As depicted in Fig. 2A, the ACBP-*V5* fusion protein significantly repressed the activity of the 617bp HMGCS1 promoter fragment by 63% and 83% in HepG2 and HeLa cells, respectively. In order to exclude possible artifacts of the V5-tag, a plasmid encoding native ACBP was also used. As shown in Fig. 2A, promoter activity of HMGCS1 was strongly repressed by native ACBP to 20% in HepG2 and to 25% in HeLa cells.

In order to provide further evidence for repressive effect of ACBP on HMGCS1 promoter activity, we performed reporter gene assays using -417/-1, -317/-1, and -217/-1 bp fragments of the HMGCS1 promoter. Since the shortest 217bp fragment showed only poor activity in HepG2 and HeLa cells, an ACBP-mediated repressive effect would

hardly be detectable. Therefore this fragment was excluded from ongoing experiments. In comparison to the -617/-1 HMGCS1 promoter fragment, -417/-1 and -317/-1 constructs showed 50-70% activity in both cell lines. As shown in Fig. 2B, these shorter HMGCS1 promoter fragments respond strongly to ACBP-*V5*. For the -417/-1 construct, a repressive effect to 27% and 21% could be observed in HepG2 and HeLa cells, respectively. ACBP-*V5* mediated decrease of HMGCS1 -317/-1 promoter activity was 62% in HepG2 cells and 47% in HeLa cells. Together, these findings show that ACBP represses the activity of different HMGCS1 promoter constructs.

ACBP decreases the levels of HMGCS1-mRNA and -protein

To analyze if the repressive effect of ACBP on HMGCS1 promoter could also be observed at the transcript level, we quantified HMGCS1 mRNA levels in HepG2 and HeLa cells 24 h and 48 h after transfection of ACBP-*V5*. As shown in Fig. 3A, transcript levels of HMGCS1 did not change significantly in dependence of ACBP expression and time in HepG2 cells. In HeLa cells mRNA levels of HMGCS1 were considerably diminished to 64% ($p=0.064$) and 70% ($p=0.057$) 24 h and 48 h after ACBP-*V5* transfection, respectively.

We further investigated if ACBP decrease HMGCS1 at protein level. For this purpose, we analyzed HMGCS1 protein levels in HeLa cells expressing ACBP-*V5*. Expression rate of the 14 kDa V5-tagged ACBP was also assessed and quantification was based on densitometric measurement. Based on a 6-fold enrichment of V5-tagged ACBP compared to endogenous ACBP levels (Fig.3B, upper panel) we observed reduced HMGCS1 protein levels to 70% compared to control cells after 48 h (Fig. 3B, middle panel). Together, mRNA as well as protein level of HMGCS1 were negatively regulated by ACBP.

The activity of a HMGCR promoter and the HMGCR mRNA is reduced by ACBP

In order to extend our analysis regarding ACBP as a transcriptional regulator, we investigated the HMGCR gene, which encodes the second rate-limiting enzyme in cholesterol synthesis. As shown in Fig. 4, ACBP-*V5* reduces the activity of a 528bp promoter fragment of HMGCR by 68% and 84% in HepG2 as well as in HeLa cells. The HMGCR-mRNA is not substantially affected by ACBP-*V5* in HepG2 cells. However, in HeLa cells ACBP decreases HMGCR mRNA levels significantly to 56% after 24 h.

Distinct HMGCR mRNA repression was not observed 48 h post-transfection. Thus, ACBP is also a negative transcriptional regulator of HMGCR.

No impact of ACBP on cellular cholesterol level

Given that ACBP-*V5* represses HMGCS1 and HMGCR at transcriptional level, we investigated the impact of ACBP on cholesterol levels in HeLa cells. As shown in Tab. 2, 48 h and 72 h after ACBP-*V5* transfection we observed no difference in total cholesterol levels between control cells and cells expressing ACBP-*V5*.

Chromatin immunoprecipitation revealed no direct interaction of ACBP with HMGCS1 promoter

To identify a possible direct binding of ACBP to HMGCS1 promoter we performed chromatin immunoprecipitation in HeLa cells transfected with ACBP-*V5* for 24 h. Precipitated DNA was subjected to PCR analyses using primers to amplify a 207 bp fragment corresponding to the HMGCS1 promoter sequence -418/-212. As depicted in Fig. 5, specific amplification of -418/-212 bp HMGCS1 promoter fragment was obtained in the presence of ACBP antibody. Amplified 207 bp HMGCS1 promoter fragment was less abundant when precipitated with non-specific IgG antibody. PCR analysis with human GAPDH primer set showed enrichment of 306 bp GAPDH amplicon with ACBP antibody precipitated chromatin when compared with IgG precipitated chromatin. Enrichment of control GAPDH PCR product in ACBP precipitated chromatin samples indicates unspecific binding of chromatin towards polyclonal ACBP antibody.

Discussion

ACBP is a multi-functional protein which is involved in basic cell metabolism such as fatty acid metabolism and steroidogenesis. ACBP functions also in highly specialized and regulated processes such as insulin [3,4] and cholecystokinin secretion [9]. The best established role of ACBP is its ability to bind long-chain fatty acid-coenzyme A esters (acyl-CoAs). ACBP acts as a cellular acyl-CoA transporter and pool former. Beside being intermediates of fatty acid biosynthesis and consumption, acyl-CoAs are described as regulatory and signaling molecules involved in gene expression and cell metabolism [23]. Therefore, ACBP is an important regulator protein for cell signaling and gene regulation. It is also known that ACBP is involved in steroid biosynthesis by increasing cholesterol transport to mitochondria [34]. Here we show by promoter-reporter assays in two different cell lines that ACBP decreases the transcription of the HMGCS1 and HMGCR gene encoding the initial enzymes of cholesterol biosynthesis. The increased ACBP expression was highly comparable to liver ACBP level achieved in transgenic rats [35]. The HMGCS1 catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA towards 3-hydroxy-3-methyl-glutaryl (HMG)-CoA. HMGCR catalyzes subsequently the conversion of HMG-CoA to mevalonate. In comparison to significantly decreased promoter activities and mRNA levels of HMGCS1, western analyses revealed only a slight reduction in protein expression level to 70%. Analogous to this weak down-regulation no changes in cholesterol levels were observed. These findings reflect the complex regulation of the cholesterol biosynthesis, a pathway that comprises numerous stages with counteracting regulatory mechanisms. In general, cholesterol synthesis is tightly and complex controlled in order to meet cellular cholesterol demands [36]. Thus, versatile transcriptional and post-transcriptional mechanisms are employed. These include not only feed-back control of target gene transcription and translation but also allosteric control of HMGCS1 and HMGCR activity as well as cholesterol uptake [37]. Regarding cholesterol levels, those regulatory mechanisms might compensate the repressing effect of ACBP on the transcription of HMGCS1 and HMGCR. Thus, it would be of interest to measure metabolites, for example HMG-CoA or mevalonate, in closer vicinity to the repressed HMGCS1 and HMGCR.

To get insight into the precise function of ACBP as a transcriptional regulator we performed ChIP analyses using the promoter fragment which showed the strongest suppressive effect of ACBP on HMGCS1. This approach revealed no genomic sites that

were directly bound by ACBP. Therefore, ACBP seems not to be a transcription factor. A way in which ACBP could regulate the expression of HMGCS1 and HMGCR is physical interaction with HNF-4α. Petrescu et al. (2003) reported direct interaction of ACBP with HNF-4α as well as a stimulatory effect of ACBP on HNF-4α mediated transactivation of an apolipoprotein B (ApoB) promoter construct [28]. Contrarily to this stimulatory effect, ACBP lowered the HNF-4α induced activity of the HMGCS1 promoter. Further, ACBP repressed the activity of the HMGCS1 and HMGCR promoter fragments in HeLa cells. This non-endodermal cell line does not express HNF-4α [33]. Thus, a profound function of HNF-4α on ACBP mediated transcriptional repression of HMGCS1 and HMGCR is not presumable. A study by Misawa et al. (2003) confirmed interaction of sterol-responsive element binding protein (SREBP)-2 with HNF-4α to effectively activate cholesterogenic genes, including low density lipoprotein receptor (LDLR), sterol isomerase (SI) and HMGCS1 [38]. Here we show that HNF-4α alone transactivates HMGCS1 promoter under sterol-independent conditions. This may provide further insights into the complex regulation of cholesterol synthesis pathway.

In principal, SREBP-2 is another transcription factor which may interact with ACBP. Supportively, our HMGCS1 promoter fragments contain two functional sterol regulatory element (SRE)-like motifs within the region -290 to -281 and -308 to -299, which are also conserved in the hamster HMGCS1 gene [39,40]. SREBPs require co-regulatory proteins such as CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) or the universal transcription factor SP-1 the for their gene regulatory function. In preliminary experiments, we tested possible interaction between SREBP-2 and ACBP using HMGCS1 promoter constructs in HeLa cells. SREBP-2 could not abolish ACBP repressive effect on basal HMGCS1 promoter activity suggesting no functional interaction.

It has been shown that overexpression of ACBP increases cellular acyl-CoA content in yeast and mouse liver [17,41,42]. Acyl-CoAs are important ligands for several enzymes and are implicated in regulation of gene transcription [43,44]. In this context, the well known transcription factor PPARα, which interacts with the retinoid X receptor (RXR), is described to directly interact with acyl-CoAs [26] and may regulate HMGCS1. Competition of acyl-CoA binding by ACBP and PPARα might be one possible mechanism of the observed ACBP effect on HMGCS1. Interestingly, ACBP repressed

317 bp HMGCS1 promoter fragment less strongly in comparison to 417 bp promoter construct. Thus, ACBP might interfere with HMGCS1 promoter activity within the region -417 and -317. Promoter analysis applying Genomatix *MatInspector* (www.genomatix.de) revealed binding sites for various transcriptional regulators. A putative recognition site for vitamin D receptor/RXR heterodimers (VDR/RXR) was identified. Conditions of heterodimeric complex formation include the presence of the cholesterol-derived 1,25-dihydroxyvitamin D3 [45]. Taken together, the contribution of PPARα/RXR and/or VDR/RXR to the suppression of HMGCS1 by ACBP needs to be investigated in further studies.

In conclusion, our findings indicate for the first time that ACBP regulates HMGCS1 and HMGCR, two enzymes in the initial steps of cholesterol biosynthesis. Further work is necessary to delineate the precise mechanisms by which ACBP represses HMGCS1 and HMGCR gene expression and consequently cholesterol synthesis.

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Figure legends

Figure 1

HNF-4α induced HMGCS1 promoter activity was suppressed by ACBP in HepG2 (A) and HeLa (B) cells.

HepG2 and HeLa cells were transfected with HNF-4α alone or together with ACBP-*V5* expression plasmid producing the 14 kDa V5-tagged ACBP. SEAP activity was measured 48 h post-transfection and normalized to luciferase containing pGl3. Data are presented as mean ± SD (n=3; Student's t-test).

Figure 2

Expression of ACBP suppressed the activity of basal 617bp HMGCS1 promoter (2A) and of 417bp and 317bp HMGCS1 promoter deletion fragments (2B) in HepG2 and HeLa cells.

(2A) Activity of basal 617bp HMGCS1 promoter was assessed in HepG2 (A) and HeLa (B) cells transiently transfected with both ACBP expression plasmids producing either the 14 kDa ACBP-*V5* fusion protein or the native 10 kDa ACBP. **(2B)** Activity of truncated -417/-1 and -317/-1 HMGCS1 promoter fragments was analyzed in ACBP-*V5* expressing HepG2 (A) and HeLa (B) cells. SEAP activity was measured after 48 h and normalised to luciferase activity. Each experiment was performed in three independent cell passages in triplicate for each sample. Data are presented as mean ± SD. Student's paired t-test (***p <0.0001, **p<0.001, *p<0.01).

Figure 3

Expression of ACBP decreased HMGCS1-mRNA and protein levels in HeLa cells.

(3A) HepG2 (A) and HeLa (B) cells were transiently transfected with ACBP-*V5* expression plasmid for 24 h and 48 h. HMGCS1 transcript levels were measured by real-time RT-PCR. Data are presented as x-fold expression in comparison to control cells incubated only with FuGene6 (mean ± SD; n=3-4). The p-value is indicated. **(3B)** A representative Western blot shows 48 h post-transfection immunodetection of ACBP, HMGCS1 and ß-actin (loading control) in ACBP-overexpressing (*ov*) and in control (*co*) HeLa cells (1) indicates the detection of V5-tagged ACBP and (2) the level of HMGCS1 in the transfectant.

Figure 4

Expression of ACBP suppressed HMGCR in HeLa cells at the transcriptional level.

HepG2 (A) and HeLa (B) cells were transiently transfected with ACBP-*V5* expression plasmid **(4A)** HMGCR SEAP activity was measured after 48 h and normalized to luciferase activity. The results are presented as mean ± SD (n=4;***p<0.0001). **(4B)** HMGCR transcript levels were assessed by real-time RT-PCR 24 h and 48 h after ACBP-*V5* expression. Data are presented as x-fold expression in comparison to control cells incubated only with FuGene6 (mean ± SD, n=3-4). The p-value is indicated.

Figure 5

Chromatin Immunoprecipitation revealed no direct binding of ACBP to HMGCS1 regulatory DNA-sequences.

Formaldehyde cross-linked chromatin was prepared from HeLa cells and immunoprecipitated with antibodies to ACBP and immunglobulin G (IgG) as negative control. PCR was performed with specific primers for the HMGCS1 promoter -418/-212 and for GAPDH as a negative control. A sample representative of the total input chromatin (input DNA) was included in the PCR analysis. Lane 5 shows a PCR negative control. Transfection experiment was performed in duplicate each with 2 independent immunoprecipitations. M, 100 bp DNA marker

Table 1. Sequence of primers used for cloning of SEAP reporter gene constructs

SEAP reporter gene constructs	**Sequence 5'→3'**
HMGCS1 reporter gene constructs	
-617 bp forward	GTATTCTGCTCCAATTTGC
-417 bp forward	GGCCCGCATCTCCTCTCAC
-317 bp forward	TCTCGTGCCACCTCACGTC
-217 bp forward	CCTAGCGAGTCATCGCCTC
reverse primer	AAGGGAGTGAGCCACGAAAGG
HMGCR reporter gene construct	
-528 bp forward	AGTTACAGGTTTGAGGTG
reverse primer	TACGCACGCTCGGAGCTGGA

Forward primers contain the nucleotides CACC at 5' end for Gateway cloning

Table 2. Total cholesterol levels did not change in HeLa cells transfected for 48 h and 72 h with ACBP-*V5*

	48 h	**72 h**
ACBP-*V5*	1.61 ± 0.42 µg/ml	3.17 ± 1.12 µg/ml
control	1.47 ± 0.92 µg/ml	2.85 ± 1.3 µg/ml

Figure 1

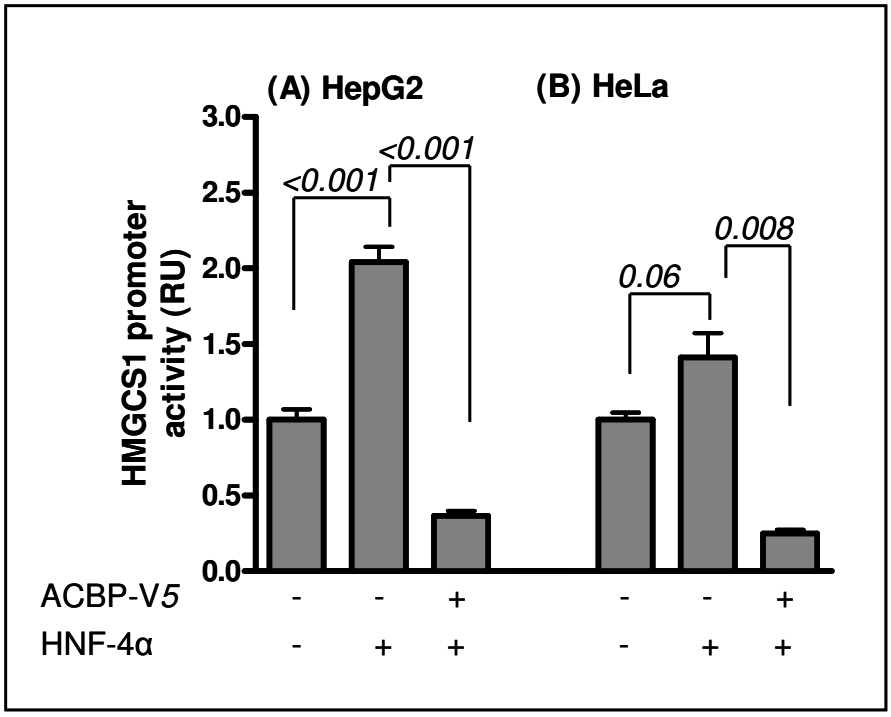

(A) HepG2
(B) HeLa
HMGCS1 promoter activity (RU)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
<0.001
<0.001
0.06
0.008
ACBP-V5
HNF-4α
- - + - - +
- + + - + +

Figure 2

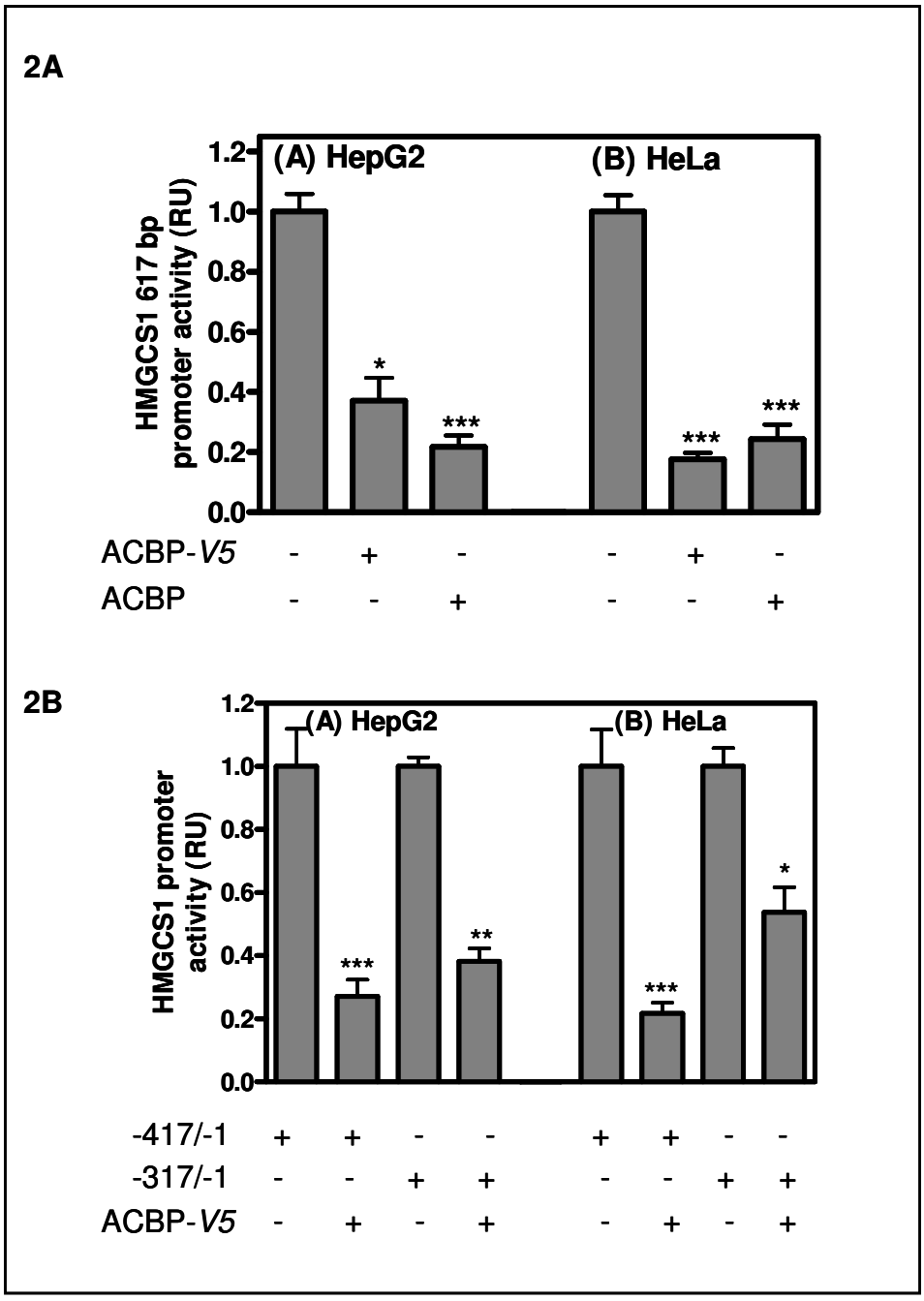

2A
(A) HepG2
(B) HeLa
HMGCS1 617 bp promoter activity (RU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
***
ACBP-V5 - + - - + -
ACBP - - + - - +
2B
(A) HepG2
(B) HeLa
HMGCS1 promoter activity (RU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
***
*
-417/-1 + + - - + + - -
-317/-1 - - + + - - + +
ACBP-V5 - + - + - + - +

Figure3

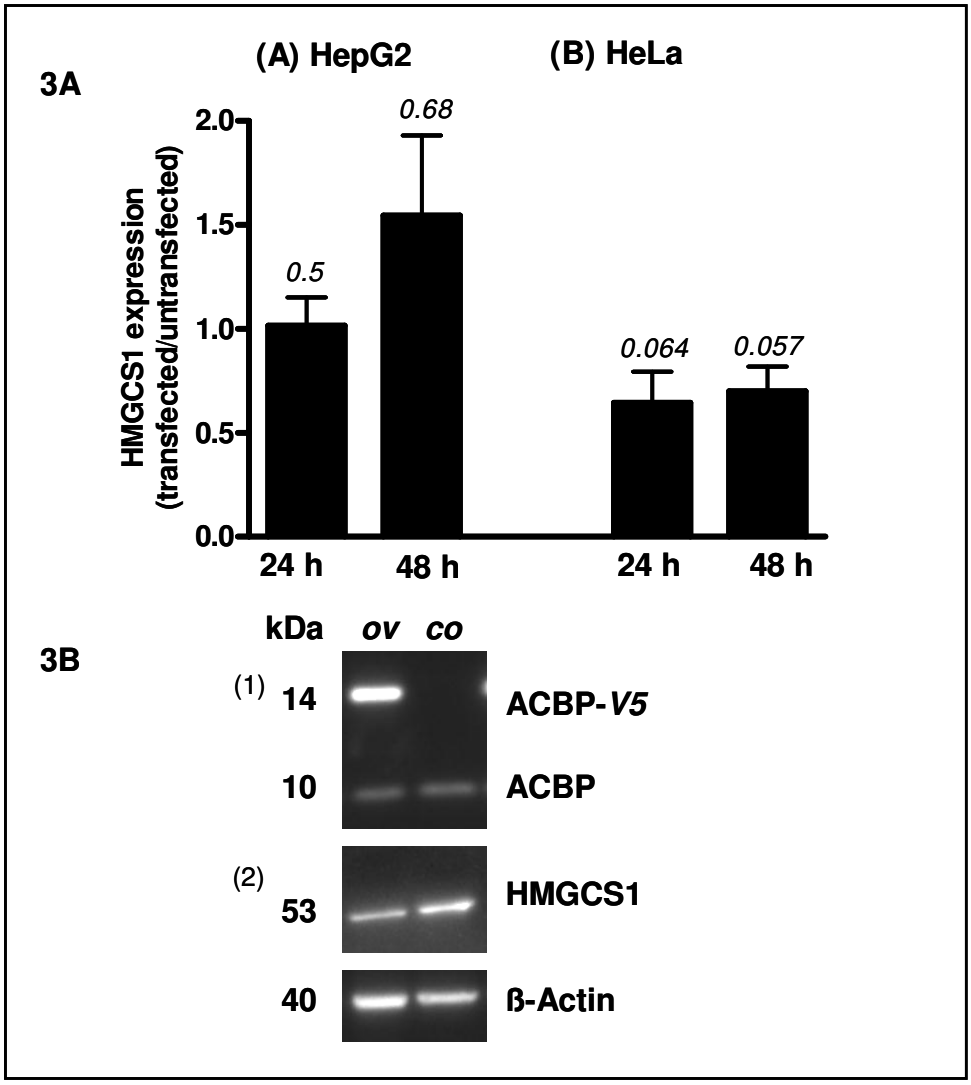

3A
(A) HepG2
(B) HeLa
HMGCS1 expression
(transfected/untransfected)
2.0
1.5
1.0
0.5
0.0
0.5
0.68
0.064
0.057
24 h
48 h
24 h
48 h
3B
kDa
ov
co
(1)
14
ACBP-V5
10
ACBP
(2)
53
HMGCS1
40
ß-Actin

Figure 4

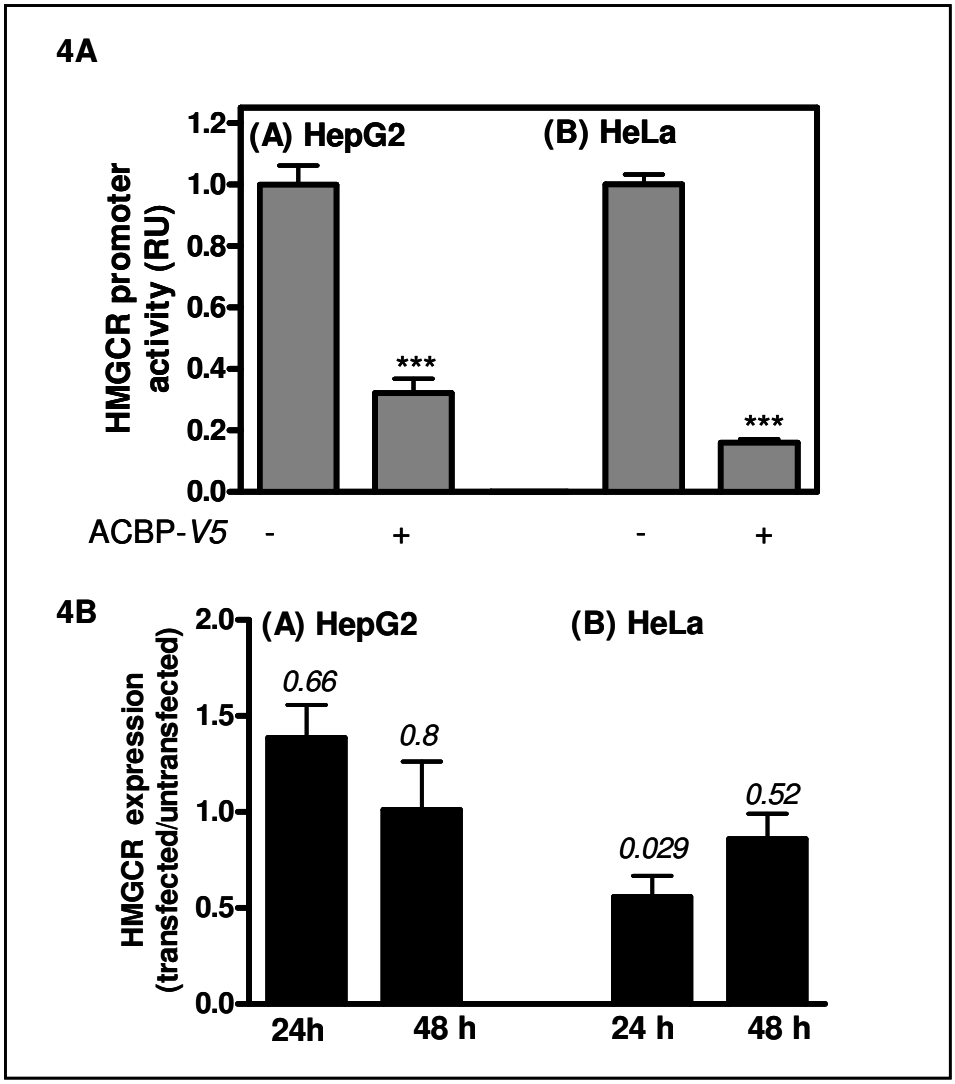
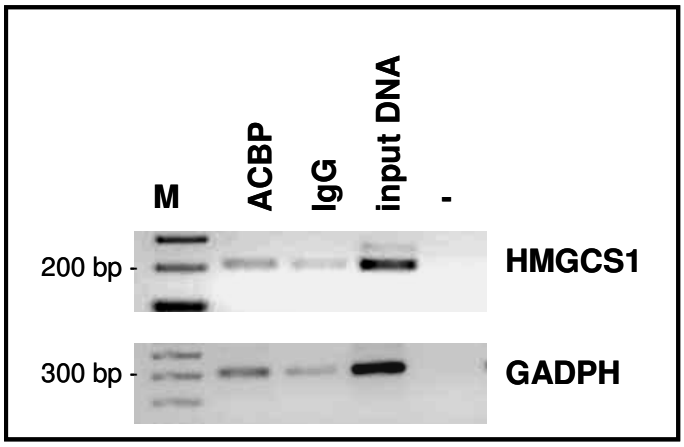

4A
(A) HepG2
(B) HeLa
HMGCR promoter activity (RU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
ACBP-V5 - + - +
4B
(A) HepG2
(B) HeLa
HMGCR expression (transfected/untransfected)
2.0
1.5
1.0
0.5
0.0
0.66
0.8
0.029
0.52
24h
48 h
24 h
48 h

Figure 5



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Lebenslauf

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Erklärung

Hiermit erkläre ich an Eides statt, daß ich die vorgelegte Dissertation mit dem Titel „Analysis of Gene Regulatory Functions of the Human Acyl-CoA-Binding-Protein in Lipid Metabolism“ selbstständig und ohne unerlaubte Hilfe angefertigt habe und daß ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorgelegen habe.

Hiermit erkläre ich, daß gegen mich kein strafrechtliches Ermittlungsverfahren schwebt.

Christina Vock