

Original Paper

Human Sterol Regulatory Element-Binding Protein 1a Contributes Significantly to Hepatic Lipogenic Gene Expression

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Key Words

Liver • Hepatocytes • SREBP1 • Lipogenesis • NAFLD

Abstract

Background/Aims: Sterol regulatory element-binding protein (SREBP) 1, the master regulator of lipogenesis, was shown to be associated with non-alcoholic fatty liver disease, which is attributed to its major isoform SREBP1c. Based on studies in mice, the minor isoform SREBP1a is regarded as negligible for hepatic lipogenesis. This study aims to elucidate the expression and functional role of SREBP1a in human liver. **Methods:** mRNA expression of both isoforms was quantified in cohorts of human livers and primary human hepatocytes. Hepatocytes were treated with PF-429242 to inhibit the proteolytic activation of SREBP precursor protein. SREBP1a-specific and pan-SREBP1 knock-down were performed by transfection of respective siRNAs. Lipogenic SREBP-target gene expression was analyzed by real-time RT-PCR. **Results:** In human liver, SREBP1a accounts for up to half of the total SREBP1 pool. Treatment with PF-429242 indicated SREBP-dependent auto-regulation of SREBP1a, which however was much weaker than of SREBP1c. SREBP1a-specific knock-down also reduced significantly the expression of SREBP1c and of SREBP-target genes. Regarding most SREBP-target genes, simultaneous knock-down of both isoforms resulted in effects of only similar extent as SREBP1a-specific knock-down. **Conclusion:** We here showed that SREBP1a is significantly contributing to the human hepatic SREBP1 pool and has a share in human hepatic lipogenic gene expression.

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Introduction

The transcription factor sterol regulatory element-binding protein (SREBP) 1 was recognized as the master regulator of lipogenesis, especially regulating genes encoding key enzymes of fatty acid and triglyceride biosynthesis in the liver [1]. An increase in *de novo* lipogenesis was recently shown to substantially contribute to the excess storage of triglycerides in non-alcoholic fatty liver disease (NAFLD) [2], which encompasses a spectrum of diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). While numerous reports showed up-regulation of SREBP1 in human NAFLD [3, and references therein], studies with SREBP1-transgenic mice [4, 5] and of SREBP1 knock-out or knock-down in murine NAFLD disease models [6, 7] further confirmed the crucial role of the gene in hepatic steatosis.

In mammals, two isoforms of SREBP1 protein, SREBP1a and SREBP1c, are known, which arise by the use of two alternative first exons. Exon 1a encodes the unique 29 amino acids of the SREBP1a NH₂-terminus, whereas exon 1c only encodes 5 unique amino acids [8]. Thus, the NH₂-terminal acidic transcriptional activation domain of SREBP1a is 24 amino acids longer and harbors 12 negatively charged amino acids, whereas the respective domain of SREBP1c contains only 6 negatively charged residues [9]. Consequently, SREBP1a proved to be the by far stronger transcriptional activator of the two isoforms, both *in vitro* [10] and *in vivo* [11]. SREBP1 proteins are produced as precursor proteins, which reside in the endoplasmic reticulum. Sequential proteolytic digests by site 1 and site 2 proteases are required to release the NH₂-terminal part of the protein, which then translocates to the nucleus and acts as the mature transcription factor [9].

SREBP1c, which is the dominant isoform in many mouse tissues, including liver [8], is mediating the nutritional control of hepatic lipogenic gene expression [12], as its expression [13] and proteolytic activation [14] are induced by insulin signaling. Thus, it is the isoform which has been implicated in NAFLD. In contrast the physiological and pathophysiological role of SREBP1a in the liver is less well understood. SREBP1a is commonly regarded as being negligible in liver, as it was previously shown in mice to be expressed at 10-fold lower levels than SREBP1c [8]. In the same study, it was further shown that 1a is 6-fold outnumbered by 1c in human liver, which however was based only on the analysis of an individual liver sample. Given the well-known interindividual variability of gene expression in humans, we re-analyzed here the contribution of SREBP1a to the total hepatic SREBP1 pool and determined its role in the control of human hepatic SREBP-target gene expression.

Materials and Methods

Plasmids

Partial sequences of exons 1a and 2 (pos. 60-423 of NM_004176) and exons 1c and 2 (pos. 341-670 of NM_001005291) of human SREBP1 were amplified by PCR out of human liver cDNA using appropriate primers and cloned into pBluescript KS M13- (Agilent Technologies, Santa Clara, CA), thereby creating pbl-SREBP1a-TQ and pbl-SREBP1c-TQ, respectively. Identity of the cloned inserts was confirmed by sequencing. The insert of pbl-SREBP1a-TQ was further subcloned into pbl-SREBP1c-TQ, thereby creating pbl-SREBP1a+1c-TQ, which contains both SREBP1a and -1c specific regions.

Human liver biobank and NAFLD grading

The human liver biobank, consisting of histologically normal, non-tumorous liver tissue samples, collected from patients undergoing liver surgery at the University Medical Center Charité (Berlin, Germany), was described previously [15]. The study was approved by the local ethical committees of the Charité, Humboldt University, Berlin, and University of Tübingen and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient. NAFLD livers, identified retrospectively by histological analysis, were graded as non-NASH, mild NASH and moderate to severe NASH as described previously [16], taking into account the increasing degrees of steatosis, hepatocellular ballooning, lobular and portal inflammation.

Primary human hepatocytes

Human hepatocytes were prepared from tissue samples of liver resections, which were obtained from patients, who underwent partial hepatectomy because of liver tumors at the University Medical Center Charité, Berlin, Germany and at the Hospital Großhadern, Munich, Germany, as described previously [17]. Donor data are available on request. The isolated cells were seeded at 1.5×10^6 cells/well into collagen type I-coated 6-well plates and treated with 0.1% DMSO for 48 h [17]. Hepatocytes from three different donors were also treated with 10 μ M PF-429242 (Tocris Bioscience, Bristol, UK).

siRNA-mediated gene knock-down

Primary human hepatocytes were seeded at 4×10^5 or 1×10^6 cells/well into collagen type-I-coated 12- or 6-well plates, respectively. After 6–12 h, cells were transfected with non-targeting negative control siRNA (Silencer Select negative control 1), Silencer Select siRNA targeting 5'-CCA TGG ACG AGC CAC CCT T-3' (pos. 193–211 of NM_004176) in exon 1a of SREBP1 or Silencer Select siRNA targeting 5'-CCA CTC CAT TGA AGA TGT A-3' (pos. 505–523 of NM_004176) in exon 2 of SREBP1 (ID# s129), which is common to SREBP1a and -1c transcripts. Silencer Select siRNAs were provided or custom-synthesized by Life Technologies (Darmstadt, Germany), and transfected at a final concentration of 20 nM using Lipofectamine RNAiMAX (Life Technologies). Culture medium (Williams' E medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1% DMSO, 0.032 I.U./ml insulin and 100 nM dexamethasone) was renewed daily and cells were harvested for RNA and protein analysis 72 h after transfection.

RNA preparation and cDNA synthesis

Total RNA was prepared using the NucleoSpin RNA kit (Machery-Nagel, Düren, Germany), including removal of contaminating genomic DNA by DNase I digest. The integrity of the isolated RNA was confirmed by formaldehyde-agarose gel electrophoresis or by Agilent2100 Bioanalyzer analysis (Agilent). First strand cDNA was synthesized from 0.32 μ g RNA with random hexamer primers and MultiScribe reverse transcriptase using TaqMan reverse transcription reagents (Life Technologies) in a volume of 16 μ l, according to manufacturer's standard protocol.

Quantitative real-time RT-PCR

Absolute quantification analyses were performed by TaqMan Real-time RT-PCR using the 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA). SREBP1a and -1c assays were set up with cDNA samples, pre-amplified for 10 cycles to shift sample C_t values into mid of standard curve range, and qPCR MasterMix (Eurogentec, Liege, Belgium). Pre-amplification was performed as described previously [16]. Assays were done in triplicate. The commercial TaqMan gene expression assay Hs00231674_m1 (Applied Biosystems), consisting of a pre-designed primer/probe set, with the probe targeting the exon 1a/2 junction (5'-GAC CGA CAT CGA AG | A CAT GCT TCA G-3'), was used to quantify SREBP1a. Oligonucleotides used for SREBP1c were as follows: 900 nM each of primers 5'- CGG AGC CAT GGA TTG CA-3' (exon 1c) and 5'- GGA AGT CAC TGT CTT GGT TGT TGA-3' (exon 2); 250 nM of 6-carboxyfluorescein labeled probe 5'- CTT TCG AAG | ACA TGC TTC A-3' (exon 1c/2). The specificity of the assays was determined using linearized pbl-SREBP1a-TQ and pbl-SREBP1c-TQ plasmids. Both assays demonstrated $> 3 \times 10^4$ -fold higher selectivity for the matching cDNA. Serial 10x dilutions of linearized plasmid pbl-SREBP1a+1c-TQ, were used to create the standard curves for both assays, ranging from 1.2×10^2 to 1.2×10^7 copies. Amplification efficiencies of SREBP1a and SREBP1c assays were 99% and 97%, respectively. These were determined using the slopes of the respective SREBP1a and SREBP1c standard curves, which were -3.346 and -3.403, with R^2 values of 0.9992 and 0.9999, respectively. Absolute gene expression levels of SREBP1a and SREBP1c were calculated by the SDS 2.3 analysis software of the 7900 Real Time PCR System, using the linear equations of the respective standard curves. Gene expression levels were normalized to corresponding 18S rRNA levels, as determined using the 18S rRNA assay previously described [17].

Relative quantification analyses were performed by TaqMan Real-time RT-PCR using the BioMark HD system and FLEXsix Gene Expression Integrated Fluidic Circuits (Fluidigm, South San Francisco, CA), as described previously [16]. Commercial TaqMan gene expression assays (Life Technologies), consisting of pre-designed primer/probe sets, were used as follows: Hs01046047_m1 (ACACA), Hs00153715_m1 (ACACB), Hs00982738_m1 (ACLY), Hs00218766_m1 (ACSS2), Hs00225412_m1 (ELOVL6), Hs01005622_m1 (FASN),

Hs01650979_m1 (INSIG1), Hs99999902_m1 (RPLP0), (Hs01682761_m1 (SCD) and Hs00930058_m1 (THRSP). Assays were performed in triplicates. Gene expression levels were normalized to corresponding 18S rRNA levels.

Protein analysis

Hepatocytes were lysed with a buffer according to Sundqvist et al. [18], which was composed of 50 mM HEPES pH 7.2, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 10 mM β -glycerophosphate, 1% (v/v) Triton-X-100, 10% (v/v) glycerol, 10 mM sodium butyrate, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1 mM PMSF, 1x HALT protease inhibitor cocktail (Pierce), by incubation on ice for 10 min and sonication in the Bioruptor ultrasonic water bath (Diagenode, Liege, Belgium). To avoid proteasomal degradation of mature SREBP1, cells were treated with 25 μ M MG132 (Santa Cruz, Dallas, TX, USA) 2 h before harvesting. Total protein lysates were quantified using the bicinchoninic acid method and further used for standard protein gel electrophoresis and Western blotting. Nitrocellulose membranes were incubated with the mouse monoclonal SREBP1-specific antibody 2A4 (Santa Cruz). Fluorescence intensity of bound IRDye® 800CW-labeled goat anti-mouse secondary antibody (LI-COR, Lincoln, NE, USA) was analyzed and quantified using the Odyssey Infrared Imaging System (LI-COR). Equal protein loading was verified by comparing to β -actin levels, which were determined using mouse monoclonal antibody AC-15 (Sigma-Aldrich, Taufkirchen, Germany) and IRDye® 680RD-labeled goat anti-mouse secondary antibody (LI-COR).

Quantification of *de novo* lipogenesis

Incorporation of 14 C-labeled acetic acid into lipids was used as a measure of *de novo* lipogenesis as described previously [16].

Data analysis

Data are presented as mean \pm SD of at least three replicate measurements, if not indicated otherwise. Analysis of the statistical significance of differences between medians or means was performed with statistical tests as indicated, using GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA).

Results

Expression of SREBP1 transcripts in human liver and NAFLD disease progression

Absolute quantification of the expression of the two alternative transcripts of SREBP1, -1a and -1c, was established to allow for the calculation of their respective contribution to the total hepatic SREBP1 pool. SREBP1a showed lower median expression than SREBP1c, both in human liver (Fig. 1A) and primary human hepatocytes (Fig. 1B). Interindividual variability of SREBP1a expression demonstrated to be smaller in liver (6-fold) and in hepatocytes (3-fold), than of SREBP1c expression (17- and 7-fold, respectively). Neither SREBP1a nor SREBP1c expression was influenced by sex or age. The median expression of both transcripts increased during NASH progression, thereby confirming our previous results [16], which were obtained by relative quantification analysis and did thus not allow comparison of expression levels of the two alternative transcripts. SREBP1a median transcript levels demonstrated to be lower than SREBP1c levels in each NAFLD subgroup (Fig. 1C). Based on the absolute expression data, the ratio of both transcripts was calculated. Figure 2A shows that median ratio of SREBP1a:SREBP1c was 0.35 (range 0.17 to 1.1) in liver and 0.59 (range 0.25 to 1.4) in primary hepatocytes. Thus, SREBP1a contributed 15-52% (median 26%) to the total SREBP1 pool in this liver cohort and 20-58% (median 37%) in hepatocytes. The ratio of both transcripts was not altered significantly in NAFLD progression, suggesting that the disease did not differentially affect their expression (Fig. 2B). In summary, these data clearly show that SREBP1a contributes considerably to human hepatic SREBP1 expression.

SREBP-dependent auto-regulation of human hepatic SREBP1 transcripts

Expression of the two SREBP1 transcripts showed to be highly correlated in human liver and primary hepatocytes (Fig. 3), suggesting co-regulation or regulation of one by

Fig. 1. SREBP1 transcript expression in human liver and primary hepatocytes. Absolute SREBP1a and -1c mRNA expression data, as copies per 10^6 copies 18S rRNA, in 65 human livers (A) and primary human hepatocytes of 22 donors (B) are presented as box and whisker plots, with boxes representing the 25th–75th percentiles, medians indicated by horizontal lines and whiskers showing minimum and maximum values. Data were analyzed by Wilcoxon matched-pairs signed rank test. (C) Absolute quantification of SREBP1a and -1c mRNA expression in NAFLD-categorized human liver samples is shown as described above. Wilcoxon matched-pairs signed rank test was used for the analysis of differences between 1a and 1c expression, whereas Kruskal-Wallis test with Dunn's multiple comparisons test was used for the analysis of differences to the non-NASH group, which was performed separately for SREBP1a and -1c. Asterisks show statistical significant differences as indicated. Daggers indicate statistical significant differences to the respective expression in the non-NASH group. †, $p < 0.05$; ††, $p < 0.01$; ***, †††, $p < 0.001$.

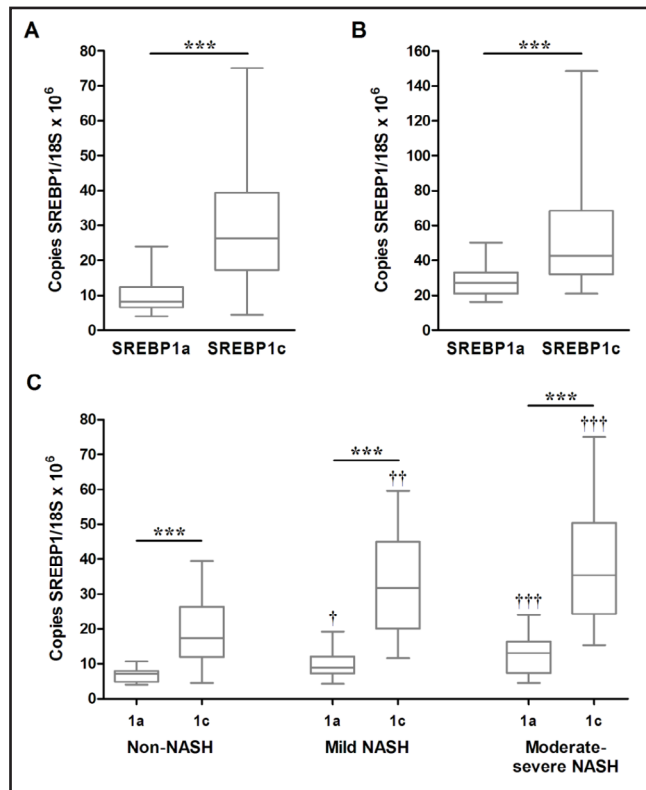
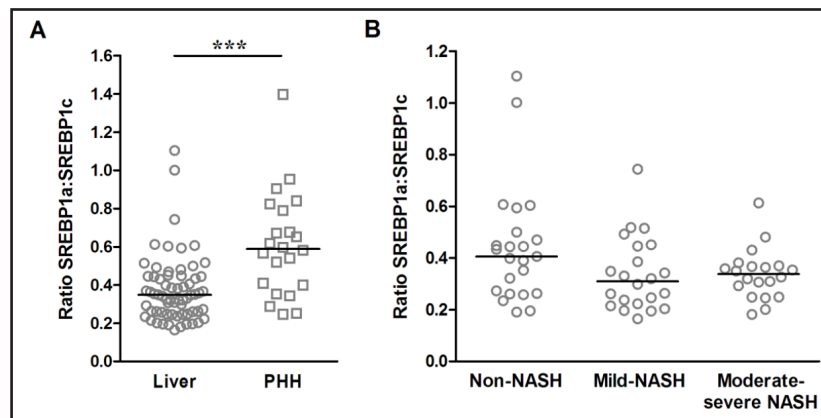


Fig. 2. Hepatic SREBP1a:SREBP1c expression ratios. (A) Scatter blots of the individual ratios of SREBP1a:SREBP1c expression in livers and primary human hepatocytes (PHH) with lines indicating medians. Data were analyzed by Mann-Whitney test. ***, $p < 0.001$. (B) Scatter plots of the individual ratios of hepatic



SREBP1a:SREBP1c expression in NAFLD progression with lines indicating medians. Absence of statistical significant differences to the non-NASH group was determined by Kruskal-Wallis test with Dunn's multiple comparisons test.

the other. The latter possibility is supported by the previously shown auto-regulation of mouse SREBP1c promoter activity by SREBP1 protein [19]. Similar auto-regulation of the 1a promoter has not been described to the best of our knowledge. To analyze a possible auto-regulation of human SREBP1 transcripts by SREBP protein, primary human hepatocytes were treated with PF-429242, a specific inhibitor of site 1 protease [20], which participates in formation of the mature, transcriptionally active SREBP proteins by site-specific cleavage of the SREBP precursor proteins [21]. Figure 4A shows that treatment with PF-429242 blocked the formation of the mature SREBP1 proteins. By comparing SREBP1 protein expression in hepatocytes with or without significant expression of the 1c transcript

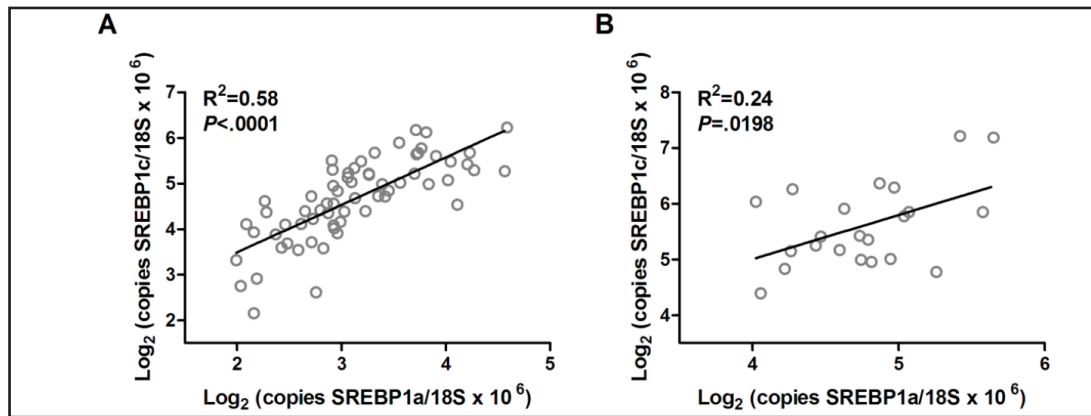


Fig. 3. Expression of SREBP1 transcripts correlates in liver and primary human hepatocytes. Correlation analysis of SREBP1a and -1c expression in human livers (A) and primary human hepatocytes (B), using the \log_2 -transformed mRNA expression data shown in Fig. 1A,B.

Fig. 4. Blocking the proteolytic activation of SREBP1 protein results in decrease of lipogenic gene expression and *de novo* lipogenesis. (A) Representative Western blot of SREBP1 protein expression in primary human hepatocytes (donor RH19), which have been treated for 48 h with 0.1% solvent DMSO (-PF) or 10 μ M PF-429242 (+PF). p-SREBP1 and m-SREBP1 indicate precursor and mature SREBP1 proteins, respectively. (B) *Top*, Western blot analysis of SREBP1 protein in hepatocytes of donors RH19 and TH04. Protein lysates of cells, transfected with control siRNA (see Fig. 5), were used. The image of the RH19 lane is a copy of the respective lane of Fig. 5D, left panel. *Bottom*, absolute quantification of SREBP1a and -1c mRNA expression in correspondingly treated hepatocytes of the same donors. (C) Relative SREBP1a and -1c mRNA expression in primary human hepatocytes (n=3, donors GH23, GH26, RH19), treated as described in (A). Statistical significant differences to respective expression levels in cells treated with DMSO only, which were designated as 1, were analyzed by one sample t-test. (D) *De novo* lipogenesis was determined in hepatocytes of donor GH-23, which were treated as described in (A). The mean value of cells (n=3) treated with solvent only, was designated as 1. Data were analyzed by unpaired t-test. (E) The relative mRNA expression of the indicated lipogenic SREBP-target genes was determined in hepatocytes (n=3, donors GH23, GH26, RH19). RNA samples as in (C). The respective expression levels in cells, treated with solvent only, were designated as 1. Data were analyzed as described in (C). Asterisks indicate statistical significant differences. *, p<0.05; **, p<0.01; ***, p<0.001.

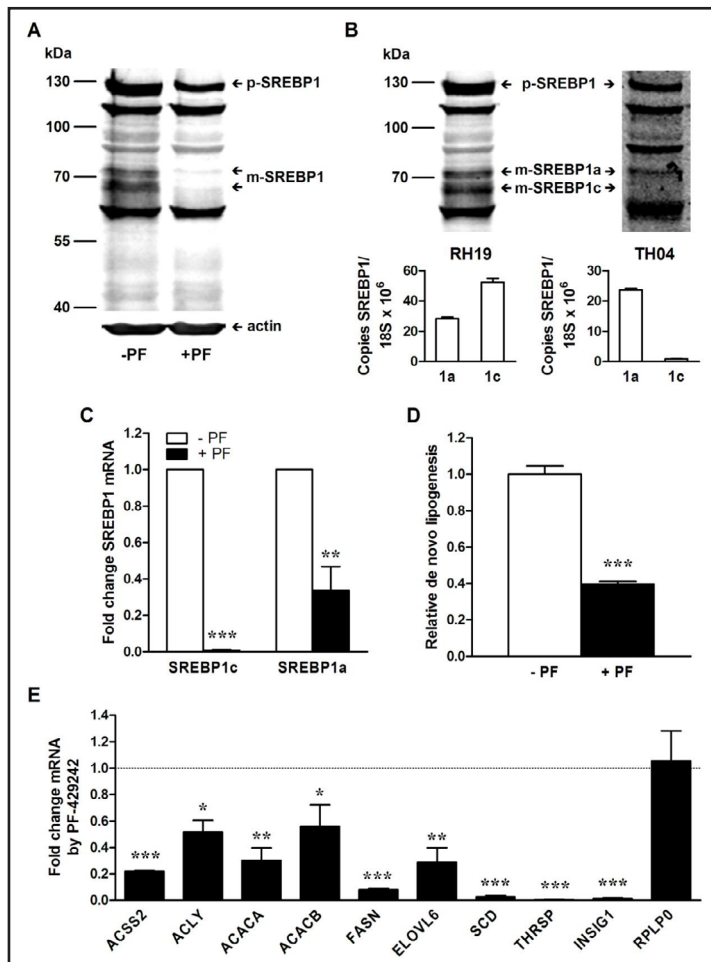
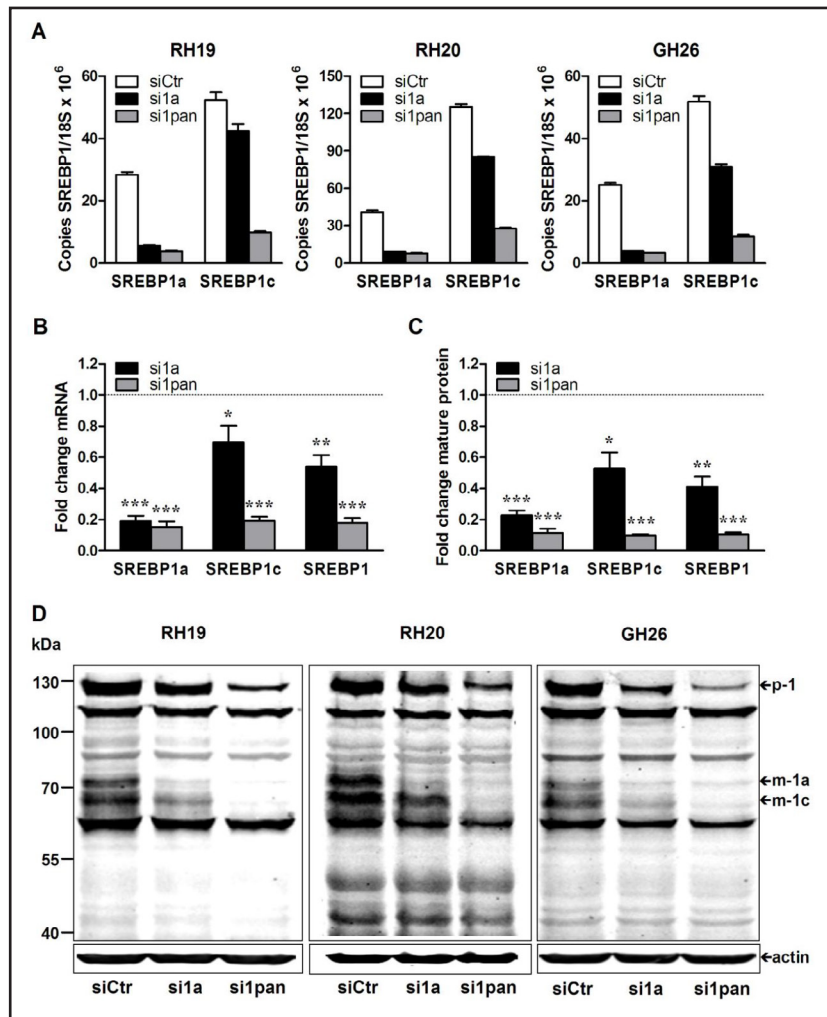


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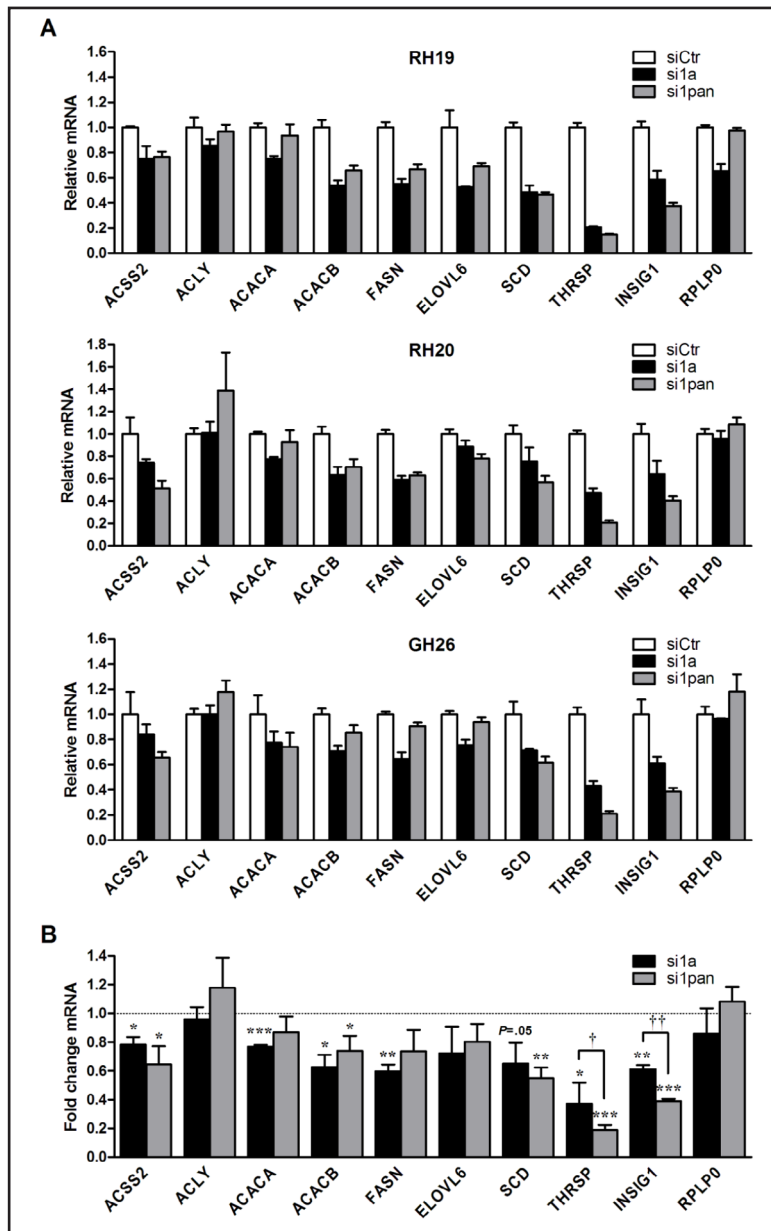
Fig. 5. SREBP1 mRNA and protein knock-down by SREBP1a-specific and pan-SREBP1 siRNAs. (A) Absolute quantification of SREBP1a and -1c mRNA expression in primary human hepatocytes from donors RH19, RH20 and GH26, transfected for 72 h with control siRNA (siCtr), siRNA specific for SREBP1 exon 1a (si1a) or siRNA specific for the SREBP1 common exon 2 (si1pan). (B) Mean fold changes \pm SD (n=3) of SREBP1 mRNA expression, calculated from the expression data shown in (A). Respective expression levels of control siRNA-transfected cells were designated as 1. Statistical significant differences to this value were analyzed by one sample t-test. (C) Relative quantification of mature SREBP1 proteins in hepatocytes, transfected with siRNAs as described in (A). Quantification was performed by densitometric analysis of the respective specific bands of the Western blots shown in (D). Data are shown as mean fold changes \pm SD (n=3). Respective expression levels in control siRNA-transfected cells were designated as 1. Data analysis was performed as described in (B). (D) Western blots of SREBP1 protein expression in hepatocytes of donors RH19, RH20 and GH26, transfected as described in (A). SREBP1 precursor (p-1), mature SREBP1a (m-1a) and mature SREBP1c (m-1c) protein bands are indicated.



variant, the bands corresponding to mature SREBP1a and -1c isoforms were unequivocally identified (Fig. 4B). Loss of 1c transcript expression was observed in hepatocytes of a single donor, which were accidentally cultured at low cell density, a culture condition promoting dedifferentiation. In accordance with the previously described auto-regulation of SREBP1c promoter activity, treatment with PF-429242 resulted in 99% decrease of SREBP1c mRNA expression. In contrast, SREBP1a mRNA was only reduced by $67 \pm 13\%$ (Fig. 4C). The strong down-regulation of total SREBP1 mRNA by PF-429242 most likely explains the decrease observed also in the expression of SREBP1 precursor protein (Fig. 4A). In agreement with the well-known role of SREBP1 in fatty acid synthesis, PF-429242 further resulted in 60% reduction of hepatic *de novo* lipogenesis (Fig. 4D), which was reflected in the consistent down-regulation of lipogenic SREBP-target gene expression (Fig. 4E). The negative control gene RPLP0 was not affected by treatment with PF-429242. These data confirm that mRNA expression of human SREBP1c is strongly auto-regulated by SREBP protein, whereas SREBP-dependent regulation of 1a mRNA appears to be variable and less prominent.

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Fig. 6. SREBP1a contributes to hepatic SREBP-target gene expression. (A) Relative quantification of lipogenic SREBP target gene expression in hepatocytes from individual donors RH19, RH20 and GH26, transfected with siRNA as described in the legend of Fig. 5. Respective mRNA expression levels of hepatocytes transfected with control siRNA (means of triplicate measurements) were designated as 1. (B) Mean fold changes \pm S.D. (n=3 donors) of gene expression by SREBP1 knock-down. Statistical significant differences to the expression levels in control siRNA-transfected cells, designated as 1, were determined by one sample t-test and indicated by asterisks. Daggers denote statistical significant differences between fold changes of gene expression by SREBP1a-specific (si1a) and pan-SREBP1 (si1pan) siRNAs, as calculated by two-way repeated measures ANOVA with Sidak's multiple comparisons test for the genes showing a significant knock-down by at least one siRNA. *, †, $p < 0.05$; **, ††, $p < 0.01$; ***, $p < 0.001$.



SREBP1a participates in human hepatic lipogenic gene expression

As we showed above that expression of the major transcript SREBP1c is largely dependent on auto-regulation by SREBP protein, we hypothesized that the 1a isoform, which is known to be the by far stronger transcriptional activator, may contribute to the transcriptional control of hepatic SREBP1c and SREBP target genes. Pan-SREBP1- and 1a transcript-specific knock-down were performed in primary human hepatocytes by transient transfection of siRNA specific for the common exon 2 or exon 1a of SREBP1, respectively. Figure 5A shows that SREBP1 mRNA knock-down efficiencies were highly comparable between the individual hepatocyte cultures. The partial reduction of SREBP1c transcript expression by SREBP1a-specific knock-down is most likely explained by decrease in SREBP-dependent auto-regulation of the 1c transcript, due to the knock-down of SREBP1a. Ratios of SREBP1a:SREBP1c transcripts of the individual hepatocyte cultures, transfected with control siRNA, were all in the same range (mean 0.45 ± 0.11). SREBP1a-specific siRNA resulted on average in 80% knock-down of the corresponding transcript, whereas pan-SREBP1 siRNA decreased the expression of both SREBP1a and -1c transcripts by $\geq 80\%$ (Fig. 5B). Due

to the limited and variable effect of SREBP1a-specific siRNA on the expression of the 1c transcript (mean reduction of 30%), this siRNA resulted on average only in 46% reduction of total SREBP1 mRNA expression. Protein expression levels of the mature SREBP1a and -1c isoforms, as well as of total mature SREBP1, were correspondingly reduced (Fig. 5C,D). Regarding the SREBP1 precursor proteins, where it was not possible to discriminate between the isoforms, SREBP1a-specific knock-down resulted on average in 60% reduction, whereas pan-SREBP1 knock-down reduced the expression on average by 84% (Fig. 5D). Thus effects on the SREBP1 precursor proteins were identical with the effects on total mature SREBP1 proteins.

Irrespective of the fact that the efficacies of SREBP1a-specific and pan-SREBP1 knock-down were highly comparable between the individual hepatocyte cultures, the effect on SREBP-dependent lipogenic gene expression differed between them (Fig. 6A). Statistical analysis using the mean values of the three donors showed that the expression of most lipogenic SREBP-target genes decreased significantly by transfection of SREBP1a-specific and/or pan-SREBP1 siRNA (Fig. 6B). However, the extent of the effect was also variable between the genes. While ACSS2, ACACA, ACACB, FASN and SCD were down-regulated by 20-40%, THRSP and INSIG1 showed 60-80% reduction by at least one siRNA. For most genes, knock-down of both SREBP1a and -1c, by transfection of pan-SREBP1 siRNA, resulted in effects of only similar extent as observed with SREBP1a-specific knock-down. Solely THRSP and INSIG1 exhibited a stronger down-regulation by pan-SREBP1 knock-down than by SREBP1a-specific knock-down (Fig. 6B). The expression of ACLY and ELOVL6 was not significantly altered by any of the two SREBP1-specific siRNAs. In summary, these data clearly indicate that SREBP1a significantly contributes to the transcriptional control of human hepatic lipogenic SREBP-target gene expression.

Discussion

Given the results here presented, SREBP1a no longer may be regarded as negligible for the control of human hepatic lipogenesis, as it contributes to up to half of the total hepatic SREBP1 pool in individual livers and participates in hepatic lipogenic SREBP1-target gene expression. The interindividual variability of hepatic SREBP1a mRNA expression was much smaller than that of 1c, both in human liver and primary human hepatocytes. In mice, SREBP1c is strongly regulated by insulin signaling [13]. Even if insulin data from the patients, of whom liver tissue samples were obtained, have not been recorded, it may be speculated that interindividual variability of insulin levels contributes to the variable expression of 1c in human livers. The variability of 1c expression in primary human hepatocytes, which was lower than in liver, has to be explained differently, as they were all cultivated in the same insulin-containing cell culture medium. It is well known that primary hepatocytes dedifferentiate in cell culture to a variable extent, a process which is already initiated during isolation [22]. Variable extent of dedifferentiation may thus contribute to the variability of SREBP1c transcript expression in cultured primary human hepatocytes. On the other hand, cultivation-dependent dedifferentiation may explain the significantly higher ratio of SREBP1a:SREBP1c in primary hepatocytes. In contrast to 1c, 1a was shown to be at best modestly affected by insulin in mice [13]. Nevertheless, expression of both transcripts proved to be highly correlated. *In vitro* analyses of mouse and human 1a and 1c promoters demonstrated that both depend on Sp1 [23-26], which is known to be also involved in insulin-dependent gene regulation [27]. Insulin-dependent control of mouse and human SREBP1c promoter activity further involves auto-regulation by SREBP1 protein [19, 26], which has not been described for the 1a promoter. We here showed that SREBP1a is also susceptible to the inhibition of site 1 protease, suggesting possible auto-regulation by SREBP protein, however much weaker than 1c and to an interindividual variable extent. Thus, both co-regulation and regulation of one by the other may contribute to the correlation of expression levels of SREBP1a and -1c transcripts.

A minor role of SREBP1a in hepatic SREBP-target gene expression had been suggested by the fact that 1a-deficient mice did not show any compromised expression of lipid metabolism genes, with the single exception of a reduced expression of *Acacb* [28]. In contrast, we here clearly showed by SREBP1a-specific knock-down using exon 1a-specific siRNA that this isoform impacts on the expression of further lipogenic SREBP-target genes in human liver cells. Apart from *ACACB*, mRNA expression of *ACSS2*, *ACACA*, *FASN*, *SCD*, *THRSP* and *INSIG1* were also affected by specific knock-down of SREBP1a.

With the exception of *ACACB*, knock-down of 1a reduced the expression of most SREBP-target genes to a smaller extent than treatment with PF-429242. This difference may be explained by the fact that inhibition of site 1 protease not only impairs the function of SREBP1 but also of SREBP2 and of further transcription factors, which reside as transmembrane precursor proteins in the endoplasmic reticulum, as e.g. ATF6 [29] and the liver-specific CREBH [30]. While ATF6 was shown to exert inhibitory effects on hepatic lipogenesis [31], murine CREBH was shown to participate in the transcriptional activation of lipogenic enzyme genes, as e.g. *Fasn*, *Acaca*, *Scd* and *Elovl6* [32]. In mice, a partial overlap of SREBP1 and -2 in the regulation of lipogenic SREBP-target genes was elegantly demonstrated by a combinatorial whole genome transcriptomic analysis in SREBP1- and SREBP2-transgenics as well as SCAP knock-out mice [33]. Except for *Acacb*, *Scd* and *Thrsp*, murine orthologues of all the other lipogenic enzyme genes, which have been analyzed in this study, proved to be regulated by both SREBP1 and SREBP2, even if half of them demonstrated a clear preference for SREBP1 [33]. Furthermore, siRNA-mediated knock-down of SREBP1 showed an efficacy of about 80%, thereby leaving residual 20% of expression. Even if the effect of siRNA may be regarded as being more specific compared to pharmacological inhibition, it is not as complete as the latter.

Strikingly, expression of *ACLY* and *ELOVL6* was not affected significantly by SREBP1 knock-down at all. In mice, *ACLY* was shown to be nearly equally well regulated by SREBP1 and -2 [33], thus the latter may compensate here most efficiently for the loss of SREBP1. In contrast to *ACLY*, expression of which was not reduced by SREBP1 knock-down in any hepatocyte culture, *ELOVL6* was clearly affected in one of them. This demonstrates that interindividual variability of effects combined with the small number of cultures, which have been used, represents a limitation of our analysis.

Surprisingly, simultaneous knock-down of both isoforms was not more efficient in reducing the expression of most SREBP-target genes than knock-down of 1a alone, even if the latter resulted only in about 60% knock-down of total mature SREBP1 protein, whereas the former achieved respective knock-down efficiency of 90% (see Fig. 5C). In mice, knock-out of SREBP1 resulted in compensatory up-regulation of SREBP2 [34, 35], which also may have happened in the case of SREBP1 knock-down by pan-SREBP1 siRNA, as this reduced total SREBP1 protein levels much stronger than 1a-specific knock-down. Alternatively, these results may indicate that SREBP1c does not contribute significantly to the regulation of these genes in cultured primary human hepatocytes, as it represents the weaker transcriptional activator of both isoforms and is not expressed in such a large surplus compared to 1a as in mice. Only *THRSP* and *INSIG1* were reduced more strongly by knock-down of both isoforms than by 1a-specific knock-down, suggesting that SREBP1c may exert a specific function in their regulation. Further studies using SREBP1c-specific siRNA are required to prove these hypotheses. In conclusion, these data clearly indicate that human SREBP1a has a much broader and, most likely due to its higher expression, stronger effect on hepatic lipogenic gene expression than its mouse orthologue.

It is already known from many studies, that SREBP1 is up-regulated in NAFLD, which is usually attributed to an increase in SREBP1c. However, following a thorough literature analysis, we determined that most of these studies had measured total SREBP1, without discriminating between the isoforms. Only two studies specifically quantified SREBP1c [36, 37], and two others specifically measured SREBP1a, apparently without noticing [38, 39], thereby confirming our results [16, and this study]. Given the here shown participation of SREBP1a in the control of hepatic expression of lipogenic SREBP-target genes, it may

be hypothesized that the 1a isoform contributes to distinct pathogenic pathways in the development of NAFLD. Besides regulating the expression of genes promoting *de novo* lipogenesis, as e.g. ACACA, FASN, SCD and THRSP, which were previously shown to be up-regulated in NAFLD progression [16, 40], SREBP1a was here shown to further regulate human ACACB. This gene encodes acetyl-CoA carboxylase (ACC) 2, which participates in the negative control of mitochondrial fatty acid β -oxidation [41]. In parallel to SREBP1a, ACACB mRNA also increased with NAFLD progression (data not shown). If this further translates into elevated ACC2 protein and activity levels, fatty acid β -oxidation should be reduced, which may contribute to hepatic steatosis.

In conclusion, we have clearly shown here that human SREBP1a contributes considerably stronger to the total hepatic SREBP1 pool and has a much stronger impact on hepatic lipogenic gene expression, both quantitatively and qualitatively, than mouse SREBP1a. Given the crucial role of lipogenesis in NAFLD etiology and progression, our data support a pathophysiological role of SREBP1a in human hepatic lipogenesis and emphasize importance of its interindividual expression variability.

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Disclosure Statement

The authors declare no conflicts of interest.

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