Stable SREBP-1a knockdown decreases the cell proliferation rate in human preadipocyte cells without inducing senescence

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ABSTRACT

Sterol regulatory element binding proteins (SREBP), encoded by the *Srebf1* and *Srebf2* genes, are important regulators of genes involved in cholesterol and fatty acid metabolism. Whereas SREBP-2 controls the cholesterol synthesis, SREBP-1 proteins (-1a and -1c) function as the central hubs in lipid metabolism. Despite the key function of these transcription factors to promote adipocyte differentiation, the roles of SREBP-1 proteins during the preadipocyte state remain unknown. Here, we evaluate the role of SREBP-1 in preadipocyte proliferation using RNA interference technology. Knockdown of the SREBP-1a gene decreased the proliferation rate in human SGBS preadipocyte cell strain without inducing senescence. Furthermore, our data identified retinoblastoma binding protein 8 and cyclin-dependent kinase inhibitor 3 genes as new potential SREBP-1 targets, in addition to cyclin-dependent kinase inhibitor 1A which had already been described as a gene regulated by SREBP-1a. These data suggested a new role of SREBP-1 in adipogenesis via regulation of preadipocyte proliferation.

1. Introduction

The sterol regulatory element-binding protein (SREBP) is a family of transcription factors that control cholesterol and lipid metabolism. This family consists of three different SREBP proteins, SREBP-1a, SREBP-1c and SREBP-2. SREBP-1a and SREBP-1c come from a single gene by alternative splicing, differing only in their N-terminal sequences, whereas SREBP-2 is codified by a separate gene [1]. SREBP-2 is constitutively expressed in most tissues and is deeply involved in the regulation of cholesterol metabolism. SREBP-1a is the predominant isoform in most cultured cell lines, spleen and intestine, whereas SREBP-1c predominates in the liver and other tissues, such as muscle and adipose tissue [2,3]. Both, SREBP-1a and SREBP-1c are responsible of governing fatty acid and triacylglycerol metabolism [4].

Adipogenesis is a particular process which involves two major events: preadipocyte proliferation and adipocyte differentiation [5]. Extensive studies have been conducted about the identification and characterization of transcription factors that are involved in lineage-specific cell determination and terminal differentiation. However, preadipocyte proliferation is still poorly understood. The role of SREBP-1 in adipogenesis has been studied in mouse preadipocytes upon exposure to hormones and growth factors. SREBP-1c is induced during differentiation of adipocytes where it activates transcription of target genes involved in both cholesterol metabolism and fatty acid metabolism [6]. Recently, it has been demonstrated that siRNA-mediated inactivation of SREBP-1 attenuates the cell growth in HEK293T, HepG2 and HeLa mitotic cells [7,8]. Moreover, in Chinese hamster ovary cell lines, SREBP-1a transactivates p21$^{WAF1/CIP1}$ expression and stabilizes p27^{Kip1} leading to cell growth arrest [9,10]. Thus, SREBP-1 may play a role in human preadipocyte proliferation.

This study was aimed to develop an experimental model for clarifying the role of SREBP-1 in the proliferation of preadipocytes, distinct from its role in the differentiation. Thus, we have generated a model cell line of permanently down-regulated SREBP-1 expression in human preadipocytes cells by virus-mediated constitutive expression of short hairpin RNA (shRNA). We have used the Simpson-Golabi Behmel Syndrome (SGBS) preadipocyte cells which offer an almost unlimited source of homogeneous human

preadipocytes with high differentiation capacity [11]. The results demonstrated that SREBP-1 knockdown specifically diminished the expression of SREBP-1a leading to a decrease in the proliferation of human preadipocytes without inducing senescence or DNA damage. Furthermore, SREBP-1a knockdown did not change the expression of lipogenic genes in human preadipocytes cells but altered the expression of genes that control the cell cycle.

2. Materials and methods

2.1. Cell culture and SGBS infection

SGBS cells, kindly provided by Dr. Martin Wabitsch (University Medical Center Ulm, Ulm, Germany), were cultured in DMEM F12 (1:1) supplemented with 10% FCS, 33 μM biotin, 17 μM pantothenic acid, and antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin). Human embryonic kidney 293T (HEK293T) cells were maintained in DMEM containing 25 mmol/l glucose, 100 U/ml penicillin, and 100 U/ml streptomycin supplemented with 10% FBS. All the cells, repeatedly shown to be mycoplasma-free, were grown at 37 °C in a humidified air 5% CO_2 atmosphere.

For lentiviral infection, HEK293T cells were transfected with 3 μg of envelope plasmid VSVg, 6 μg of packaging vector PSPAx2 and 9 μg of pLKO shRNA GFP (control plasmid) or five different shRNA sequences to SREBP-1 (Mission shRNA Bacterial Glycerol Stock, Sigma Aldrich, St. Louis, MO, USA), using the phosphate calcium standard transfection protocol. Cell culture supernatants were collected during 2 days and added to SGBS cells in presence of 8 μg/ml of polibreno for 24 h. 48 h later, the medium was replaced by fresh medium supplemented with 1 μg/ml puromycin. SGBS/shSREBP-1 cells with stable inhibition of SREBP-1 and SGBS/pLKO cells without expression of shSREBP-1 RNA were used in subsequent research.

To evaluate cellular growth rate, stable cells were seeded at low density (4000 cell/cm²) and the cell proliferation rate was measured using a MTT-based proliferation assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega Corporation, Madison, USA), according to the manufacturer's instructions.

2.2. Total RNA isolation and PCR analysis

Total RNA from shSREBP-1 or pLKO cells was extracted after 72 h of culture by using Trizol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. 1 μg RNA was reverse transcribed using random hexamer and expand reverse transcriptase (Roche Diagnostics, Manheim, Germany). The cDNA was used as template for real-time PCR using the ABI7500 fast instrument. The relative gene expression changes were calculated employing the second derivative comparative Ct method. Taqman technology (Assays-on-demand gene expression, Life Technologies) was used to determine the SREBP-1 and fatty acid synthase (FAS) mRNA levels, whereas to discriminate between SREBP-1a and 1c gene expression, the cDNA was used as a template for quantitative PCR using Sybr Green reagent (Life Technologies) with specific primers for each isoform (primers are available upon request). Human GAPDH was used as control in both cases.

For PCR array experiments, human cell cycle RT2 Profiler PCR array (PAHS-020; Qiagen Hilden, Germany) was used to examine the mRNA levels of 84 genes in a 96-well PCR array plate according to the manufacturer's protocol. Using RT2 First Strand kit, 1 μg of total RNA was reverse transcribed and real-time PCR was performed using SYBR Green PCR Master Mix. The Cell Cycle PCR Array was repeated with four different pLKO cDNA samples and seven different shSREBP-1 cDNA samples and the data were analyzed using Excel-based PCR Array Data Analysis Templates (SABio-

science, Qiagen). For each plate, quality tests for PCR reproducibility, reverse transcription efficiency, and level of genomic DNA contamination were all successfully passed (data not shown). The data were analyzed using the free software for RT2 Profiler PCR Array data analysis based on the comparative threshold cycle (Ct) method (www.sabiosciences.com/pcrarraydataanalysis.php), normalized to GAPDH and expressed as relative fold differences in shSREBP-1 compared to pLKO samples. Genes showing a change in expression more than 1.5-fold were considered differentially expressed. The results were validated by real-time qRT-PCR in another set of stable shRNA SREBP-silenced SGBS cells using different primers and PCR enzymes from those used for the PCR array. Relative gene expression was calculated by using the comparative ($2^{-\Delta\Delta Ct}$) method. All reactions were carried out in triplicate.

2.3. Senescence-associated β-galactosidase assay

SA-β-galactosidase activity at pH 6.0 was assayed in the SGBS cells after three days in culture following the manufacturer's recommended protocol (Senescence Cells Histochemical Staining Kit-Sigma). The percentage of the β-galactosidase-positive cells (of the total cell number) was obtained to estimate the degree of senescence-associated cells.

2.4. DNA damage analysis

To analyze the presence of DNA damage, the cells were fixed in 4% (wt/vol) paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% TritonX-100 in PBS, and were blocked with 1% (wt/vol) bovine serum albumin in PBS for 45 min. Then, the cells were incubated with anti H2AX (1:300) (Millipore, Concord Road Billerica, MA, USA) and H3K9me3 (1:500) (Abcam, Cambridge, UK) overnight at 4 °C. After washing, the plates were treated with goat anti-rabbit Alexa 488 secondary antibody (1:800) (Life Technologies) for 45 min. Then, the nucleus were stained with 8 μg/ml Hoechst (Sigma) 10 min room temperature, and the plates were mounted with Aqua-Poly/Mont (Polysciences, Valley Road Warrington, PA, USA) and were visualized by fluorescence microscopy.

2.5. 5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

For BrdU labeling, shSREBP-1 and pLKO cells were maintained in culture for 24 h and, then, they were labeled with 10 μM 5-bromo-2′-deoxyuridine (Sigma). After 48 h of incubation, cells were fixed in 4% (wt/vol) paraformaldehyde for 20 min at room temperature, washed three times with PBS, and were treated with HCl 2 N for 10 min at 37 °C followed by two washes for 5 min each with sodium borate pH 8.5 0.1 M. Then, the cells were washed twice in PBS and blocked with 0.2% Triton X-100 in PBS and FBS 10% for 45 min at room temperature, after which they were incubated in the same buffer at 4 °C overnight with anti 5-BrdU primary antibody (1:600) (Dako, Rhode Island, USA). Next day, the cells were washed with PBS five times and were incubated with the secondary DyLight 594 (1:800) (Abcam) for 45 min. After staining the cells with 8 μg/ml Hoechst for 10 min at room temperature, the coverslips were mounted with Aqua-Poly/Mont (Polysciences) and were visualized by fluorescence microscopy.

2.6. Statistical analysis

Statistical significance was estimated with the Student's two-tailed *t*-test for unpaired observations, One-way or Two-way ANOVA tests. A *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. SREBP-1 knockdown affects growth of human preadipocytes

To elucidate the role of SREBP-1 in the preadipocyte state, we studied the effects of lentivirus-mediated SREBP-1 knockdown in SGBS cells. We tested five shRNA targeting different regions of the gene sequence. SGBS cells were also infected with the pLKO non-targeting control vector to generate SGBS-infected control cells. 72 h after lentivirus infection, SREBP-1 expression was reduced by all the used shRNA-SREBP-1 lentivirus with a variable knockdown efficiency as compared with the control cells (Fig. 1A). The sh4 (TRCN0000020605) and sh5 (TRCN0000020607) lentiviral particles, whose sequences are located in the exon 6 and are thus common to the SREBP-1a and 1c isoforms, decreased SREBP-1 expression by 70% and 85%, respectively, so we used these lentiviruses to obtain SGBS cells with a stable loss of SREBP-1 expression after antibiotic selection. As both SREBP-1 variants were target of the lentiviral particles, we analyzed by real-time PCR the SREBP-1a and SREBP-1c mRNA levels. SGBS cells mostly expressed the SREBP-1a variant and, consequently, shRNA-SREBP-1 lentivirus specifically decreased the SREBP-1a mRNA levels without modifying the already low levels of SREBP-1c (Fig. 1B).

Furthermore, we assessed the effect of shRNA-mediated stable knockdown of SREBP-1 expression on cell proliferation by the MTT assay. SREBP-1a knockdown led to a marked decrease (>60%) in the number of cells at 72 h (Fig. 2A). The impact on SGBS proliferation was also evaluated by BrdU assay. The preserved S-phase activity was not significantly altered in shSREBP-1 cells after 72 h in culture as indicated by the retained incorporation of the thymidine analog (Fig. 2B).

Lipogenesis is activated during cell growth and proliferation to provide membranous components for subcellular organelle formation [12]. To examine whether a deteriorated lipogenesis was involved in the attenuation of proliferation rate between pLKO and shSREBP-1 cells, the mRNA levels of FAS were also monitored. As shown in Fig. 1B, SREBP-1a knockdown did not disturb the mRNA levels of the lipogenic enzyme.

3.2. Blocking SREBP-1 expression does not induce senescence in human preadipocytes

Previous report observed that SREBP-1 activation is involved in senescence [13]. Senescence-associated-β-galactosidase (SA-β-gal) activity was assayed after 72 h of culture. Some shSREBP-1 cells exhibited a large, extended and flattened cell shape typical of senescence. However, quantitative analysis showed that there was not a significantly different percentage of cells stained positively for SA-β-gal activity as compared to that of pLKO control cells (Fig. 2C). Moreover, changes in chromatin structure have been implicated in cellular senescence [14]. Upon the induction of senescence, senescence-associated heterochromatin foci (SAHFs) are enriched for heterochromatin markers as H3K9me3 [15]. Analysis of heterochromatin distribution using immunostaining of H3K9me3 showed no change between the control group and SREBP-1 knockdown cells without distinguishing heterochromatin clusters (Fig. 3A). Taken together, all the data seems to indicate that SREBP-1 knockdown does not progress toward senescence in human preadipocytes.

Finally, to further understand the mechanism underlying shSREBP-1-induced slow proliferation, we measured the cellular content of DNA damage, which is known to perturb cell proliferation and compromise cell survival. To understand changes in the DNA damage response in SGBS cells, H2AX foci were examined in the pLKO and shSREBP-1 cells, without detecting their formation (Fig. 3B).
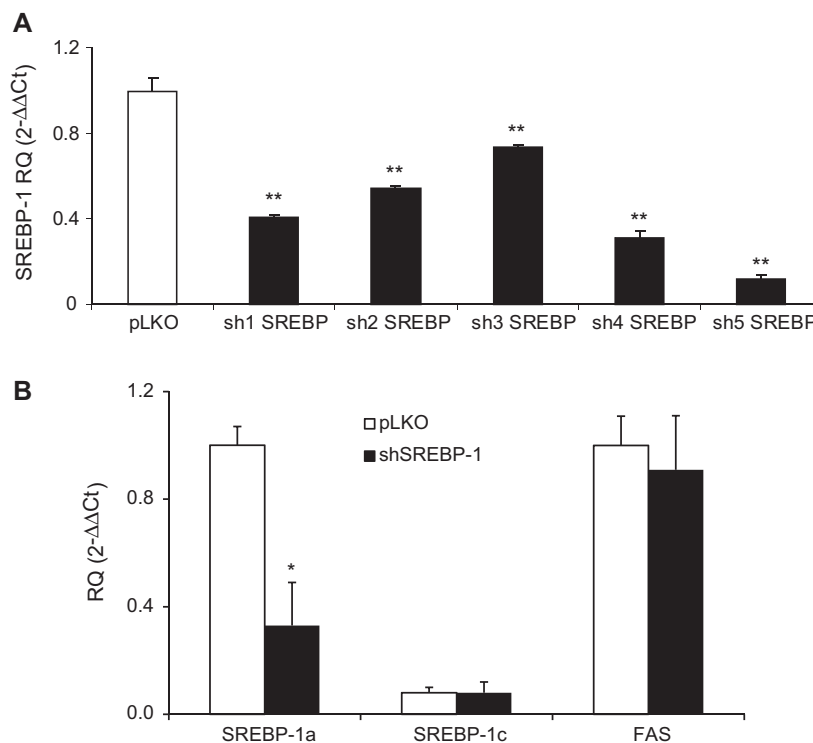

Fig. 1. Inhibition of SREBP-1 expression in SGBS cells by shRNA expression vectors. SGBS cells were infected with different SREBP-1-shRNAs lentiviral particles or pLKO negative control vector and 48 h after infection RNA was extracted and synthesized to cDNA. qRT-PCR analysis was performed to analyze the levels of SREBP-1 mRNA (A), SREBP-1a, SREBP-1c, and FAS mRNAs (B) using GAPDH as the normalization control. Results are reported as means ± SD and represent fold change relative to negative control condition (pLKO). *$p < 0.05$ vs pLKO/SREBP-1a; **$p < 0.01$ vs pLKO.
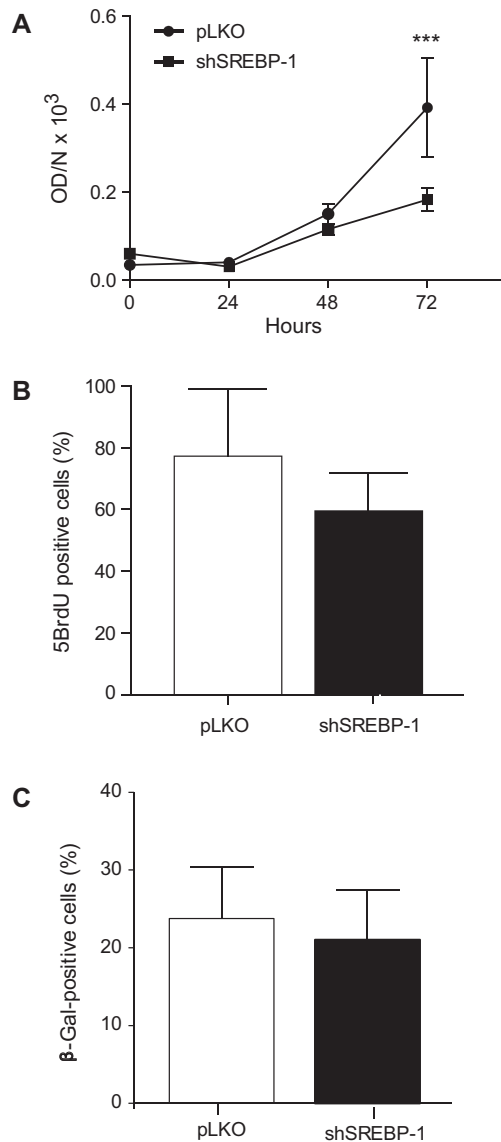
Fig. 2. SREBP-1 knockdown slowed growth of SGBS cells without affecting the S-phase activity or inducing senescence of human preadipocytes. (A) SGBS cells were stably transfected with shSREBP-1 (■) or pLKO (●) vectors. The effect of cell proliferation was determined by MTT assay at different times of culture. Absorbance at 570 nm was recorded using a 96-well plate reader and was normalized by the initial number of cell per well (N). Each point represents the mean ± SD of 6 independent experiments. (B) At 48 h of BrdU pulse, BrdU positive cells were counted and data are shown as means ± SD (3 independent experiments) of the percentage of positive cells out of the total number of cells. (C) SGBS cells stably transfected with shRNA SREBP-1 or pLKO vectors were maintained in culture during 72 h. Then, SA-®-galactosidase activity was monitored. Cells were counted in different microscopic fields and the percentage of SA-®-gal-positive cells were calculated. The histograms represent the mean ± SD of 3 independent experiments.

3.3. Gene expression profiling of cell cycle regulatory genes

We next examined if SREBP-1 controlled the expression of cell cycle regulatory genes in preadipocyte cells. To investigate potential changes in the levels of mRNAs encoding cell cycle-regulatory proteins, a PCR array of 84 genes involved in cell cycle progression was used. Across the screened panel, the expression of twelve genes was down or up-regulated by 1.5-fold or more by SREBP-1a knockdown (Fig. 4A). PCR-array findings were further validated by quantitative RT-PCR. In addition to CDKN1A (p21$^{\text{Waf1/Cip1}}$) which has already been described as target of SREBP-1 [9,10], we
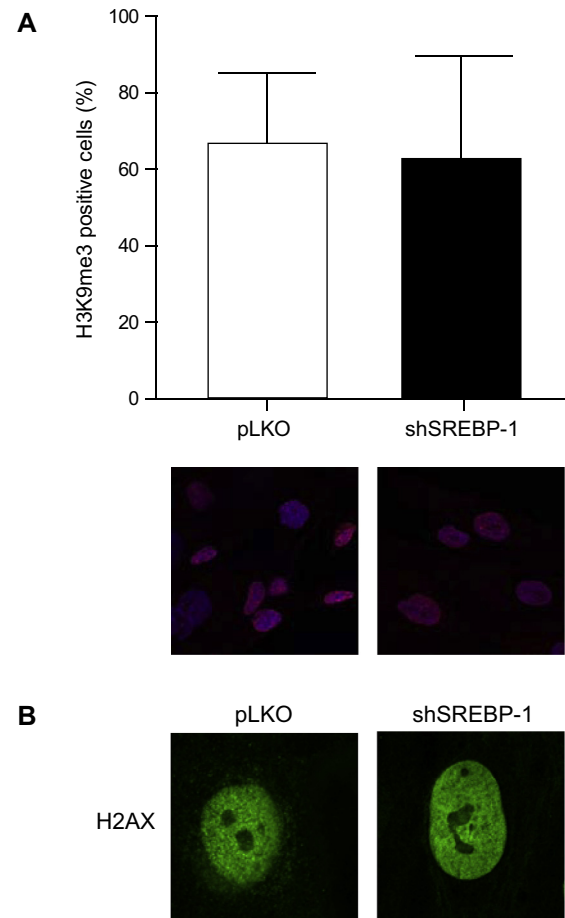
Fig. 3. Nuclear staining of heterochromatin marker and DNA damage foci in stably transfected shRNA SGBS cells. (A) Confocal images of immunofluorescence for H3K9me3 histone mark. The histogram represents the proportion of SGBS cells displaying H3K9me3 staining. Data are shown as mean ± SD from 3 independent experiments. (B) Representative images of H2AX staining in nuclei of stably SREBP-1 knockdown and control SGBS cells.

statistically validated another two targets: RBBP8 (Retinoblastoma binding protein 8) and CDKN3 (Cyclin-dependent kinase inhibitor 3), which were down and up-regulated by SREBP-1a knockdown, respectively (Fig. 4B).

4. Discussion

Adipose tissue growth is the result of hypertrophy and hyperplasia of adipocytes. The increase of the adipocyte number accounts for the adipose tissue expansion observed in obesity and during normal tissue development [5]. Adipocyte hyperplasia in adults requires the generation of new adipocytes from precursor cells (preadipocytes) and stem cells resident in the stromal-vascular compartment of white adipose tissues [16]. Thus, understanding the mechanisms controlling preadipocyte proliferation and conversion to adipocyte provides insights into the etiology and prevention of obesity and its associated pathologies. Here, we show a new role of SREBP-1 in adipogenesis via regulation of preadipocyte proliferation.

The differentiation of adipose cells has been studied in immortal preadipocyte cell lines of murine origin as 3T3-L1 cells which are all aneuploidy, or in freshly human isolated preadipocytes which are diploid cells but have a limited life span and variability dependent of the donors. SGBS cells, originated from an adipose tissue of a patient with Simpson-Golabi-Behmel Syndrome, repre-
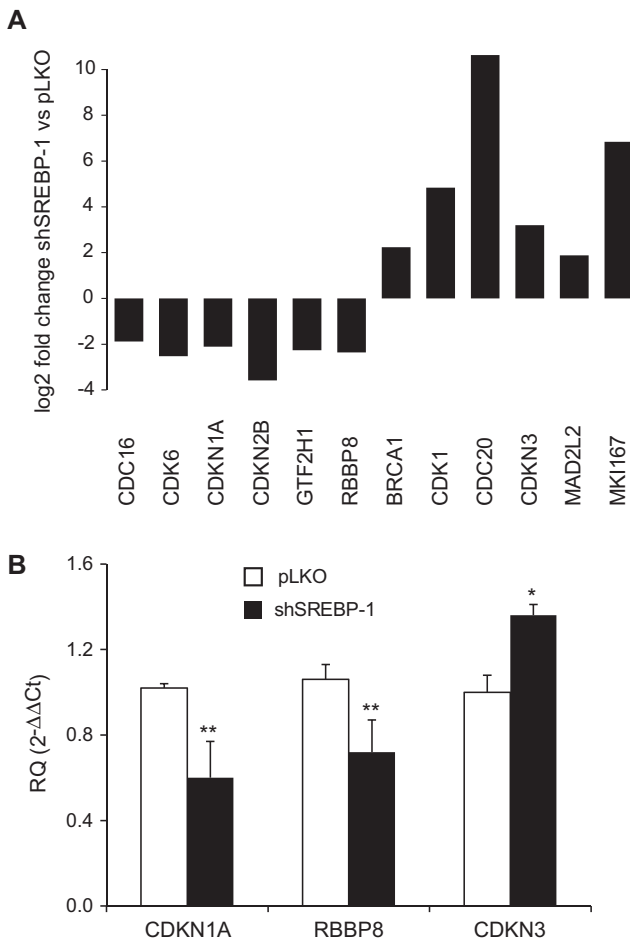
Fig. 4. Identification of the cell cycle-related gene regulated by suppression of endogenous SREBP-1 expression in human preadipocytes. (A) Analysis of differential gene expression profiling between shSREBP-1 and pLKO cells was assessed by quantitative PCR array. Columns represent averaged changes (fold $\geqslant 1.5$) of individual gene expression on stably SREBP-1 knockdown SGBS cells in comparison with cells transfected with pLKO control vector. (B) RNA samples from stably SREBP-1 knockdown SGBS cells and pLKO negative control cells were analyzed by qRT-PCR quantification of CDKN1A, RBBP8, and CDKN3 transcript levels. mRNA amounts were calculated as RQ and normalized to the expression of GAPDH levels. Values represent fold change relative to pLKO samples. Data are reported as means ± SD of at least 3 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$ vs pLKO.

sent a neither transformed nor immortalized human cell strain with high homogeneity between passages [11,17].

SREBPs have been analyzed for their role in lipid metabolism and the regulation of genes directly involved in terminal adipocyte differentiation. Some studies have indicated a cell cycle-dependent activity for SREBP-1 and have suggested a link to the regulation of cell proliferation and growth [7,8]. In the present study we focused on the role of SREBP-1 in the proliferative burst of human preadipocytes generating a cell model that stably knocked down the intracellular SREBP-1 in SGBS cells by a high efficiency transfection system of lentivirus vectors. Although shSREBP-1 lentiviral particles were able to target both SREBP-1 variants, SREBP-1a expression was the only one significantly decreased (Fig. 1B). Moreover, SGBS cells showed a high SREBP-1a/SREBP-1c ratio which shows that SREBP-1a is the predominant transcript in these cells.

The inhibition of SREBP-1a expression in SGBS cells induced a decrease of the proliferation with a minimal cell detachment, indicative of absence of cell death. Previous reports showed that the transient downregulation of SREBP-1 in HeLa cells resulted in G_1 phase arrest and failed to progress through the G_1/S boundary

[7]. However, the proliferative impairment observed in SGBS cells was not correlated with a reduced S phase activity as measured by uptake of BrdU, pointing to a discrepancy upon the experimental conditions and cell types. SREBP-1 is highly phosphorylated and activated in G_2/M arrested cells, suggesting that SREBP-1 may also play a role in other stages of the cell cycle, further strengthening our results [8].

SREBP-1 regulates pivotal enzymes in fatty acid synthesis. As lipogenesis is activated during cell growth to provide components for organelle formation, we asked whether the anti-growth effects of SREBP-1 knockdown were mediated through inhibition of lipogenesis. SREBP-1a knockdown did not alter the FAS mRNA levels, one of the key enzymes in fatty acids synthesis, according to the central role of Sp1 and SREBP-1c as the two major transcription activators of FASN [18,19] (Fig. 1B).

Previous results have shown a role of SREBP-1 in both Chang cells and primary young human diploid fibroblasts to which senescence were induced [13]. We tested the percentage of senescent cells associated to SREBP-1 knockdown in SGBS cells using the SA-β-galactosidase activity as marker, as well as senescence-associated heterochromatin foci. None of these markers showed increases of senescence after the decrease of SREBP-1 expression. These results would agree with the unmodified number of cells in S phase, as senescent cells arrest growth usually with a DNA content that is typical of G1 phase [20].

The first stage of regulation of gene expression occurs at the transcriptional level. Considering that transcriptional activity of SREBP-1 is modulated during the cell cycle [8], we identified the cell cycle genes regulated by SREBP-1a in SGBS cells using PCR array. Cell cycle analysis revealed that 12 genes could be modulated by SREBP-1. Three genes were confirmed by quantitative PCR.

SREBP-1a and SREBP-2 bind directly to a SRE element in the promoter of the CDKN1A (p21[WAF1/CIP1]) gene and strongly activate its promoter activity leading to cell arrest. Moreover, p21 expression was reduced in SREBP-1 deficient mice [9]. In agreement with this data, we also detected a decrease of p21 gene expression in SREBP-1a knockdown SGBS cells (Fig. 4), although also detected a decrease of proliferation (Fig. 2). Despite its profound role in halting cellular proliferation and its ability to promote differentiation and cellular senescence, recent studies suggest that, under certain conditions, p21 can promote cellular proliferation and oncogenicity [21]. In fact, p21 is necessary for certain processes that positively regulate cell cycle progression: cyclin D assembly with CDK4, its stability and its nuclear localization [22].

In eukaryotes, the cell cycle proceeds through two major checkpoints, one before the transition from G_1 to S, the other before G_2 to M. RBBP8 encodes CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains that plays a role in G_2/M transition [23]. Available evidence suggests that CtIP might mediate its known functions by recruitment of transcriptional regulators such as the CtBP corepressor complex or through direct interaction with the core transcription machinery [24]. CDKN3 encodes a kinase-associated phosphatase (KAP) which is an important regulator of cell cycle progression. Moreover, CDKN3 orchestrates mitosis through direct pathways (SAC, spindle assembly checkpoint) and indirect mechanisms (centrosome maintenance) [25]. siRNA-mediated silencing of the YB-1 gene inhibited cell proliferation and induced cell cycle arrest in T-47D breast by up-regulation of the CDKN3 gene and down-regulation of ten genes associated with positive regulation of the cell cycle, among which it is the RBBP8 [26]. Hence, decreased cell proliferation observed with knockdown of SREBP-1 could be due in part to the regulation of the expression of these genes. Sequence analysis of these genes promoters using TRANSFAC algorithm revealed the existence of at least 8 putative binding sites for SREBP between positions −1 to −1000 of the promoters of both genes, warranting further inves-

tigation into a SREBP-1a-dependent cell cycle control in human preadipocytes.

Recently, Ayala-Sumuano et al. have shown the importance of SREBP-1a during early adipogenesis acting as a link between the GSK3β-dependent signaling pathway and the beginning of the transcriptional cascade that results in the expression of *Pparg2* [27]. These results suggest that SREBP-1a is a key regulator of transcriptional control for adipogenesis. Here, we have shown an additional role of SREBP-1a before differentiation. We have demonstrated that there is a significant association between SREBP-1a and cell proliferation in human preadipocytes. Moreover, cell cycle analysis revealed that SREBP-1a could regulate cell cycle progression by modulating expression of potential new SREBP-1a targets of cell cycle-related genes. However, the physiological roles of SREBP-1 in the regulation of cell growth may be complex, and should be investigated carefully.

Conflict of interest

None declared.

Acknowledgments

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