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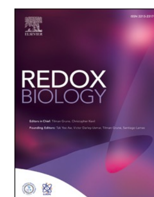

Research Paper

Permanent cystathionine-β-Synthase gene knockdown promotes inflammation and oxidative stress in immortalized human adipose-derived mesenchymal stem cells, enhancing their adipogenic capacity

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ABSTRACT

In the present study, we aimed to investigate the impact of permanent cystathionine-β-Synthase (CBS) gene knockdown in human telomerase reverse transcriptase (hTERT) immortalized human adipose-derived mesenchymal stem cells (ASC52telo) and in their capacity to differentiate into adipocytes. *CBS* gene KD in ASC52telo cells led to increased cellular inflammation (*IL6, CXCL8, TNF*) and oxidative stress markers (increased intracellular reactive oxygen species and decreased reduced glutathione levels) in parallel to decreased H_2S production and rejuvenation (*LC3* and *SIRT1*)-related gene expression. In addition, *CBS* gene KD in ASC52telo cells resulted in altered mitochondrial respiratory function, characterised by decreased basal respiration (specifically proton leak) and spare respiratory capacity, without significant effects on cell viability and proliferation. In this context, shCBS-ASC52telo cells displayed enhanced adipogenic (*FABP4, ADIPOQ, SLC2A4, CEBPA, PPARG*)-, lipogenic (*FASN, DGAT1*)- and adipocyte (*LEP, LBP*)-related gene expression markers, decreased expression of proinflammatory cytokines, and increased intracellular lipid accumulation during adipocyte differentiation compared to control ASC52telo cells. Otherwise, the increased adipogenic potential of shCBS-ASC52telo cells was detrimental to the ability to differentiate into osteogenic linage. In conclusion, this study demonstrated that permanent *CBS* gene KD in ASC52telo cells promotes a cellular senescence phenotype with a very increased adipogenic potential, promoting a non-physiological enhanced adipocyte differentiation with excessive lipid storage.

1. Introduction

Recent studies in 3T3-L1 cells pointed to a relevant role of transulfuration pathway in adipogenesis [1–4]. Even though Tsai et al. reported that both transulfuration pathway enzymes [cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH or CSE)] were important in adipocyte differentiation [1], the latter studies demonstrated that only CTH exerted a relevant role in adipogenesis increasing PPARγ activity during adipocyte differentiation [2,3]. In fact, in a previous study, in which *Cth* and *Cbs* gene expression in 3T3-L1 cells was analysed and compared, we found that *Cth* was highly expressed in preadipocytes and increased during adipocyte differentiation, whereas expression of *Cbs* gene was almost undetectable in preadipocytes and did not change during adipocyte differentiation, even tending to decrease in the first two days of the process [4].

Human adipose-derived mesenchymal stem cells (hAMSC), the adipocyte precursor cells located in adipose tissue stromal vascular fraction, are required for adipose tissue hyperplasia and functionality [5,6]. A previous study reported a useful human telomerase reverse transcriptase (hTERT) immortalized hAMSC (ASC52telo) cell model to study

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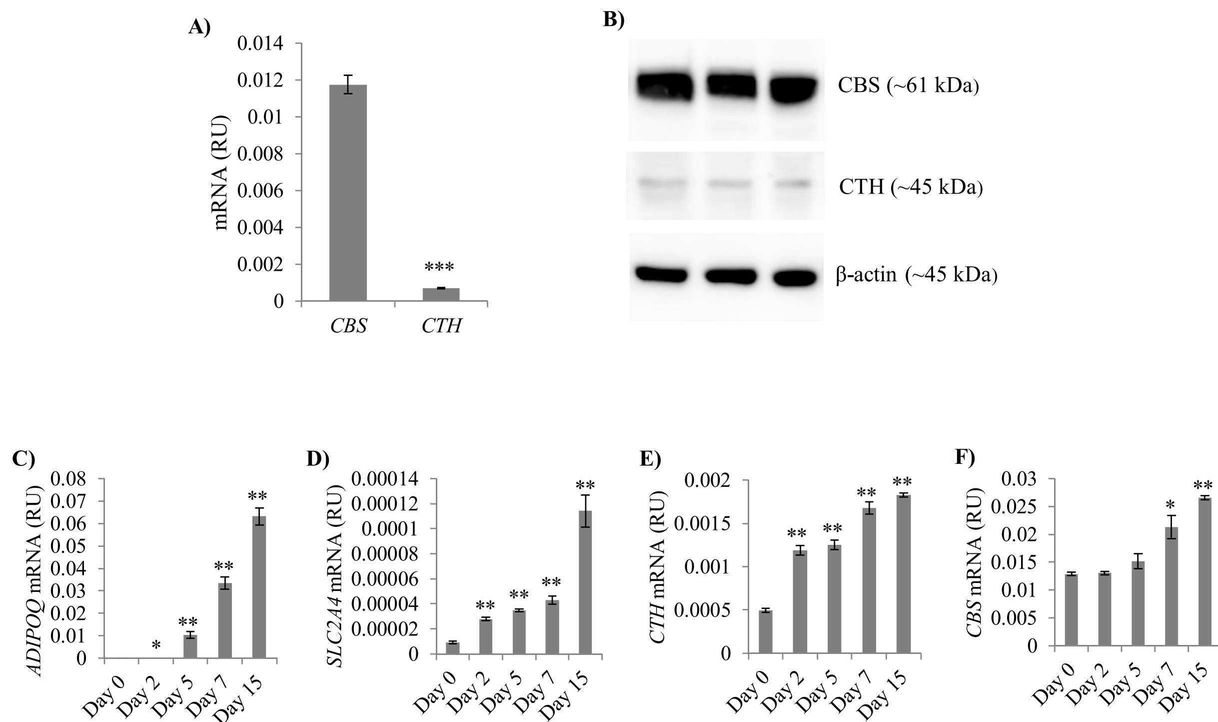

Fig. 1. A) *CBS* and *CTH* mRNA levels in ASC52telo cells,***$p < 0.001$. **B)** Representative Western blot of CBS and CTH protein in ASC52telo cell lysates. **C–F)** *ADIPOQ, SLC2A4, CTH* and *CBS* mRNA levels during adipocyte differentiation of ASC52telo cells. *$p < 0.05$ and **$p < 0.01$ *vs* day 0.

adipocyte differentiation [7].

To the best of our knowledge, the importance of transulfuration pathway in hAMSC adipogenesis has not been yet examined. Taking advantage of ASC52telo cells, in the present study, we aimed to investigate the possible role of CBS enzyme in hAMSC during adipocyte differentiation.

2. Material and methods

2.1. Differentiation of human immortalized adipose-derived mesenchymal stem cells

Human telomerase reverse transcriptase immortalized adipose-derived MSC (ASC52telo, SCRC-4000, ATCC, LGC Standards SLU, Barcelona, Spain) cells were cultured in Mesenchymal Stem Cell Basal Medium (ATCC PCS-500-030) plus FBS (2%), rhFGF basic (5 ng/ml), rhFGF acidic (5 ng/ml), rhEGF (5 ng/ml), L-Alanyl-L-Glutamine (2.4 mM) and G418 (0.2 mg/ml) at 37 °C in a 5% CO2 in air atmosphere.

Adipogenic differentiation. ASC52telo cells were cultured in three repetitive cycles of 72 h in adipogenic differentiation medium composed of DMEM/Nutrient Mix F-12 medium, FBS (10%), penicillin, streptomycin, human insulin (10 μg/mL), DXM (1 μmol/L), isobutylmethylxanthine (0.5 mmol/L), and PPARγ agonists (rosiglitazone, 1 μmol/L), followed by 72 h in adipogenic maintenance medium composed of DMEM/Nutrient Mix F-12 medium, FBS (10%), penicillin, streptomycin, and human insulin (10 μg/mL).

Osteogenic differentiation. ASC52telo cells were cultured with osteogenic differentiation medium composed of DMEM/Nutrient Mix F-12 medium, FBS (10%), 50 μg/ml ascorbic 2-phosphate, 10 nM dexamethasone and 10 mM β-glycerophosphate. Osteogenic medium was refreshed every 3 days. After 9 days, the cells were harvested.

2.2. Short hairpin (sh) RNA-mediated knockdown of CBS gene

Permanent silencing was performed using *CBS*-targeted and control (scrambled) shRNA lentiviral particles (sc-60335-V and sc-108080, Santa Cruz Biotechnology, CA, USA) and following the manufacturer instructions. Positive hAMSC harboring shRNA cassette for *CBS* were selected by puromycin (3 μg/mL) selection 60 h after infection.

2.3. In vitro measurements

Intracellular reactive oxygen species were measured using Fluorometric Intracellular ROS Kit (Cat. nº MAK142, Sigma, Madrid, Spain), reduced and total glutathione was measured using Glutathione Colorimetric Assay Kit (Cat. nº K261-100, Biovision, CA, USA). LDH activity and 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were performed with specific commercial kits, Cytotoxicity Detection Kit (LDH) (Cat. nº 11644793001, Roche Diagnostics SL, Barcelona, Spain) and Cell Proliferation Kit (MTT) (Cat. nº 11465007001, Roche Diagnostics SL, Barcelona, Spain). H_2S concentration in cultured medium was assessed as detailed elsewhere [4], using a naphthalimide-based fluorescent sensor 6-Azido-2-[2-[2-(2-hydroxyethoxy)ethoxy]ethyl]benzo[de]isoquinoline-1,3-dione (L1), which was chemically synthesised in Institute of Computational Chemistry and Catalysis (Chemistry Department, University of Girona) as described previously [8]. Intracellular lipid accumulation was assessed by Oil Red O staining as detailed elsewhere [4].

2.4. Mitochondrial respiratory function

Mitochondrial respiratory function was assessed using Seahorse XFp Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies) using Seahorse XFp Cell Mito Stress Test Kit as detailed elsewhere [9].

2.5. RNA expression

Briefly, RNA purification was performed using RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa SA, Barcelona, Spain) and the integrity was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Gene expression was assessed by real time PCR using an LightCycler® 480 Real-Time PCR System (Roche Diagnostics SL, Barcelona, Spain),

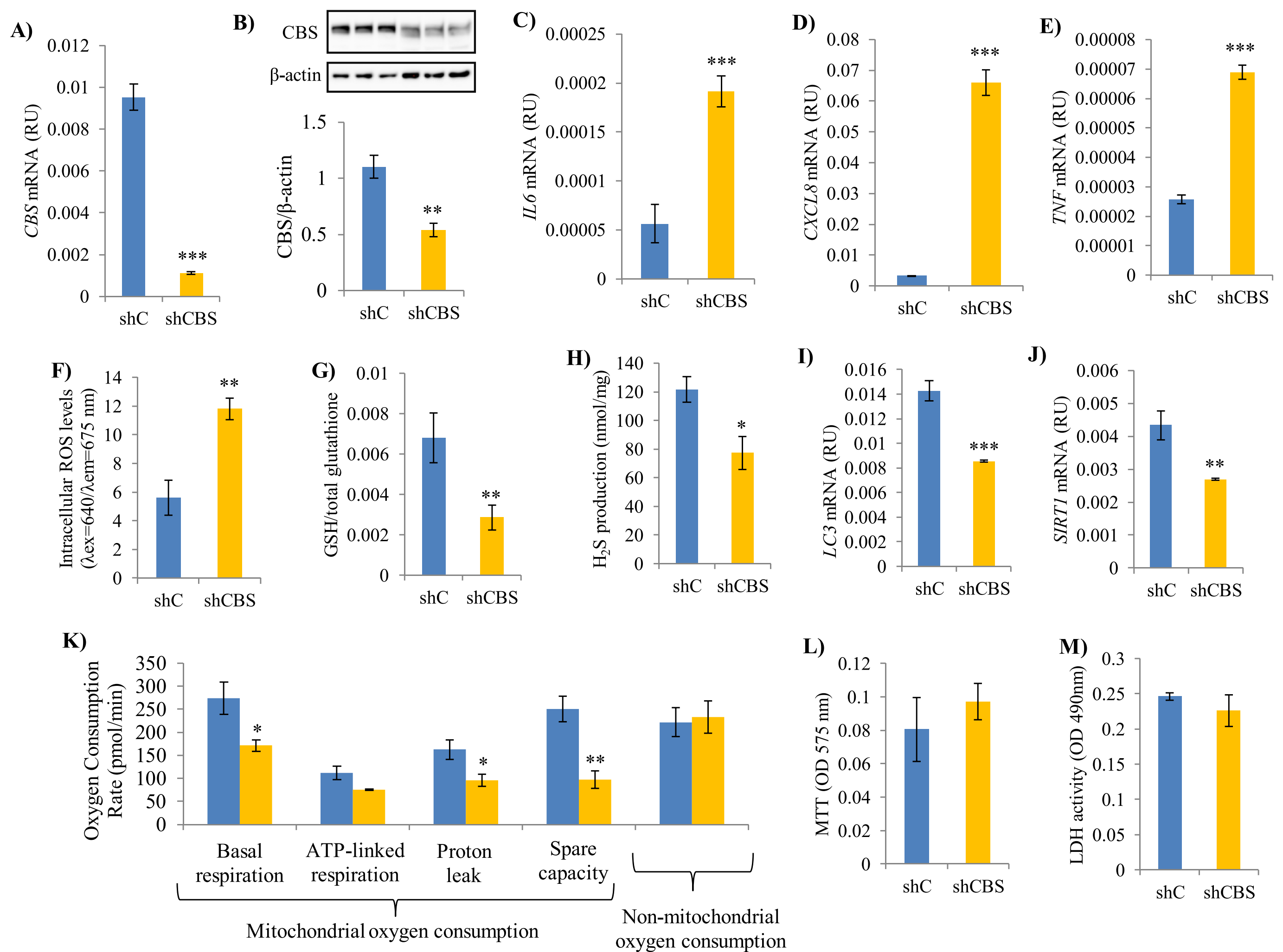

Fig. 2. A-M) Effect of permanent *CBS* gene knockdown (shCBS) on *CBS* mRNA **(A)** and CBS protein **(B)** levels, *IL6* **(C)**, *CXCL8* **(D)** and *TNF* **(E)** mRNA levels, intracellular ROS levels **(F)**, GSH/total glutathione **(G)**, H_2S production **(H)**, *LC3* **(I)** and *SIRT1* **(J)** mRNA levels, mitochondrial respiration **(K)**, MTT assay **(L)** and LDH activity **(M)** in ASC52telo cells. *p < 0.05, **p < 0.01 and ***p < 0.001 *vs* shC-ASC52telo cells.

using TaqMan® and SYBRgreen technology suitable for relative genetic expression quantification. The commercially available and pre-validated TaqMan® primer/probe sets used were as follows: Peptidylprolyl isomerase A (cyclophilin A) (4333763, PPIA as endogenous control), cystathionine γ-lyase (CTH, Hs00542284_m1), cystathionine β-synthase (CBS, Hs00163925_m1), adiponectin (ADIPOQ, Hs00605917_m1), peroxisome proliferator-activated receptor gamma (PPARG, Hs00234592_m1), fatty acid synthase (FASN, Hs00188012_m1), diacylglycerol O-acyltransferase 1 (DGAT1, Hs01020362_g1), CCAAT/enhancer binding protein alpha (CEBPA, Hs00269972_s1), solute carrier family 2 member 4 (SLC2A4 or GLUT4, Hs00168966_m1), insulin receptor substrate 1 (IRS1, Hs00178563_m1), Leptin (LEP, Hs00174877_m1), lipopolysaccharide binding protein (LBP, Hs01084621_m1), interleukin 6 (interferon, beta 2) (IL6, Hs00985639_m1), tumor necrosis factor (TNF, Hs00174128_m1), C-X-C motif chemokine ligand 8 (CXCL8 or IL8, Hs00174103_m1), C–C motif chemokine ligand 2 (CCL2 or MCP1, Hs00234140_m1), microtubule associated protein 1 light chain 3 alpha (MAP1LC3A or LC3, Hs00738808_m1), sirtuin 1 (SIRT1, Hs01009005_m1) and fatty acid binding protein 4, adipocyte (FABP4, Hs01086177_m1).

2.6. Protein preparation and Western blot analysis

Cellular protein were extracted directly in radioimmnuno precipitation assay (RIPA) buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, and 50 mM Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 13000 rpm for 10 min at 4 °C, recovering the soluble fraction. Protein concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). RIPA protein extracts (25 μg) were separated by SDS-PPGE and transferred to nitrocellulose membranes by conventional procedures. Membranes were immunoblotted with anti-CTH, CBS and β-actin (sc-365382, sc-133154, sc-47778, Santa Cruz Biotechnology, CA, USA). Anti-mouse IgG coupled to horseradish peroxidase was used as a secondary antibody. Horseradish peroxidase activity was detected by chemiluminescence, and quantification of protein expression was performed using scion image software.

2.7. Statistical analyses

Statistical analyses were performed using SPSS 12.0 software. Unpaired *t*-test and nonparametric test (Mann Whitney test) was used to analyse *in vitro* experimental data. Levels of statistical significance were set at p < 0.05.

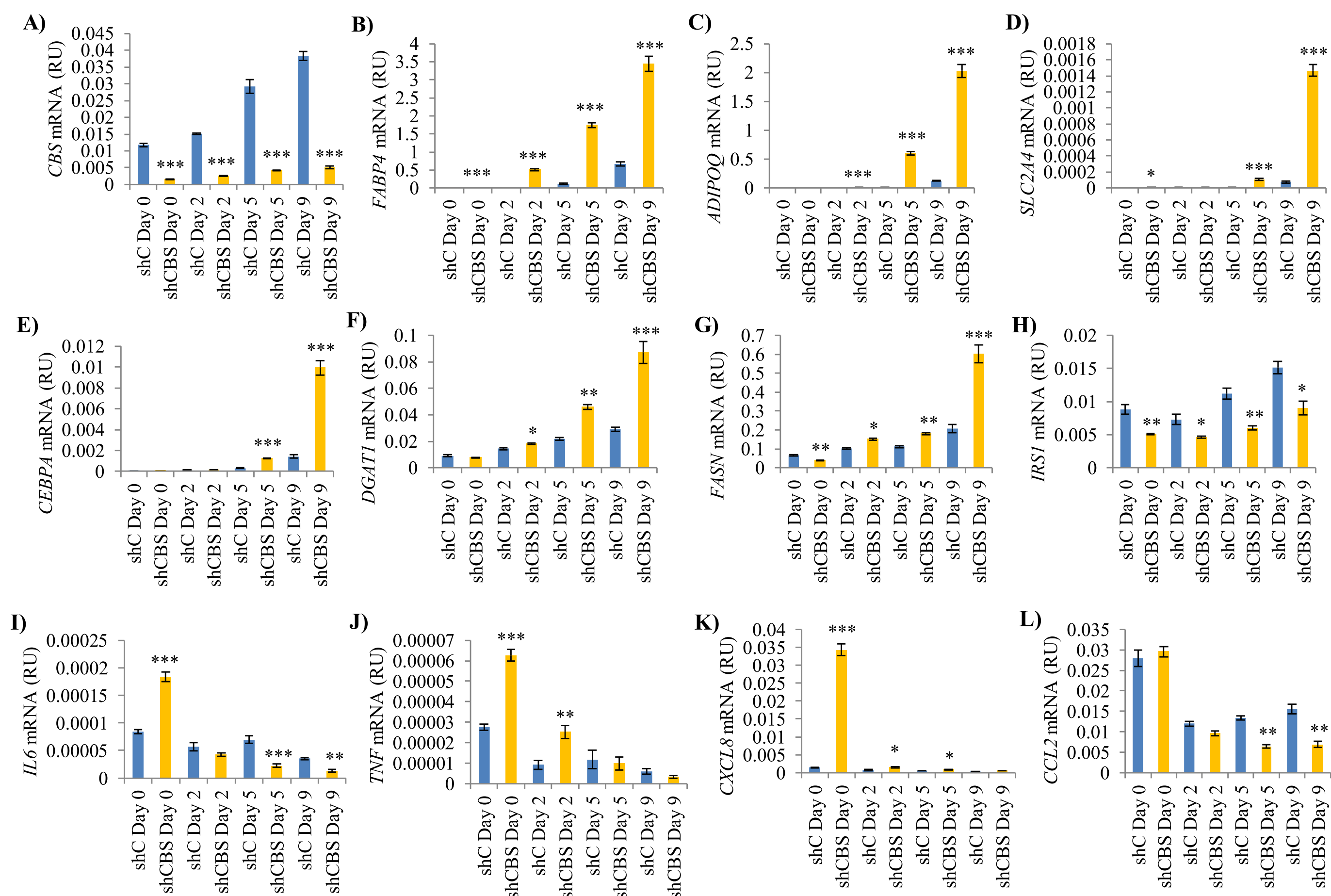

Fig. 3. A-L) Effect of permanent *CBS* gene knockdown (shCBS) on *CBS, FABP4, ADIPOQ, SLC2A4, CEBPA, DGAT1, FASN, IRS1, IL6, TNF, CXCL8* and *CCL2* mRNA levels in a time course experiment during ASC52telo cell adipocyte differentiation. *p < 0.05, **p < 0.01 and ***p < 0.001 *vs* shC-ASC52telo cells.

3. Results

3.1. CBS is much more abundant than CTH in ASC52telo cells

In ASC52telo cells, and contrary to 3T3-L1 cells [4], *Cbs* gene expression and CBS protein levels were significantly increased compared to CTH (Fig. 1A and B).

When adipocyte differentiation was examined in ASC52telo cells, we found a significant increased of adipogenic (*ADIPOQ* and *SLC2A4*) gene expression (Fig. 1C and D) in a time course experiment, confirming that adipogenesis has been appropriately induced in these cells. Similar to the observations in 3T3-L1 cells [4], *CTH* gene expression increased during hAMSC adipocyte differentiation in parallel to adipogenic genes (Fig. 1C–E), whereas *CBS* gene expression also increased but only in the last days of adipocyte differentiation process (Fig. 1F).

3.2. CBS gene KD in ASC52telo cells promotes inflammation and oxidative stress

Considering the higher CBS compared CTH protein levels, the effect of permanent *CBS* gene knockdown (KD) on ASC52telo cells was tested. *CBS* gene KD in these cells (Fig. 2A and B) led to a significant increase in cellular inflammation (*IL6, CXCL8, TNF*) and oxidative stress markers, including increased intracellular reactive oxygen species and decreased intracellular reduced glutathione levels, in parallel to decreased H_2S production (Fig. 2C–H). In addition, *SIRT1* and *LC3* mRNA were also decreased (Fig. 2I and J). Importantly, when mitochondrial respiration was evaluated, we found that *CBS* gene KD in ASC52telo cells resulted in decreased basal respiration (specifically proton leak) and spare respiratory capacity (Fig. 2K). Otherwise, no significant effects *CBS* gene knockdown on cell proliferation (MTT assay, Fig. 2L) or necrosis (LDH activity, Fig. 2M) were found.

3.3. CBS gene KD in ASC52telo cells enhances differentiation into adipocytes

In this context, *CBS* gene KD during ASC52telo adipocyte differentiation (Fig. 3A) led to enhanced adipocyte differentiation, with increased expression of adipogenic genes (*FABP4, ADIPOQ, SLC2A4, CEBPA, DGAT1, FASN*), but decreased *IRS1*, in a time course experiment (Fig. 3B–H; Suppl Figure 1). Consistent with previous findings (Fig. 2C–E), proinflammatory cytokines (*IL6, TNF* and *CXCL8*), but not the chemokine *CCL2*, were significantly increased at day 0 (Fig. 3I-L). Of interest, *IL6, TNF, CXCL8* and *CCL2* mRNA were strongly reduced during adipocyte differentiation, being significantly decreased *IL6* and *CCL2* at day 5 and 9 in shCBS-ASC52telo cells compared to shC-ASC52telo (control) cells (Fig. 3I-L).

To confirm these enhanced adipogenic capacity of shCBS-ASC52telo, in an independent experiment, we evaluated these cells in adipogenic conditions at day 15 (Fig. 4A). Reinforcing previous observations, shCBS-ASC52telo cells displayed enhanced adipogenic (*FABP4, ADIPOQ, SLC2A4, CEBPA, PPARG*)-, lipogenic (*FASN, DGAT1*)- and adipocyte (*LEP, LBP*)-related gene expression markers (Fig. 4B–J), decreased expression of proinflammatory cytokines (*IL6* and *TNF*) (Fig. 4K-L), and increased intracellular lipid accumulation (Fig. 4M–N) at day 15.

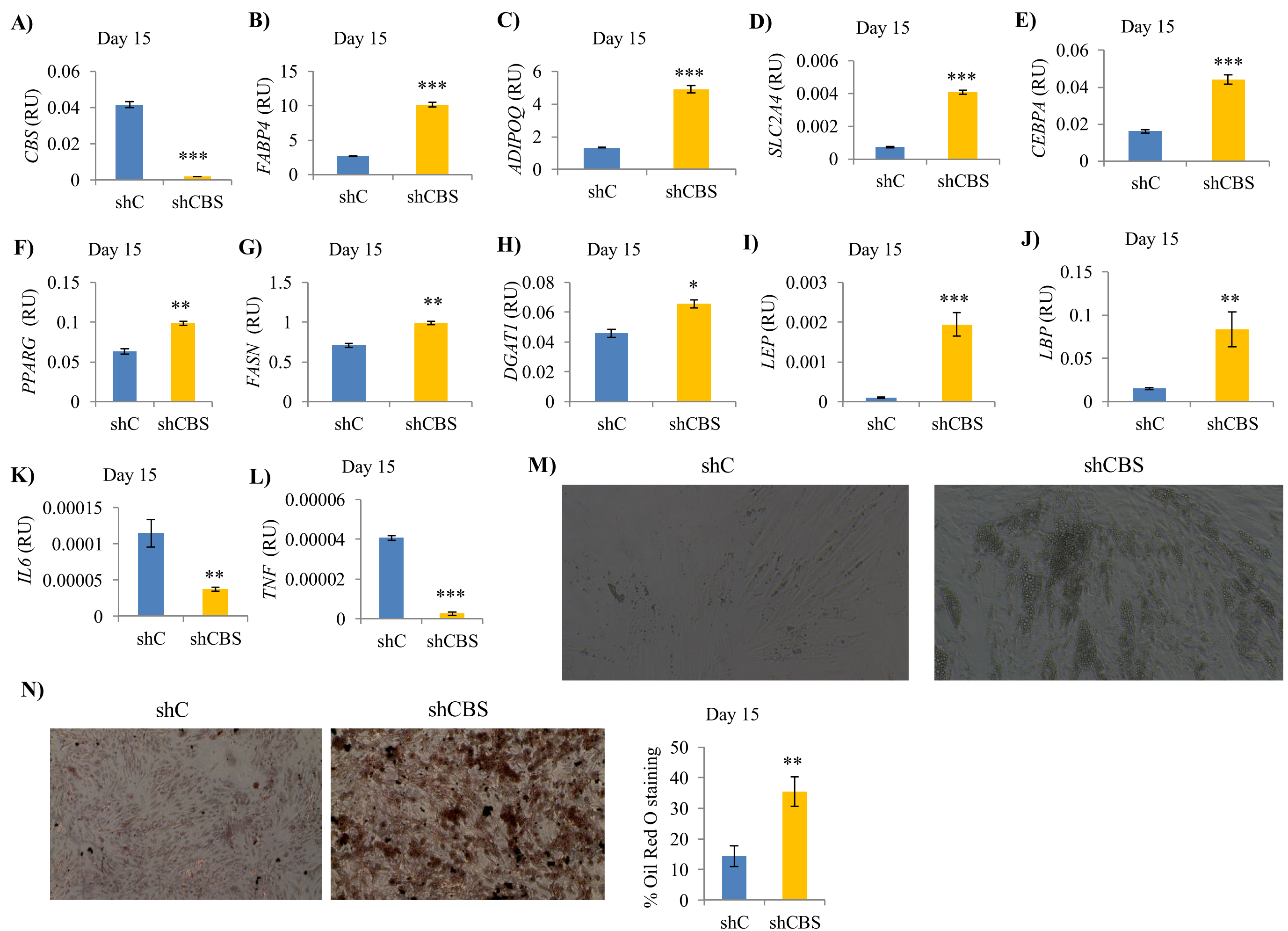

Fig. 4. A-L) Effect of permanent *CBS* gene knockdown (shCBS) on *CBS, FABP4, ADIPOQ, SLC2A4, CEBPA, PPARG, FASN, DGAT1, LEP, LBP, IL6* and *TNF* mRNA levels. **M-N)** Effect of permanent *CBS* gene knockdown (shCBS) on intracellular lipid accumulation, visually assessed at 10x magnifications **(M)** and measured by Oil Red O staining **(N)** at day15 of ASC52telo cell adipocyte differentiation. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* shC-ASC52telo cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. CBS gene KD in ASC52telo cells decreased osteogenic markers

Next, to evaluate the capacity of shCBS-ASC52telo cells to differenciate into another cell type, these cells were cultured in osteogenic conditions during 9 days. shCBS-ASC52telo cells (Fig. 5A) displayed reduced osteogenic markers (such as *RUNX2, BGLAP* and *TFGB1*) (Fig. 5B–D) in parallel to decreased rejuvenation (*LC3* and *SIRT1*)-related genes (Fig. 5E and F), but increased *FABP4* (Fig. 5G) and proinflammatory cytokines (*IL6, TNF* and *IL8)* (Fig. 5H–J).

4. Discussion

The current study demonstrated that, contrary to 3T3-L1 cells in which CTH levels were significantly increased but CBS was almost undetectable [4], ASC52telo cells showed an increased CBS/CTH ratio, characterized by increased CBS but very low levels of CTH mRNA and protein. In line with these findings, previous studies also demonstrated increased CBS *vs* CTH mRNA and protein levels in human mesenchymal stem cells [10,11].

These data indicate a major role of CBS in intracellular H_2S biosynthesis compared to CTH in ASC52telo cells. In fact, *CBS* gene KD in ASC52telo cells resulted in a significant increased in cellular inflammation and oxidative stress in parallel to decreased capacity to produce endogenous H_2S. A large number of studies demonstrated anti-inflammatory and anti-oxidant effects of H_2S in mesenchymal stem cells [10,12] and other cells [13–15]. However, the pro-oxidant and pro-inflammatory effects of CBS depletion might be also explained by other causes, including:

i) Altered mitochondrial respiratory function, characterized by decreased proton leak and spare respiratory capacity, two functional measures of mitochondrial function [16,17]. Supporting these findings, a recent study demonstrated the importance of CBS on mitochondrial function in endothelial cells [18]. It is important to note that proton leak (uncoupled respiration), which was significantly decreased in CBS KD ASC52telo (shCBS-ASC52telo) cells (Fig. 2K), is key mitochondrial process in the prevention of oxidative stress [19,20].
ii) Decreased expression of rejuvenation (*LC3* and *SIRT1*)-related genes. The protective effects of increased *SIRT1* [21–27] and *LC3* [28–31] mRNA levels in the prevention of cellular inflammation, oxidative stress and mitochondrial function are widely reported.
iii) Increased homocysteine/cysthationine ratio. CBS enzyme inhibition results in increased homocysteine in detrimental of cysthationine levels [32,33]. The pro-oxidant and pro-inflammatory effects of excess homocysteine have been demonstrated in *in vitro* and *in vivo* experiments [33–38]. Otherwise, similar to ASC52telo cells, breast tumor tissue displayed increased CBS, but very low CTH mRNA levels, resulting in increased intracellular levels of cysthationine, and in consequence preserving mitochondrial

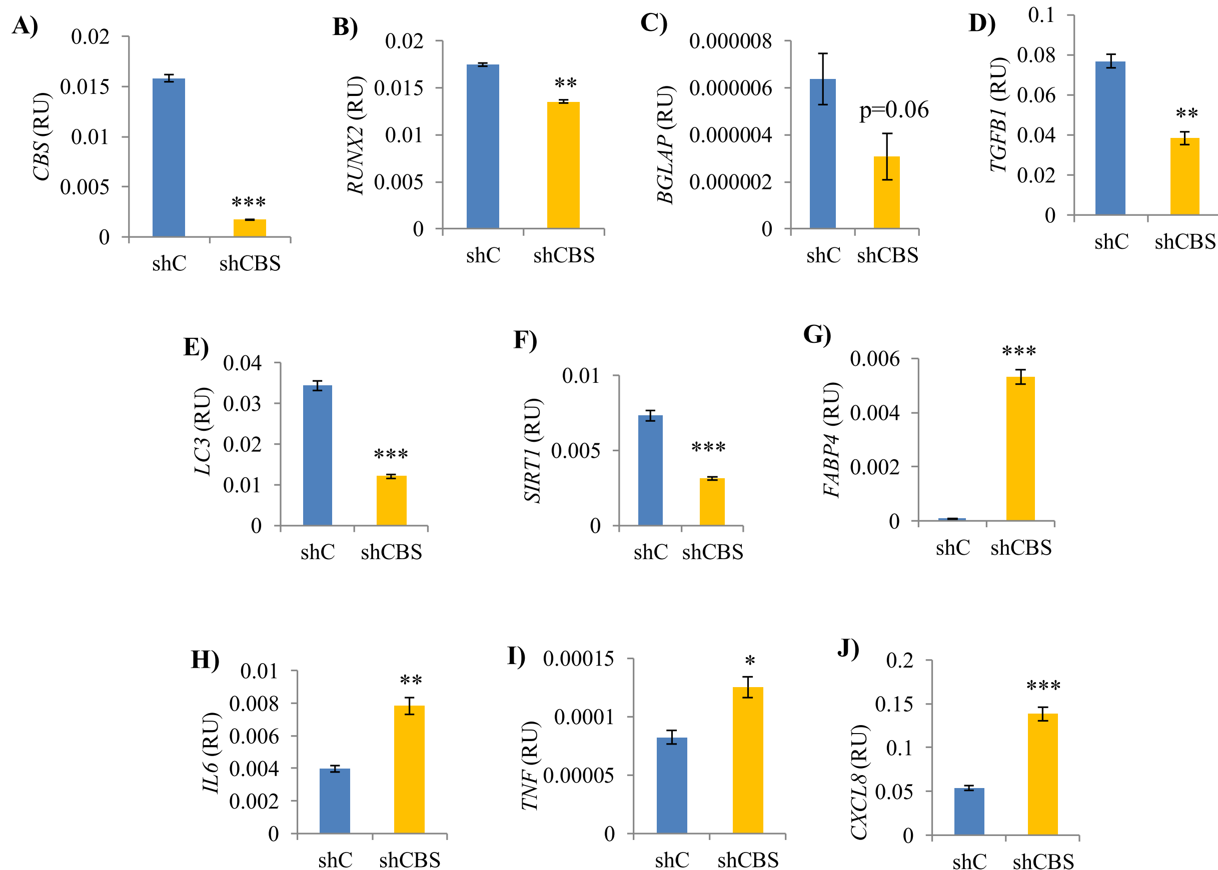

Fig. 5. A-J) Effect of permanent CBS gene knockdown (shCBS) on *CBS* mRNA levels **(A)**, expression of osteogenic-related genes (*RUNX2, BGLAP, TGFB1*) **(B–D)**, rejuvenation-related genes (*LC3, SIRT1*) **(E–F)**, *FABP4* **(G)** and proinflammatory cytokines (*IL6, TNF, CXCL8*) **(H–J)** during ASC52telo cell osteocyte differentiation (at day 9). $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* shC-ASC52telo cells.

function and preventing oxidative and endoplasmic reticulum stress [39].

Another important finding of current study was that *CBS* gene knockdown in ASC52telo cells greatly enhanced their capacity to differentiate into adipocytes in detrimental to the ability to differentiate into osteogenic linage. It is well-stablished that increased oxidative stress (ROS levels) in adipose-derived mesenchymal stem cells promotes a cellular senescence and inflammation phenotype characterised by increased capacity for differenciate into adipocyte at the expense of decreased stemness capacity [40–42]. In fact, antioxidant treatment in mesenchymal stem cells prevented adipocyte differentiation [42]. In agreement with current data, the administration of diallyl disulfide, a slow H_2S donor [43], restored hAMSC stemness via inhibition of intracellular ROS levels [44]. The relevance of CBS on osteogenesis has been previously reported [45–47]. Interestingly, one of these studies demonstrated that exogenous homocysteine administration increased intracellular homocysteine levels, and resulted in increased ROS, altered mitochondrial function, reduced expression of *CBS* gene and intracellular H_2S level, and in consequence, inhibiting its capacity to differentiate into osteogenic linage [47]. Otherwise, to the best of our knowledge, the impact of CBS depletion enhancing hAMSC adipogenesis has not been previously shown. These findings could seem controversial with one previous study in 3T3-L1 cells, in which CBS gene knockdown decreased adipogenesis [1], whereas other studies demonstrated that CTH was the only enzyme of transulfuration pathway with adipogenic properties [2–4]. In line with these studies, increased CTH/CBS ratio was associated to increased adipogenic potential in ASC52telo cells, which was characterised with very high expression of adipogenic genes (such as FABP4 and SLC2A4), even before adding the adipogenic media (at day 0). Moreover, supporting the cellular inflammation-associated adipogenic potential observed in shCBS-ASC52telo cells, two recent studies demonstrated that cellular inflammation promoted the generation of new adipocytes and adipose tissue expansion [48,49]. Otherwise, *IRS1* gene expression was significantly decreased in shCBS-ASC52telo cells at day 0 and during adipocyte differentiation. In line with this, even though IRS-1 is important for lipid storage in adipose tissue [50], a recent study reported that IRS-1 knockdown in bone marrow mesenchymal stem cells increased their capacity to differentiate into adipocytes [51]. Current findings and these studies [45,46,48] suggest that cellular senescence and inflammation might promote a non-physiological increased adipocyte differentiation.

5. Conclusions

Altogether these findings demonstrated that permanent *CBS* gene KD in ASC52telo cells promotes a cellular senescence phenotype, characterized by increased cellular inflammation and oxidative stress, reduced cellular rejuvenation-related gene expression markers and a very increased adipogenic potential, which resulted in a non-physiological enhanced adipocyte differentiation with excessive lipid storage.

Author contributions

FC, JL, FO, NO-C and AL researched data; WR and JMF-R contributed to the discussion and reviewed the manuscript; JMMN researched data and wrote the manuscript.

Declaration of competing interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101668.

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