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ORIGINAL ARTICLE

Dysregulated lipid metabolism in hepatocellular carcinoma cancer stem cells

Alicia Bort[1] · Belén G. Sánchez[1] · Irene de Miguel[1] · Pedro A. Mateos-Gómez[1] · Inés Diaz-Laviada[1,2]

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Abstract

According to the stem cell theory for cancer, hepatocellular carcinomas are sustained by a group of cancer stem cells (CSCs) which are responsible for resistance to chemotherapy. In the present study we aimed to examine lipid metabolism in cancer stem cells induced by long-term treatment with sorafenib and its relationship with acquisition of a CSC-like phenotype. Two cell lines (HepG2SF1 and Huh7SF1) were generated by incubation with a step-wise increase of sorafenib concentrations for 10 months. These cell lines displayed stem-like characteristics like increase in the expression of ABCB1A, Nanog and Oct4 as well as an E-cadherin/N-cadherin switch. HepG2SF1 and Huh7SF1 cells showed intracellular accumulation of neutral lipids, assessed by flow cytometry and confocal microscopy. The exam of lipid metabolism revealed that HepG2SF1 and Huh7SF1 cells increased the expression of the enzymes involved in de novo lipid synthesis ATP–citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN) and that of the fatty acid transporter CD36. In addition, these CSC-like cells had enhanced expression of the lipogenic transcription factor SREBP1c. Analysis of the key metabolic sensor AMP-activated kinase (AMPK) demonstrated that both AMPK phosphorylation and levels were decreased in the CSC-like cells compared to their parental cells. Interestingly, transfection of HepG2SF1 and Huh7SF1 cells with AMPK, restored the levels of the lipogenic enzymes and SREBP1c and decreased the intracellular lipid accumulation. Furthermore, AMPK transfection decreased the stemness markers and inhibited the E-cadherin/N-cadherin switch. Targeting AMPK and lipid metabolism of hepatocellular cancer stem cells is a promising strategy to face stemness and chemotherapy resistance.

Keywords HepG2 · Huh7 · Sorafenib · cancer stem cells · Hepatocellular carcinoma · Lipid metabolism

Introduction

Hepatocellular carcinomas are sustained by the presence of cancer stem cells (CSCs), a subpopulation of cells within the tumor, responsible for self-renewing, differentiation and tumorigenicity and one of the major causes of therapy failure and recurrence [1, 2]. Current theories support the notion that CSC are highly adaptable and that stemness is a dynamic property of tumor cells [3]. Altered conditions within the tumor such as epigenetics, hypoxia, paracrine factors or chemotherapy, could trigger the gain of stemness [4]. Additionally, intracellular programs like the epithelial-mesenchymal transition could confer increased metastatic potential and therapeutic resistance to tumor cells [5]. Recent theories state that the metabolic changes in tumor cells can cause signaling dysregulation that can participate in tumor development, resistance, and recurrence and therefore targeting metabolic enzymes may be a strategy to fight against cancer. In fact, the metabolic reprogramming in cancer represents one of the major fields of research [6, 7]. This is especially relevant in hepatocellular carcinoma since the liver plays an integral role in the coordination of fuel homeostasis and it is the major site for storage and release of glucose and lipids. Recent research has suggested that the metabolic adaptations in CSCs are different from that in bulk cancer cells and quite similar to that in normal tissue

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✉ Inés Diaz-Laviada
ines.diazlaviada@uah.es

1 Biochemistry and Molecular Biology Unit, Department of System Biology, School of Medicine and Health Sciences, University of Alcalá, Ctra A-2, Km 32, 28871 Alcalá de Henares, Madrid, Spain

2 Chemical Research Institute "Andrés M. del Río" (IQAR), Alcalá University, 28871 Alcalá de Henares, Madrid, Spain

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stem cells [8, 9]. Studies of cancer stem cell metabolism have been mainly focused on and oxidative phosphorylation (OXPHOS) in the mitochondrion. Cancer cells use basically glycolysis instead of mitochondrial OXPHOS to obtain ATP even under oxygen-rich conditions, which is known as the "Warburg effect"[10]. However, CSCs seems to rely on mitochondrial respiration and oxidative phosphorylation for survival as well as a decreased dependence on glycolysis [11, 12]. Regarding lipid metabolism, there is a general consensus that cancer cells display increased de novo lipid synthesis and enhanced lipid accumulation compared to normal cells [13] in order to satisfy energy demand and biomass production. However, few studies have focused on the status of lipid metabolism and enzymes accompanying CSC differentiation. Therefore, a research on this line will shed light on mechanisms that sustain CSC growth and would potentially uncover key targets to impact CSC. The master metabolism regulatory protein is the AMP-activated kinase, AMPK, which activates catabolic routes while inhibits ATP-consuming pathways [14]. AMPK can regulate the activation of several transcription factors involved in growth and metabolism but its role on CSC reprogramming has yet to be investigated.

Here, we analyzed lipid content and lipid metabolic enzymes in a model of HCC CSCs induced by long term-treatment with sorafenib. We hypothesize that CSC can be reprogrammed by switching off energy metabolic pathways through AMPK up-regulation and that targeting key players of lipid metabolism might be a promising therapeutic strategy to fight against recurrence and chemoresistance in HCC.

Materials and methods

Materials

The compound A-769662 were purchased from Tocris Bioscience (Bristol, UK). BODIPY (493/503) were purchased to Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased to Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The human hepatocellular carcinoma HepG2 cell line was purchased from the American Type Culture Collection (ATCC HB-8065, Rockville, MD, USA). The human hepatoma cell line Huh7 was kindly provided by L. Boscá (Instituto de Investigaciones Biomédicas Alberto Sols, Madrid). Cells were routinely grown in DMEM/10% fetal bovine serum supplemented with 1% nonessential amino acids, 100 IU mL^{-1} penicillin G sodium, 100 μg mL^{-1} streptomycin sulphate, and 0.25 μg mL^{-1} amphotericin B (Invitrogen, Paisley, UK). Cell lines were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

To generate cancer stem-like cells, HepG2 and Huh7 cells were cultured continuously for 12 months with a step-wise increase in the sorafenib concentration (starting at 0.75 µm and increasing the concentration by 0.15 µm at each passage up to a final concentration of 8 µm). Surviving cells were selected and designated as HepG2SF1 and Huh7SF1 cells. HepG2 and Huh7 parental cells were cultured in parallel without sorafenib and served as controls.

Western blot

Proteins for Western blotting were isolated by lysing cells in lysis buffer [50 mM Tris pH 7.4, 0.8 M NaCl, 5 mM MgCl2, 0.1% Triton X-100] containing protease inhibitor and phosphatase inhibitor cocktail (Roche, Diagnostics; Mannheim, Germany), incubated on ice for 15 min and cleared by microcentrifugation. Twenty micrograms of total protein/lane were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane. Membranes were incubated overnight at 4 °C with the primary antibodies. After washing in TTBS, membranes were incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies for 2 h at room temperature. The immune complex was visualized with an ECL system (Cell Signaling Technology). Protein expression levels were quantified using Scion Image 4.0 (Scion Corporation, Chicago, Illinois, USA), normalized relative to the indicated housekeeping protein, and expressed as fold changes relative to the control treatment. Primary antibodies anti-p-AMPKα1-thr172 (#2531, working dilution 1:1000), p-ACC-ser79 (#3661, working dilution 1:1000), p-Akt-ser473 (#4060, working dilution 1:1000), p-ACLY-ser455 (#4331, working dilution 1:1000), p-SIRT-ser47 (#2314, working dilution 1:500) and the antibodies against the corresponding total forms and p-SRBPIC-ser372 (#9874, working dilution 1:1000), PPARγ (#2443, working dilution 1:1000), FAS (#3180, working dilution 1:2000), N-cadherin(#3195, working dilution 1:500) and E-cadherin (#14215, working dilution 1:1000) were obtained from Cell Signaling Technology (Danvers, MA, USA). PGC1α (NBP1-04676, working dilution 1:1000) was obtained from Novus (St. Louis, MO, USA) and CD36 (ab133625, working dilution 1:1000) was obtained from Abcam (Cambridge, UK). Peroxidase labeled secondary anti-mouse IgG (A9044, working dilution 1:5000) was from Sigma-Aldrich (St. Louis, MO, USA) and anti-rabbit IgG (#7074, working dilution 1:1000) was from Cell Signaling Technology (Danvers, MA, USA).

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RNA extraction and reverse transcription quantitative polymerase chain reaction

Total cellular RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (2–4 µg) underwent cDNA synthesis using SuperScript™ RT (Roche, Basel, Switzerland) according to the manufacturer's protocol. qPCR was performed in a 10 µL volume using SYBR-Green PCR Master Mix (Takara Bio, Inc., Kusatsu, Japan) on a 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocols. PCR amplification was carried out using the following primer sequences: Nanog-F 5′-TTTGTGGGCCTG AAGAAAACT-3′, Nanog-R 5′-AGGGCTGTCCTGAAT AAGCAG-3′; Oct4-F 5′-GACAGGGGGAGGGGAGGA GCTAGG-3′, Oct4-R 5′-CTTCCCTCCAACCAGTTGCCC CAAAC-3′; and ABCB1A-F 5′-TTGCTGCTTACATTC AGGTTTCA-3′, ABCB1A-R 5′-AGCCTATCTCCTGTC GCATTA-3′.

Quantification of intracellular lipid accumulation by flow cytometry

A total of 3×10^5 HCC cells was seeded into 6-well plates. HCC cells were stained for intracellular neutral lipids with BODIPY (493/503) at 5 ng/mL for 30 min. The cells were then harvested in 0.5% trypsin, collected and centrifuged at 430×*g* for 5 min at 4 °C. Subsequently, the cells were resuspended in 300 µL ice-cold PBS and analyzed on a FACSCalibur flow cytometry system (BD Biosciences, San Jose, CA, USA) using CYFLOGIC software V1.2.1 (Perttu Terho, Mika Korkeamaki, CyFlo Ltd., Turku, Finland). A total of 10^4 events were collected for each sample. Experiments were performed at least in triplicate. Mean fluorescence values of the unstimulated controls were set to 100%.

Fluorescence microscopy

Cells were seeded on coverslips, washed on serum-free EMEM medium and stained with BODIPY (493/503) at 5 ng/mL, for 30 min. The cells were fixed in 4% paraformaldehyde in PBS. Coverslips were then mounted with Mowiol mounting medium (Sigma-Aldrich). Imaging was performed with a Leica DM100 microscope with LAS V4.6 IMAGING software using a 65 × oil objective.

Transient transfections

Plasmids encoding the full-length human AMPK-α1, AMPK-β1 and AMPK-γ1 were kindly provided by G. Hardie (University of Dundee, UK). Sensitive and resistant HepG2 and Huh7 cells were co-transfected with 4 µg recombinant α1 (pcDNA5-FRT α1-Flag), β1 (pCMV β1-untagged) and γ1 WT (pcDNA5-Flpln-T10 γ1 WT-Flag) plasmids using 6 µL Lipofectamine 3000 (Thermo Fisher) seeded into 6-well plates. After 48 h of transfection, the cells were collected and assayed the protein expression by western blotting.

Statistical analysis

Statistical significance was estimated with Graphpad 6.0 (La Jolla, CA, USA) software using 1-way or 2-way ANOVA and Tukey's multiple comparison test or the unpaired Student's *t*-test when indicated. Data are presented as the mean ± SD.

Results

To induce hepatocellular carcinoma cancer stem cells, we generated two cell lines by long-term treatment with increasing concentrations of sorafenib as described in the methods section. Those cells, named HepG2SF1 and Huh7SF1, had a different cell morphology compared with their parental cells (Fig. 1a) as well as enhanced clonogenicity, differentiation ability and tumorigenic capacity as previously described [15]. qPCR analysis demonstrated CSCs features like higher expression of the efflux transporter ABCB1A and high levels of the stemness transcription factors Oct4 and Nanog which are characteristics of the stem cells [16–18] (Fig. 1b). In addition, HepG2SF1 and Huh7SF1 cells, decreased the expression of E-cadherin while increased the expression of N-cadherin a hallmark of endothelial mesenchymal transition and a feature of aggressive tumors (Fig. 1c).

The phosphoinositide 3-kinase and AKT (PI3K–AKT) signaling pathway is essential for survival and progression of hepatocellular cancer stem cells [19, 20]. It has also been implicated in the rewiring of specific metabolic processes, including glycogen biosynthesis and degradation, glycolysis through phosphorylation of the transcription factor FOXO1 and protein synthesis through the phosphorylation of mTOR. In addition, AKT regulates neutral lipid metabolism through sterol regulatory element-binding protein 1c (SREBP1c), which increases the expression of enzymes involved in fatty acid production and accumulation [21]. We therefore checked the status of Akt in the sorafenib-induced cancer stem-like cells. Western blot analysis revealed that phosphorylation of AKT was upregulated in HepG2SF1 and Huh7SF1 cells compared with their parental cells (Fig. 1c), suggesting that these cancer stem-like cells had an increase in the anabolic routes.

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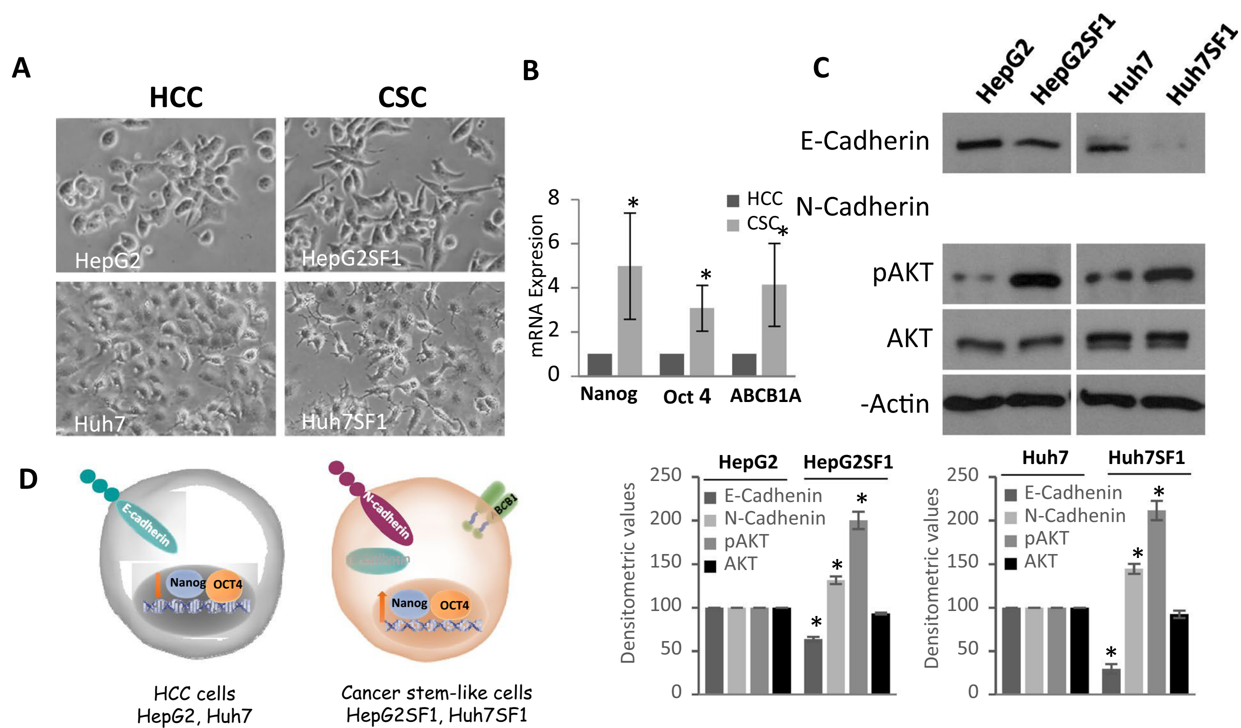


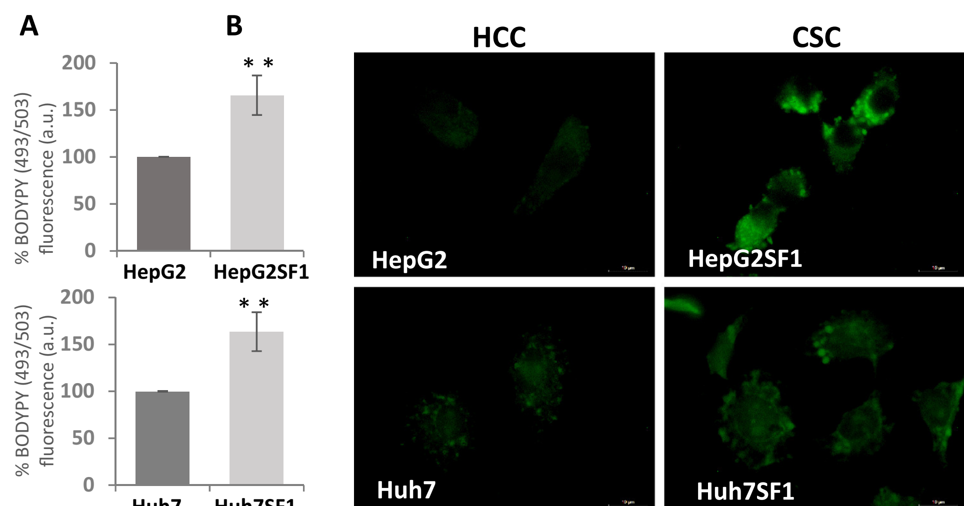
Fig. 1 .

Lipid metabolism is dysregulated in cancer stem-like cells

In order to investigate the lipid metabolism, we first examined neutral lipid content by flow cytometry in the cancer stem-like cells. As observed in Fig. 2a, HepG2SF1 and Huh7SF1 cells showed higher lipid content compared with their parental cells (HepG2 and Huh7). Neutral lipids as TAGs and cholesterol esters are accumulated in highly dynamic cytoplasmic organelles named lipid droplets. Fluorescence microscopy analyses revealed that the cancer stem-like cells had increased and enlarged cytoplasmic lipid droplets, confirming the lipid accumulation in those cells (Fig. 2b).

As exposed above, sterol regulatory element binding proteins (SREBPs) are transcription factors that play a central role in the synthesis of fatty acids, triglycerides and cholesterol. Specifically, SREBP1c governs de novo lipogenesis by stimulating the expression of enzymes involved in triglyceride and fatty acid synthesis. In addition to proteolytic cleavage, SREBP1c activity can be regulated by phosphorylation in different residues. We

Fig. 2 .



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257 then, analyzed the phosphorylation of SREBP1c in Ser 372 which suppresses its activity [22, 23]. Figure 3a shows that SREBP1c phosphorylation is dramatically reduced in the cancer stem-like cells (HepG2SF1 and Huh7SF1) pointing to the activation of this transcription factor. To confirm this finding, its target lipogenic enzymes, ATP–citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), which work consecutively to synthetize fatty acids, were determined by Western blot. As shown in Fig. 3a, either the activating phosphorylation at S455 and the levels of ACLY were increased in both cancer stem-like cells. In line with this, the inhibitory phosphorylation of ACC was decreased, indicating ACC activation. Likewise, total levels of FASN were higher in CSCs (Fig. 3a). These results indicated that de novo fatty acids synthesis was increased in the cancer stem-like cells compared with their parental cells. 273

274 In addition to *de novo* lipogenesis, extracellular lipid uptake was also increased contributing to LDs accumulation. In mammalian cells, fatty acids can be obtained through direct exogenous uptake by specialized transporters required to facilitate efficient flux across the plasma membrane like the fatty acid translocase CD36 [24]. Therefore, we determined the levels of CD36 and found that CSC differentiation significantly promoted the expression of CD36 (Fig. 3b), indicating that both *de novo* biosynthesis and the uptake of fatty acids were increased in the cancer stem-like cells. 283

284 Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), initially described as a peroxisome proliferator-activated receptor gamma (PPARγ) interacting 286

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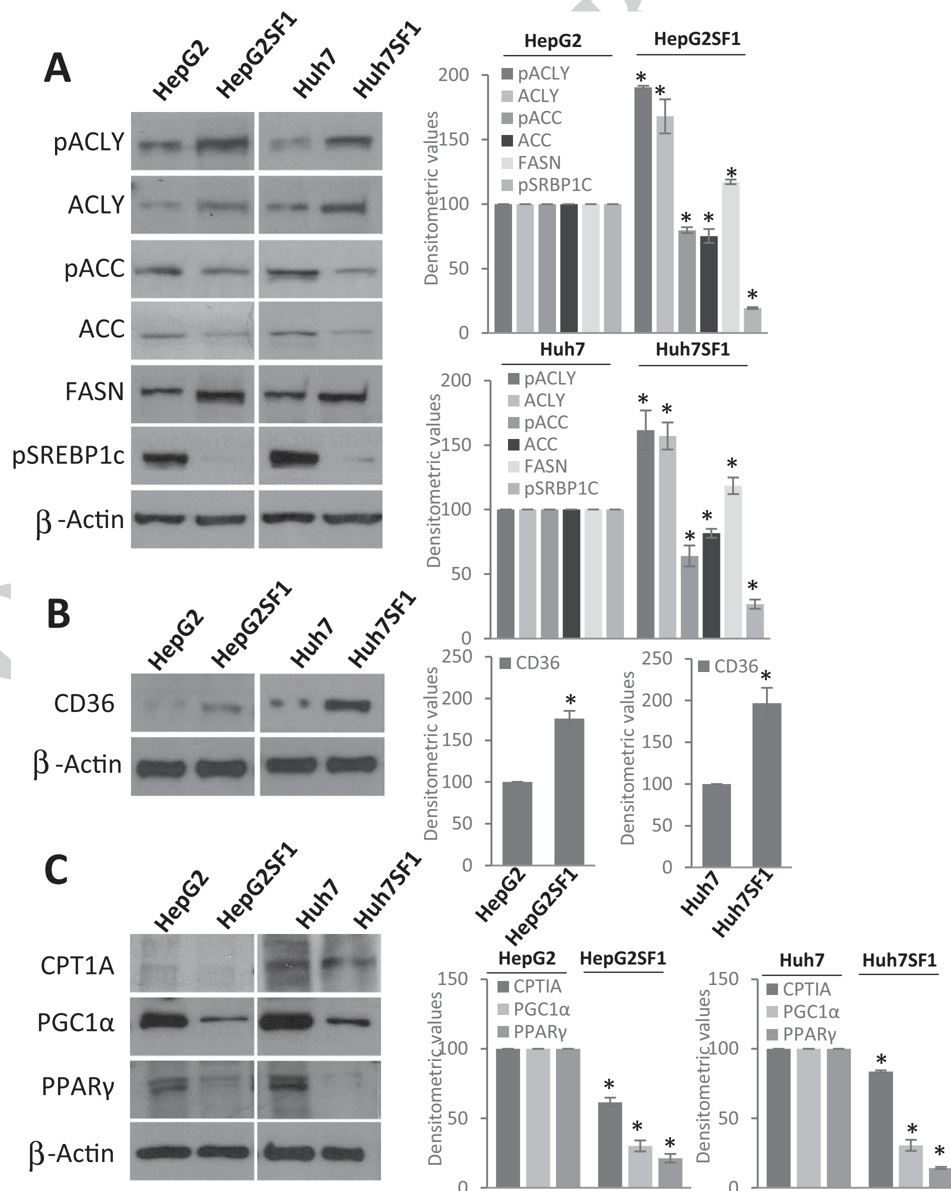
Fig. 3 .



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287 protein, is a transcription cofactor that interacts with numerous transcription factors and has been shown to be a potent activator of mitochondrial biogenesis and fatty acid oxidation. Its activation increases the expression of carnitine palmitoytransterase-1 (CPT-1) which catalyzes the transfer of long-chain fatty acids into mitochondria and is the rate-limiting enzyme of fatty acid β-oxidation. PPARγ signaling has been shown to regulate lipogenesis and lipid accumulation in liver [25]. As shown in Fig. 3c, levels of CPT1A as well as PGC1α and PPARγ were decreased in HepG2SF1 and Huh7SF1 cells compared with HepG2 and Huh7 cells. Since PPARγ activates the expression of many genes governing fatty acid β-oxidation, these results suggested that β-oxidation might be inhibited in the cancer stem-like cells.

These results indicated that the enhanced lipid droplets accumulation observed in the cancer stem-like cells was due, at least in part, by the increase in lipid uptake, *de novo* biosynthesis and by a decrease in β-oxidation.

Increased lipogenesis in the cancer stem-like cells relies on AMPK inhibition

The master intracellular energy-sensing molecules are adenosine monophosphate (AMP)-activated protein kinase (AMPK) and the histone/protein deacetylase Sirtuin 1 (SIRT1) that function as key regulators of metabolism. SIRT1 is a nicotinamide adenine dinucleotide-dependent deacetylase activated by calorie restriction [26, 27]. In recent years, SIRT1 has emerged as a pivotal molecule controlling the pathways of hepatic lipid metabolism by modifying the acetylation status of several target molecules, including PGC1α. Deacetylation of PGC-1α by SIRT1 is tightly linked with enhanced PGC-1α transcriptional activation. In contrast, SIRT1 regulates SREBP-1c activity by inhibiting its transcriptional activity resulting in downregulation of its target lipogenic genes [28]. AMPK in turn, is activated when the ratio AMP/ATP increases, and its activation restores energy balance by activating catabolic pathways while inhibiting anabolic routes. Accumulating evidences suggest that SIRT1 and AMPK regulate each other [29]. SIRT1 may promote AMPK phosphorylation through activation of its upstream kinase LKB1 while AMPK increased cellular NAD^+ levels, which subsequently activates SIRT1 [30].

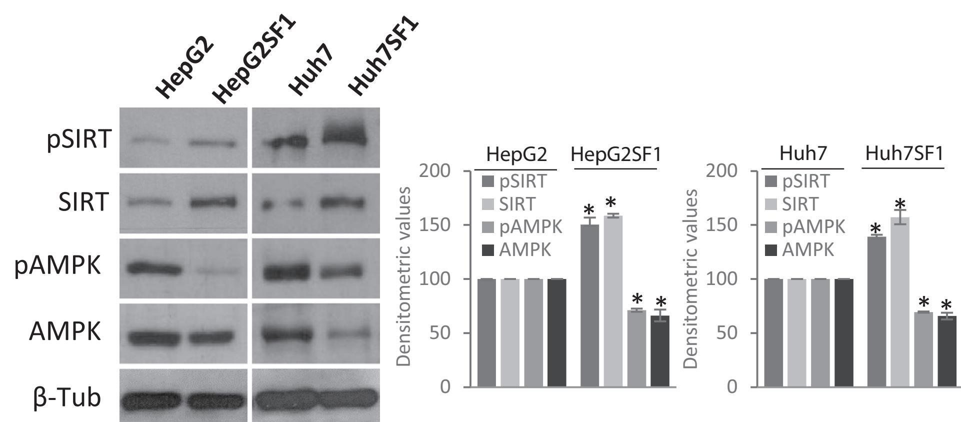
To address the involvement of these proteins on the metabolic changes observed in the cancer stem-like cells, we determined phosphorylation and total levels by Western blot. Results in Fig. 4 showed that while phosphorylation in S47 and total levels of SIRT1 were increased in HepG2SF1 and Huh7SF1 CSCs compared to their parental cells, whereas the phosphorylation in T172 and levels of AMPK were diminished. The decrease of AMPK levels could explain the lipid accumulation observed in the cancer stem-like cells since AMPK phosphorylates and inhibits SREBP1c and enzymes involved in lipid biosynthesis whilst activates β-oxidation.

To corroborate this idea, we transfected HCC and the cancer stem-like cells with a plasmid coding for AMPK and subsequently, checked the status of the lipogenic enzymes and that of the transcription factor SREBP1c. Overexpression of AMPK in the cancer stem-like cells (Supplementary Fig. 1) inhibited the activation of ACLY, ACC and FAS observed and decreased the inhibitory phosphorylation of SREBP1c (Fig. 5a). In addition, levels of the lipid transporter CD36 were restored in the cancer stem-like cells with the transfection of AMPK (Fig. 5b). Moreover, CPT1A, PGC1α and PPARγ increased in AMPK-transfected cells, suggesting a recovery of β-oxidation.

Restoration of intracellular lipid levels correlates with a decrease in epithelial mesenchymal transition and inhibits CSCs features

We next examined the effects of AMPK transfection on lipid accumulation and on the expression of stem markers. To analyze the anti-hyperlipidemic effects of AMPK on the cancer stem-like cells, intracellular neutral lipids

Fig. 4 .



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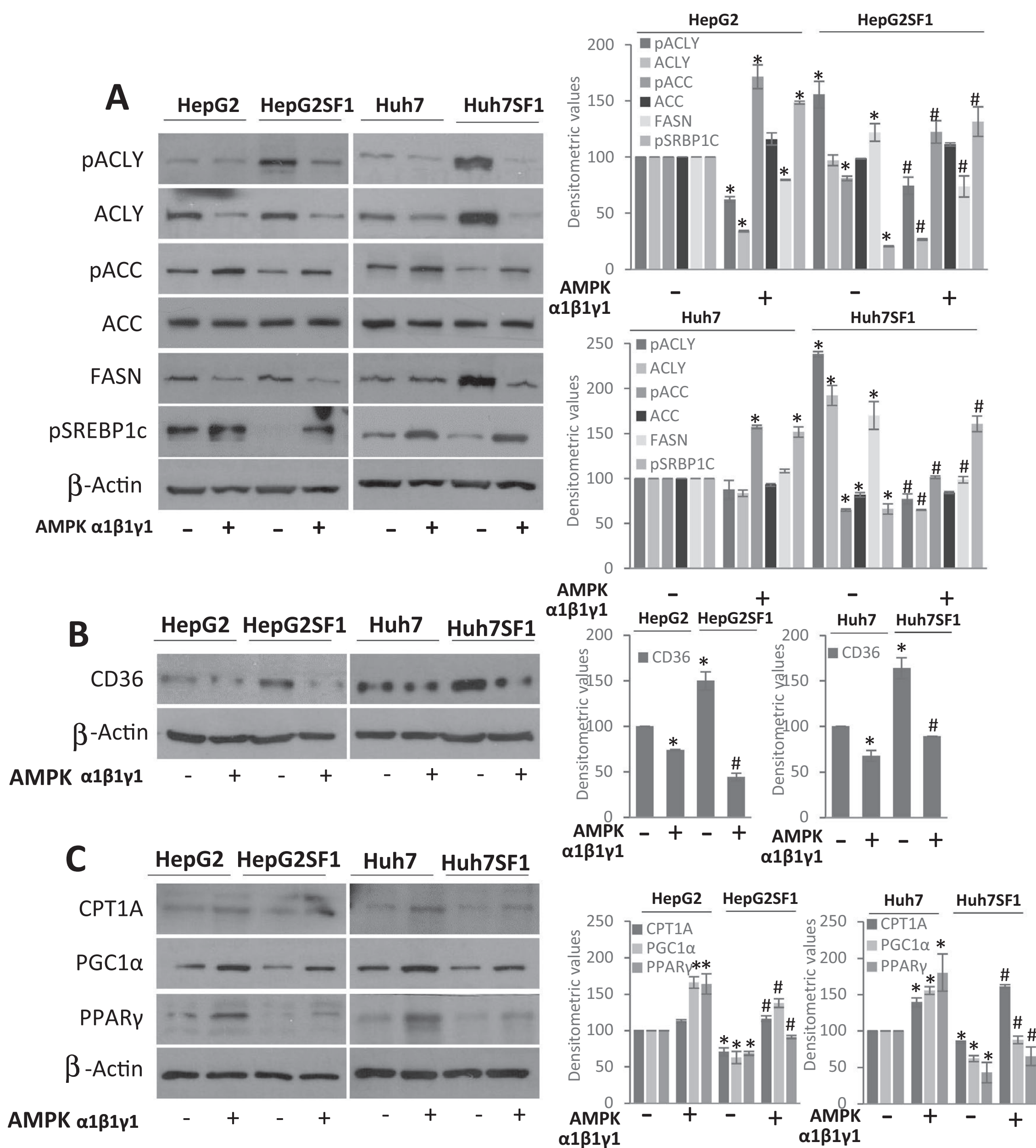

Fig. 5 .

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were determined in AMPK-transfected cells. As shown in Fig. 6a, overexpression of AMPK reduced lipid content in HepG2SF1 and Huh7SF1 cells. Accordingly, pharmacological activation of AMPK by A-769662 induced a decrease in the level of neutral lipids (Fig. 6a). The diminution of intracellular lipids in AMPK transfected cells and in A-769662 treated cells could also be observed by fluorescence microscopy (Fig. 6b).

Next, we analyzed the expression of stem cell markers in this new situation. As observed in Fig. 7a, the expression of Nanog, Oct4 and ABCB1A decreased in CSCs transfected with AMPK. Notably, transfection with AMPK inhibited the E-cadherin N-cadherin switch observed in HepG2SF1 and Huh7SF1 cells (Fig. 7b).

These results indicate that high lipid accumulation correlates with the expression of stem features and that targeting AMPK can reduce lipid content and stem cell markers as well as epithelial mesenchymal transition.

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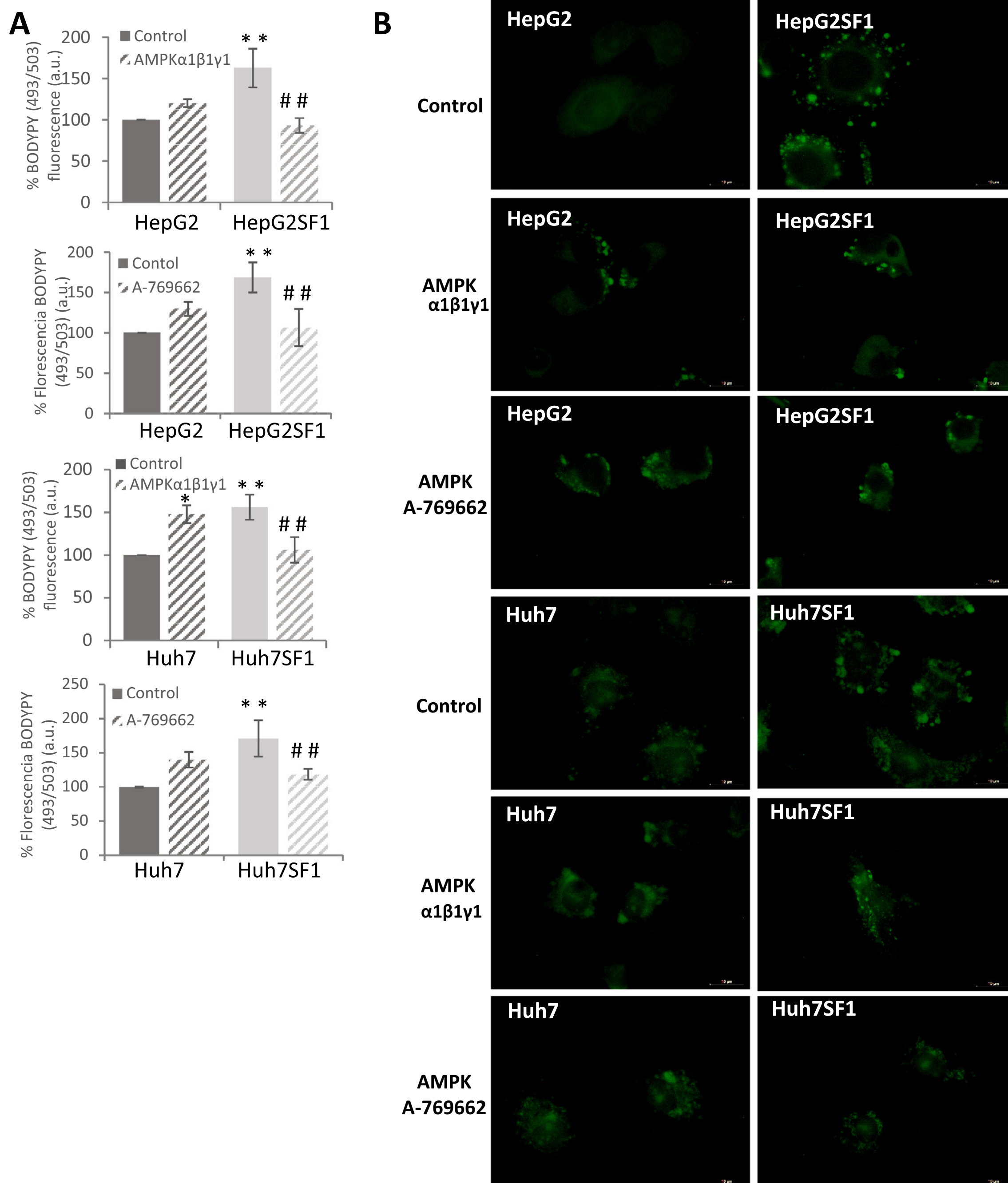

Fig. 6 .

Discussion

The importance of altered fatty acid metabolism in hepatocellular carcinoma has received renewed interest since altered lipid homeostasis has been identified as a contributing factor to hepatocellular carcinoma, and liver cancer cells rely upon lipid metabolism for fulfilling their biomass and energy demands [31]. In addition, emerging evidence indicates that like bulk cancer cells, cancer stem cells also exploit aberrant lipid metabolism to boost their survival [32]. In this context, we have shown in this study, in a model of hepatocellular carcinoma cancer stem cells, that CSCs depended on lipids to maintain their stemness and that decreasing lipid content by targeting AMPK, diminished stem cell markers as well as E-cadherin/N-cadherin switch.

After several months of treatment with sorafenib, HCC cells were enriched in CSCs, as demonstrated by the high expression of stem markers as Nanog, Oct4 and ABCB1A.

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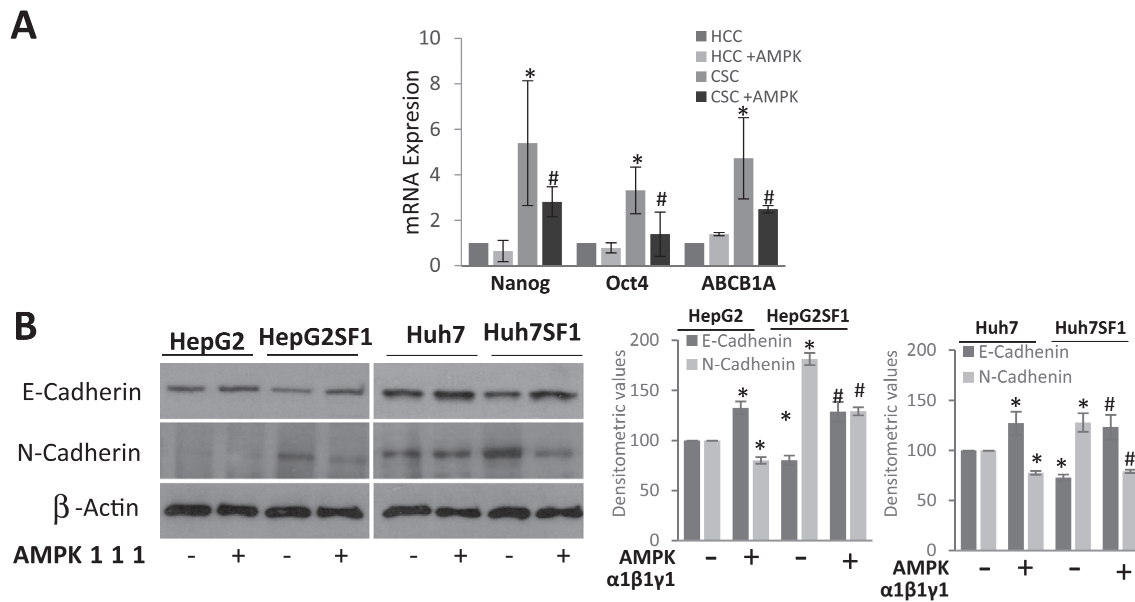

Fig. 7 .

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These cells also exhibited a decrease in the expression of the adhesion molecule E-cadherin as well as an increase in N-cadherin, fact that indicated epithelial to mesenchymal transition. Interestingly, these cancer stem-like cells had enhanced intracellular lipids accumulation. Enzyme phosphorylation and levels analysis provided evidence for an upregulation of key enzymes of fatty acid synthesis, such as ATP-citrate lyase and acetyl-CoA carboxylase in the cancer stem-like cells and a strong expression of the lipogenic transcription factor SREBP1c. In addition, gene expression of CD36, responsible for inducing lipid uptake and catabolism, was increased in the cancer stem-like cells. By contrast, CPT-1, the step-limiting enzyme of fatty acid β-oxidation and the transcription modulators PGC1α and PPARγ involved in mitochondrial biogenesis, were found to be suppressed in CSCs (Fig. 8). Our results are in good agreement with recent data describing enhanced lipogenesis in CSCs of different origins. For instance, in melanosphere-derived CSCs, increased lipid accumulation and SREBP1 overexpression was observed and correlated with AMPK inhibition and lower autophagy activity [33]. A higher lipid content and higher rate of de novo lipogenesis has been observed as well in colon cancer CSCs and glioma CSCs (reviewed in [32]). Data from TGCA database [34] reveal significant alterations in genes likely to result in metabolic reprogramming in HCC. One of the lipogenic proteins up-regulated is FAS, which is enhanced in 13.48% of HCC cases and negatively correlates with AMPK phosphorylation [35]. Other metabolic genes overexpressed in the HCC cases analyzed in TCGA, are ACLY (2.7%), SREBP1 (2.43%) and CD36 (3.23%) [34]. Previous research demonstrated that highly metastatic breast cancer cells had an increased β-oxidation rate and a diminished expression of CD36 with respected to low metastatic cells, indicating that metastatic cells depend upon intracellular fatty acids for their metabolism [36]. In our model, the stem-like cells showed an increase in CD36 expression and a decrease in β-oxidation involved proteins. According to this, our lipid profile correlates to cells in a low metastatic grade. However, we observe an increase in the mesenchymal marker N-cadherin in the stem-like cells suggesting that our stem-like cells are shifting from epithelial to mesenchymal phenotype. In line with this, recent research indicates that CD36 promotes the epithelial-mesenchymal transition and metastasis in cervical cancer [37] and in hepatocellular carcinoma [38]. Data derived from the TCGA liver cancer dataset [34] and further confirmation in HCC samples [38] revealed that elevated expression of fatty acid uptake proteins including CD36, was associated with EMT progression in HCC. Moreover, elevated FFAs enhance EMT rates in the liver cancer cells [38]. In accordance, ligands of CD36 enhanced self-renewal and proliferation of glioblastoma stem cells which express high levels of CD36 in comparison with tumor non-stem cells, in which CD36 ligands did not have effect [39]. The observed increase in CD36 and the dependence from exogenous fatty acids could be directly associated with the activation of intracellular programs as fatty acids may be used to generate lipid signaling mediators. For instance, in HCC cells elevated palmitate uptake via CD36, -triggered TGF-beta and Wnt signaling pathways independently of lipogenic and inflammatory networks [38].

Although the elevated levels of Sirt1 found in our study correlated with previous data showing increased

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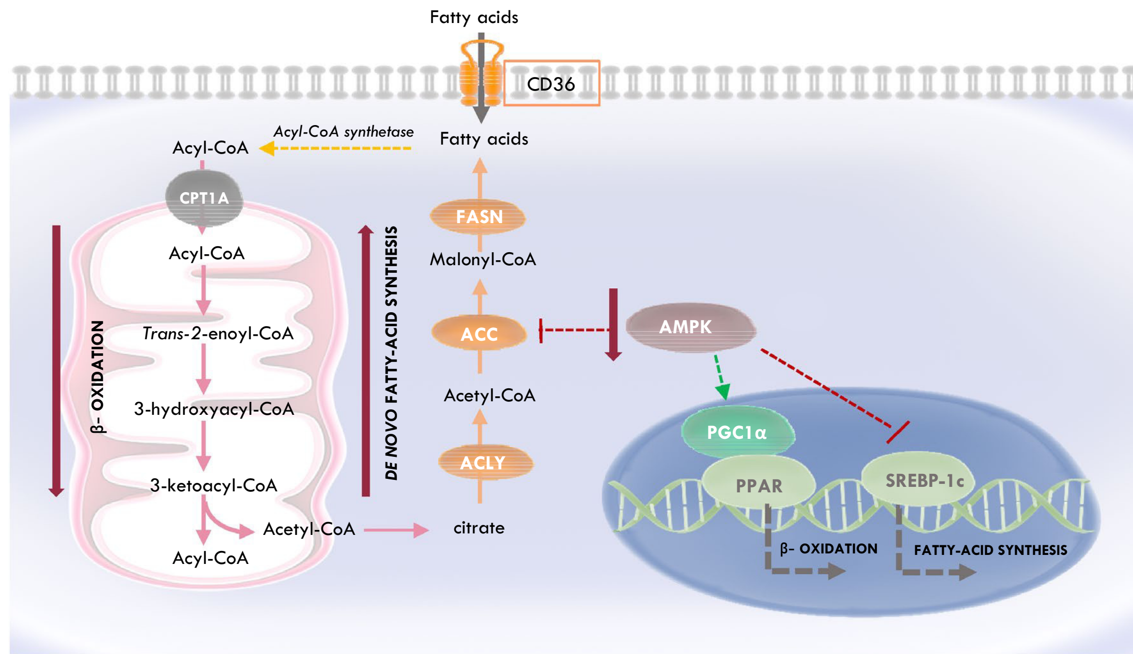

Fig. 8 .

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expression of Sirt1 in patient-derived CSCs [40], did not explain the activation of fatty acid synthesis or the lipid accumulation observed. However, the down-regulation of AMPK produced in HepG2SF1 and Huh7SF1 cells, could offer an explanation for the dysregulated lipid metabolism. The fact that overexpression of AMPK (or its activation by the compound A-967662) decreased the lipid accumulation in CSCs and reestablished the levels of proteins involved in lipid homeostasis, confirmed the role of AMPK in HepG2SF1 and Huh7SF1 CSCs lipid metabolism.

The importance of AMPK as a therapeutic target in cancer is beginning to be unveiled since recent data suggest a greater benefit of metformin, an anti-diabetic drug which can indirectly activate AMPK, as anticancer therapy, although these findings vary depending on the intrinsic properties of the tumor, as well as the systemic physiology of patients. In line with our results it has been demonstrated that metformin suppressed CD133 expression in HCC cell lines mainly via AMPK signaling and independently of the mammalian target of rapamycin (mTOR) [41]. Moreover, it has been described that metformin suppress tumor-initiating hepatocellular carcinoma cells [42] and reverses multidrug resistance [27]. In osteosarcoma stem cells, metformin suppressed tumorigenicity and self-renewal ability [43]. In addition, AMPK decreased prostate cancer cell stemness by inducing Nanog phosphorylation and degradation [44]. In line with this, activation of AMPK reduced cell growth in human prostate cancer cells by inhibiting de novo lipogenesis [45].

Alterations on lipid metabolism could not only satisfy the energy demands and biomass production of CSCs but could also contribute to maintain their stemness properties. Our results showed that targeting lipid accumulation and biosynthesis decreased stem cells markers and reversed the E-cadherin/N-cadherin switch, supporting the notion that lipid metabolism sustained stemness in our model of CSCs. It is worthy to note that in the human HCC cases analyzed in the TCPA dataset, AMPK and ACC expression positively correlates with E-cadherin and negatively correlates with N-cadherin [35], which is in agreement with our observations in the cell lines. Nevertheless, the crucial question then was how metabolic status can exert an influence over the transcriptional factors that maintain the self-renewal and differentiation capacities of cancer stem cells. We have seen changes in SREBP1c as well as in PCG1α and PPARγ. PCG1α has a powerful transcriptional activity by interacting with many different transcription factors including, among others, PPARγ, PPARα, estrogen receptor–related α (ERRα), FoxO1, hepatocyte nuclear factor 4α (HNF4α), and nuclear respiratory factor 1 (NRF1) which could govern cell differentiation. The PPARγ transcription factor controls the expression of key regulators of inflammation, and also PPARγ ligands have been shown to have an anti-proliferative and antitumor effects on a variety of cancers [46, 47]. In brain tumor stem cells, PPARγ agonists inhibited the expression of stemness and increased differentiation genes [48]. Treatment of brain CSCs with the PPARγ agonist ciglitazone, decreased the expression of CD133 and

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induced differentiation of cells [48]. In addition, HNF4α regulates genes involved in hepatic progenitors differentiation [49] and FoxO1 modulates differentiation of chondrocytes [50]. All this transcription factors could link the cell metabolic status with cell fate. Other molecules involved in lipid homeostasis could also exert a role in proliferation or differentiation. For instance, it has been demonstrated that overexpression of CD36 is a requisite for tumor metastasis [51]. In addition, in lung cancer cells, knockdown of ACLY inhibits epithelial–mesenchymal transition and tumorsphere formation [52], highlighting the central role of lipid regulating enzymes in tumor aggressiveness. Moreover, a role of FASN and other enzymes involved in lipid metabolism on the regulation of signal transduction pathways and their involvement in the fate of CSCs have gained particular interest [53]. Future research is needed to shed light to this interesting point.

Our results illuminate the role of lipid biosynthesis in the maintenance of stemness. The finding that AMPK activation or overexpression could reprogram cancer stem cells and reverse epithelial-mesenchymal transition, provides new tools to restrain cancer aggressiveness. It is well known that CSC phenotype is an underlying mechanism of sorafenib resistance in HCC. Since we have generated CSCs from cells adapted to grown with the chemotherapeutic sorafenib, our results are a promising strategy to face therapy resistance in cancer.

Authors have consulted data generated by the TCGA Research Network: https://www.cancer.gov/tcga for discussion.

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Author contributions IDL conceived and supervised the study; AB and IDL designed experiments; BS, IdeM and AB performed experiments; BS and PMG analyzed data; IDL wrote the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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