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ORIGINAL ARTICLE

Cholangiocarcinoma progression depends on the uptake and metabolization of extracellular lipids

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Abstract

Background and Aims: Cholangiocarcinoma (CCA) includes a heterogeneous group of biliary cancers with a dismal prognosis. We investigated if lipid metabolism is disrupted in CCA and its role in tumor proliferation.

Abbreviations: ACADM, acyl-CoA dehydrogenase medium chain; CCA, cholangiocarcinoma; CE, cholesteryl ester; CFSE, carboxyfluorescein succinimidyl ester; CL, cholesterol; eCCA, extrahepatic cholangiocarcinoma; FA, fatty acid; FABP, fatty acid–binding protein; FAO, fatty acid oxidation; FASN, fatty acid synthase; iCCA, intrahepatic cholangiocarcinoma; LPL, lipoprotein lipase; NHC, normal human cholangiocyte; Nicd1, NOTCH1 intracellular cytoplasmic domain; PC, phosphatidylcholine; PCNA, proliferating cell nuclear antigen; PG, prostaglandin; TG, triglyceride.

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Approach and Results: The in vitro and in vivo tumorigenic capacity of five human CCA cell lines was analyzed. Proteome, lipid content, and metabolic fluxes were evaluated in CCA cells and compared with normal human cholangiocytes (NHC). The *Akt1*/NOTCH1 intracellular cytoplasmic domain (*Nicd1*)-driven CCA mouse model was also evaluated. The proteome of CCA cells was enriched in pathways involved in lipid and lipoprotein metabolism. The EGI1 CCA cell line presented the highest tumorigenic capacity. Metabolic studies in high (EGI1) versus low (HUCCT1) proliferative CCA cells in vitro showed that both EGI1 and HUCCT1 incorporated more fatty acids (FA) than NHC, leading to increased triglyceride storage, also observed in *Akt1*/*Nicd1*-driven CCA mouse model. The highly proliferative EGI1 CCA cells showed greater uptake of very-low-density and HDLs than NHC and HUCCT1 CCA cells and increased cholesteryl ester content. The FA oxidation (FAO) and related proteome enrichment were specifically up-regulated in EGI1, and consequently, pharmacological blockade of FAO induced more pronounced inhibition of their tumorigenic capacity compared with HUCCT1. The expression of acyl-CoA dehydrogenase *ACADM*, the first enzyme involved in FAO, was increased in human CCA tissues and correlated with the proliferation marker *PCNA*.

Conclusions: Highly proliferative human CCA cells rely on lipid and lipoprotein uptake to fuel FA catabolism, suggesting that inhibition of FAO and/or lipid uptake could represent a therapeutic strategy for this CCA subclass.

INTRODUCTION

Cholangiocarcinoma (CCA) includes a diverse group of biliary malignant tumors and represents the second most common primary liver cancer.[1] According to the anatomical location, these cancers are classified as intrahepatic (iCCA), perihilar (pCCA), or distal (dCCA) tumors, although pCCA and dCCA were previously considered as extrahepatic (eCCA).[2] CCA is a rare cancer globally (0.3–6 cases per 100,000 people); however, its incidence and associated mortality have been significantly rising over the last few decades.[1,2] Currently, the only treatment with curative intent is the surgical resection of the tumor. Nevertheless, patients with CCA are usually asymptomatic until late, unresectable stages of the disease. This highlights the need to

determine risk factors, implement screening policies, and investigate accurate diagnostic methods and therapeutic strategies for this cancer. However, the high heterogeneity of CCAs extremely compromises the finding of common and effective treatments for all patients but opens a door to precision-targeted therapies.[2] CCA subclasses differ not only on their anatomical location but also on their histological features,[3,4] risk factors,[5] putative cell of origin,[6] and mutational landscape.[7–9] In this sense, two different molecular subclasses (i.e., proliferative or inflammatory) have been proposed for iCCA based on gene expression profiling.[7] A comparable genomic and molecular profiling for eCCA was also performed, proposing four biological subclasses for eCCA (i.e., proliferative, mesenchymal, metabolic, or immune).[8]

Metabolic reprogramming is a hallmark of cancer, and targeting metabolism has been proposed for cancer therapy.[10] Cancer cells need a great amount of energy and biomaterials for the abnormal cell growth and division that characterizes the disease. Therefore, they adapt their mechanisms of nutrient uptake, energy production, and biosynthesis of complex molecules to suit these demands.[11] Genetic, epigenetic, and molecular alterations in tumor cells modulate their metabolic state. Moreover, differences in the microenvironment and the supply of nutrients and oxygen also contribute to the metabolic heterogeneity of the tumor.[11] The cancer-specific metabolic adaptability also contributes to the spread and survival to treatments.[11] Thus, there is a need to identify specific metabolic rewiring of each cancer type. Regarding lipid metabolism, cancer cells require higher amount of lipids mainly for the generation of new membranes and for energy production. Consequently, most cancer types are characterized by increased *de novo* lipogenesis through overexpression of lipogenic enzymes, such as fatty acid synthase (FASN) or acetyl-CoA carboxylase (ACC). However, they can also proliferate relying on extracellular lipid sources.[11]

Little is known about the rewiring of lipid metabolism in CCA.[12,13] However, previous data showed a remarkable downregulation of FASN and ACC levels in human iCCA tumors compared with surrounding nontumorous tissue. *Fasn* expression was found also diminished in different mouse CCA models.[14–16] Consistently, *Fasn* silencing did not abrogate CCA development in the *Akt1*/NOTCH1 intracellular cytoplasmic domain (*Nicd1*) and *Akt1/Nras* models.[16] Therefore, although still unclear, these data suggest that the uptake of exogenous lipids, rather than the *de novo* biosynthesis, should preferentially play a predominant role in CCA progression.

The main aim of the present study was to gain further insights into the identification of the source, metabolic fate, and role of lipids in the proliferation of CCA cells.

MATERIALS AND METHODS

Animal models

To promote the generation of iCCA in mice, 8-week old male wild-type mice (mixed background C57BL/6J and 129/Sv) were subjected to hydrodynamic tail vein injection of plasmids, as described previously and detailed in Supporting Materials and Methods.[14,15]

For the xenograft animal models, CCA cells were subcutaneously injected in flanks of immunodeficient CD1 nude mice as described in Supporting Materials and Methods. Etomoxir (30 mg/kg, MedChemExpress) was administered every 2 days for 32 days as described in Supporting Materials and Methods.

The Ethics Committee of the University of the Basque Country (UPV/EHU) (CEEA M20/2019/60) or the Biodonostia Health Research Institute (CEEA21-10/OH-21-027) approved all the procedures.

Human cohorts

Gene expression microarray data from the Copenhagen cohort including 104 CCA surgical specimens,[17] and the Jusakul cohort including 118 CCA surgical specimens[18] were used for gene expression analysis and correlation. Besides, 23 pairs of matched tumor stroma and epithelium were obtained.[17] 182 tissue samples from human iCCAs collected at the Medical University of Regensburg (Regensburg, Germany) were used for the immunohistochemistry studies. Institutional Review Board approval was provided by the local Ethical Committee of the Medical University of Regensburg (approval # 17-1015-101) in compliance with the Helsinki Declaration. Informed consent was obtained from all individuals. No donor organs were obtained from executed prisoners or other institutionalized persons.

Cells

Human CCA cell lines (EGI1, TFK1, WITT, HUCCT1 and TKKK) were used in the experiments, as well as primary cultures of normal human cholangiocytes (NHC; i.e., NHC2, NHC3, NHC-SS, and NHC324). NHCs were isolated from normal liver tissue specimens as previously described.[19–21] Additional information has been added in Supporting Materials and Methods.

Metabolic fluxes

Oleate and palmitate uptake

The analysis of oleate and palmitate uptake was performed in vitro, in the EGI1 and HUCCT1 CCA cell lines,

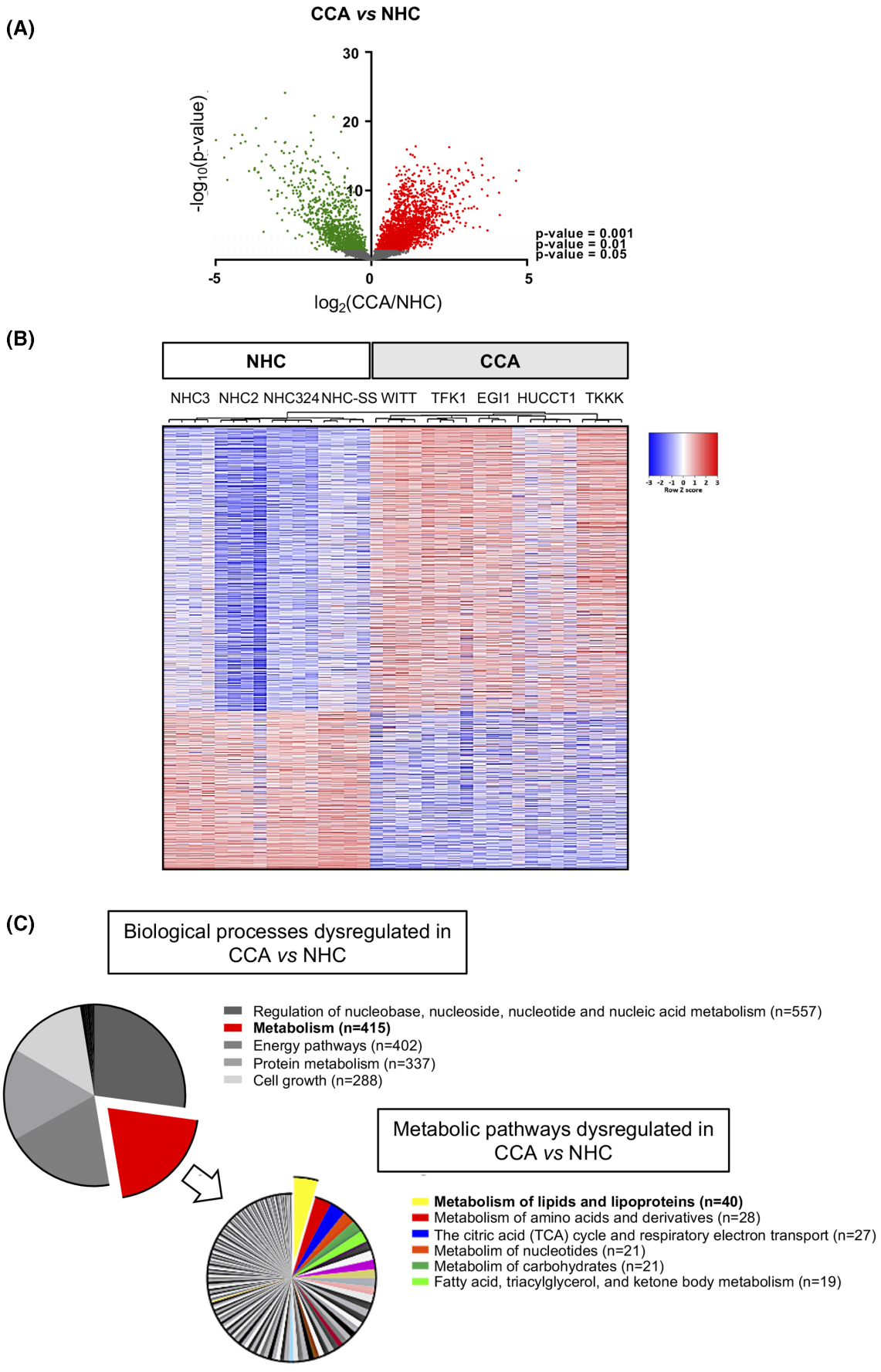

(A)
CCA vs NHC
-log10(p-value)
30
20
10
-5
0
5
log2(CCA/NHC)
p-value = 0.001
p-value = 0.01
p-value = 0.05
(B)
NHC
CCA
NHC3 NHC2 NHC324 NHC-SS WITT TFK1 EGI1 HUCCT1 TKKK
Row Z score
(C)
Biological processes dysregulated in CCA vs NHC
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (n=557)
Metabolism (n=415)
Energy pathways (n=402)
Protein metabolism (n=337)
Cell growth (n=288)
Metabolic pathways dysregulated in CCA vs NHC
Metabolism of lipids and lipoproteins (n=40)
Metabolism of amino acids and derivatives (n=28)
The citric acid (TCA) cycle and respiratory electron transport (n=27)
Metabolim of nucleotides (n=21)
Metabolim of carbohydrates (n=21)
Fatty acid, triacylglycerol, and ketone body metabolism (n=19)

FIGURE 1 The profile of proteins involved in lipid metabolism is dysregulated in CCA. (A) Volcano plot showing differentially expressed proteins between CCA and NHCs. (B) Heatmap showing differentially expressed proteins between CCA and NHCs. (C) Enrichment analysis of biological processes was performed with differentially expressed proteins between NHC primary cultures and CCA cell lines (up) and with metabolism-related, differentially expressed proteins between NHC and CCA cells. Only pathways with $p < 0.05$ are shown (hypergeometric test). The number of proteins categorized in each process/pathway is displayed next to the name.

and in the normal NHC3 cholangiocytes as control, as described previously[22] and detailed in Supporting Materials and Methods.

Lipoprotein isolation, labeling, and uptake

Lipoprotein uptake was determined in NHC3, EGI1, and HUCCT1 cells by fluorescent labeling and flow cytometry, as described in Supporting Materials and Methods.

[^{3}H]-oleate incorporation into lipids

The analysis was performed *ex vivo*, in fresh mice liver pieces, and in vitro, in NHC3, EGI1, and HUCCT1 cells as previously described.[23] Lipids from liver or cell samples were extracted and separated. Additional information can be found in Supporting Materials and Methods.

[^{14}C]-palmitate oxidation

The analysis of FA oxidation (FAO) rate was performed *ex vivo*, in fresh mice liver pieces, and in vitro, in NHC3, EGI1, and HUCCT1 cells, as described previously.[24,25] Additional information can be found in Supporting Materials and Methods.

[^{3}H]-oleate blood clearance and liver uptake

The analysis was performed in vivo in the *Akt1*/*Nicd1*-driven CCA mouse model using untreated animals as control. Animals were injected with 1 μCi [^{3}H]-oleate. Blood samples were collected and liver uptake of [^{3}H]-oleate was measured as detailed in Supporting Materials and Methods.

Lipid quantification

Lipids from liver samples and from NHC3, EGI1, and HUCCT1 cells were quantified as described in Supporting Materials and Methods.

Statistical analysis

Data are represented as mean ± SEM. Normal distribution assessments were carried out with the Shapiro-Wilk test. Differences between groups were analyzed with a two-tailed Student's *t* test, a Tukey's multiple comparison test, Wilcoxon test, or with a two-way ANOVA test. Association between two variables was assessed by Pearson correlation coefficient or by Spearman test. Significance was defined as $p < 0.05$. Results were statistically analyzed using GraphPad Prism version 8.01 software (San Diego, CA, USA) and SPSS 22 software (IBM, Ehningen, Germany).

RESULTS

CCA cells are enriched in proteins involved in lipid and lipoprotein metabolism

To assess the relevance of lipid metabolism in CCA, the proteomic profiles of 5 different human CCA cell lines (HUCCT1, TKKK, EGI1, TFK1, and WITT) and 4 primary cultures of NHCs (NHC2, NHC3, NHC-SS, NHC324)[19–21] were determined. The analysis of the proteome clearly differentiated CCA and NHC cells (Figure 1A,B and Figure S1A) and indicated that 2066 proteins were up-regulated and 1148 down-regulated in CCA compared with NHC. Enrichment analysis showed that the most represented biological processes related to those dysregulated proteins in CCA versus NHC cell cultures were "regulation of nucleic acid metabolism," "metabolism," and "energy pathways." Among the 415 proteins inside the "metabolism" biological process, the most represented specific biological pathway was "metabolism of lipids and lipoproteins" (Figure 1C).

Validation of proteins involved in different lipid metabolic pathways by immunoblotting showed that levels of the acyl-CoA synthetase long chain family member 5 (ACSL5), which participates in the activation of fatty acids (FAs) to acyl-CoA (Figure S2A,E), and levels of the FA-binding protein 5 (FABP5), relevant for the malignant progression of CCA[26] and involved in FA uptake, intracellular transport, and intracellular metabolism (Figure S2B,E), were increased in CCA cells when compared with NHC (Figure S2A,B). Regarding lipoprotein lipase (LPL) and CD36, the levels of both proteins decreased in CCA cell lines compared with most NHC, except in the EGI1 CCA cell line (Figure S2C–E).

CCA cells display different proliferative and migration capacities

Given the different origin and mutational profiles of the human CCA cell lines (HUCCT1, TKKK, EGI1, TFK1, and

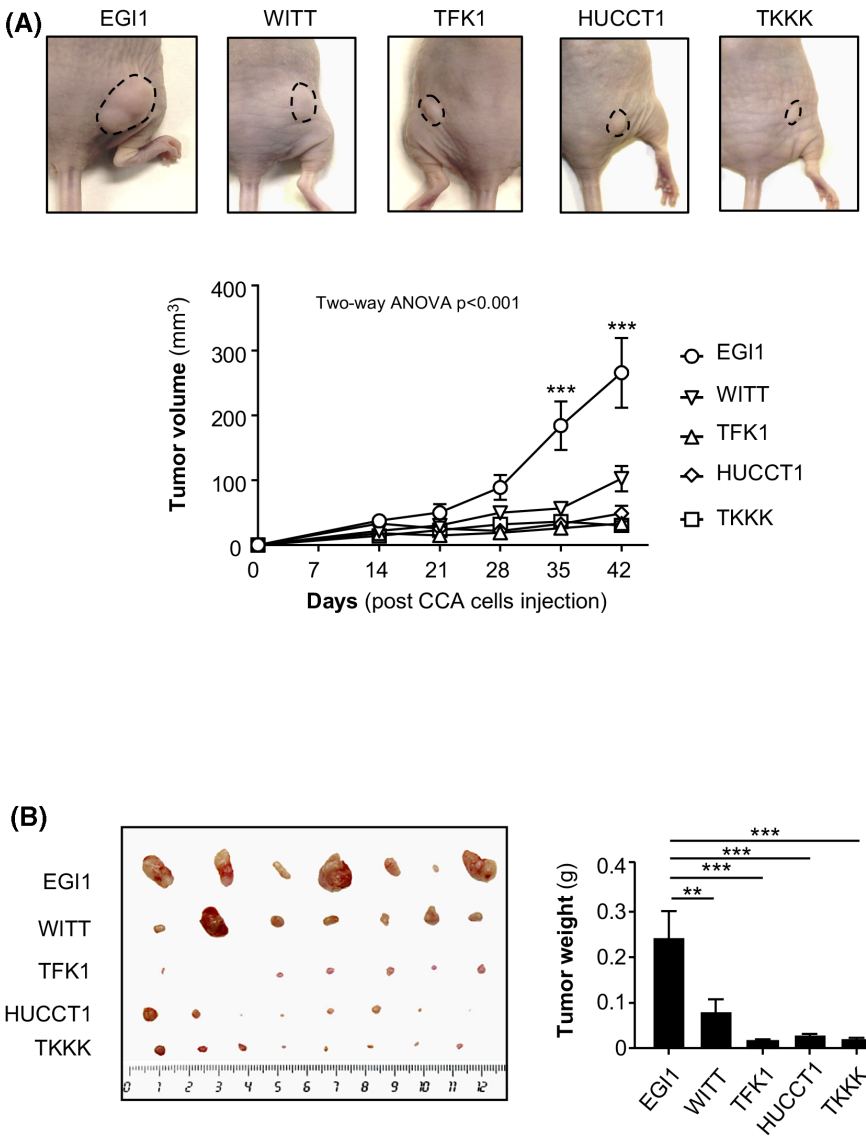


FIGURE 2 In vivo tumorigenic capacity of human CCA cell lines. (A) Tumor volume was measured 7, 14, 21, 28, 36, and 42 days after subcutaneous injection of EGI1, WITT, TFK1, HUCCT1, or TKKK CCA cells in the xenograft mouse model. Representative images of tumors in mice are shown below (n = 5-7). (B) Tumor weight was measured 42 days after injection of EGI1, WITT, TFK1, HUCCT1, or TKKK cells in the subcutaneous xenograft mouse model. Representative images of resected tumors (left) and quantification (right) are shown. Values are means ± SEM. Statistical analysis was determined by Tukey's multiple comparison test and by two-way ANOVA test. Significant differences are denoted as $^{**}p < 0.01$, $^{***}p < 0.001$

WITT),[27] we functionally characterized them by measuring the proliferation and migration rates in baseline conditions. EGI1 cells displayed the highest growth rate in a subcutaneous xenograft mouse model (Figure 2A), leading to increased tumor size (Figure 2A,B) and tumor weight (Figure 2B). A flow cytometry-based proliferation assay (carboxyfluorescein succinimidyl ester [CFSE]) also pinpointed EGI1 as the most proliferative CCA cell line in vitro (Figure 3A). We additionally performed a wound healing assay to compare the migration properties of the cells. Among the CCA cell lines, EGI1 cells displayed the highest migration capacity (Figure 3B), whereas no significant differences were found among the other cell lines (Figure 3B).

The uptake of extracellular free FAs, and very low- and high-density lipoproteins, is increased mostly in highly proliferative CCA cells

The results mentioned above showed that EGI1 is the most proliferative cell line, with the highest migration and tumorigenic capacity, whereas the HUCCT1 cell line is among the less proliferative ones. Thus, taking into account the evidence suggesting a role of extracellular lipids on CCA progression,[16] the uptake of exogenous free FAs and lipoproteins was analyzed in these CCA cell lines and compared with NHC (i.e., NHC3).

Notably, the uptake of extracellular FAs (oleic acid and palmitic acid) was increased in both EGI1 and HUCCT1 cell lines compared with NHC3 cells (Figure 4A). Differences were also observed between tumor cell lines, as EGI1 cells presented higher uptake compared with HUCCT1 cells (Figure 4A). To study lipoprotein uptake, VLDLs, LDLs, and HDLs from serum samples were isolated, purified, and fluorescently-labeled. The results showed that VLDL and HDL uptake was markedly increased in EGI1 compared with HUCCT1 or NHC3 cells, whereas LDL uptake was decreased significantly only in HUCCT1 cells compared with both EGI1 and NHC3 cells (Figure 4B). As cholesteryl ester (CE) is a major component of HDLs, we investigated whether the increased uptake of lipoproteins resulted in differences in the CE concentration in the highly proliferative and migrative EGI1 CCA cells. As expected, the CE content was higher in EGI1 cells than NHC3 or HUCCT1 cells (Figure 4C), closely resembling the observed changes in HDL uptake. Nevertheless, the increased CE levels in EGI1 cells were not linked to changes in free cholesterol (CL) levels (Figure S3A). The same profile was observed in the CCA lesions from *Akt1*/*Nicd1* mice in which liver CE content was found elevated (Figure S3B), whereas CL remained unaltered (Figure S3B). Given that most circulating lipoproteins in mice are HDL[28] and that liver is a main organ involved in HDL uptake,[29] total serum CL and serum HDL-CL levels were quantified. Accordingly, lower serum levels of total CL and of HDL-CL were observed in *Akt1*/*Nicd1* mice compared with control mice (Figure S3C), suggesting an increased liver uptake of serum HDLs in the cancer model. The endogenous CE synthesis from exogenous oleic acid (oleate) in CCA cell lines (Figure 4D) and in liver tumors from *Akt1*/*Nicd1* mice (Figure S3D) remained unchanged compared with corresponding normal controls. The *de novo* synthesis of CL and CE also remained unaltered (Figure S3D), suggesting all together that the increased HDL uptake is a source of CEs in specific CCA cell subclasses.

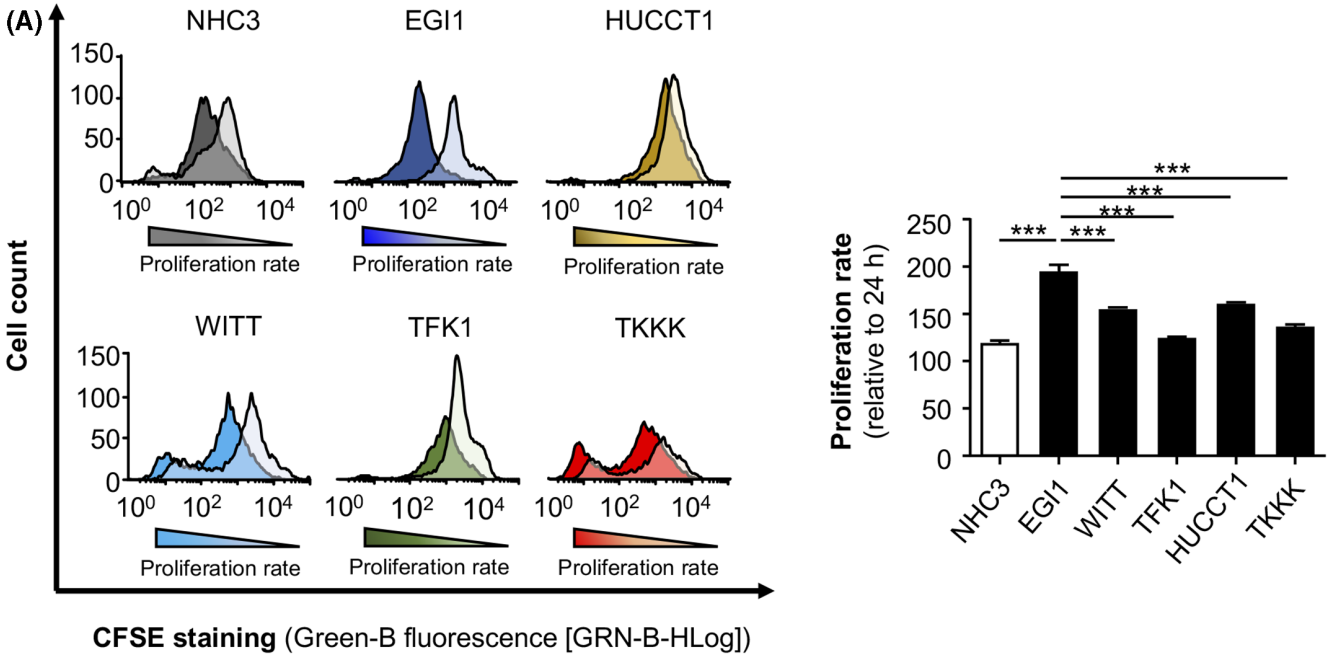
Rewiring of glycerolipid metabolism in CCA cells

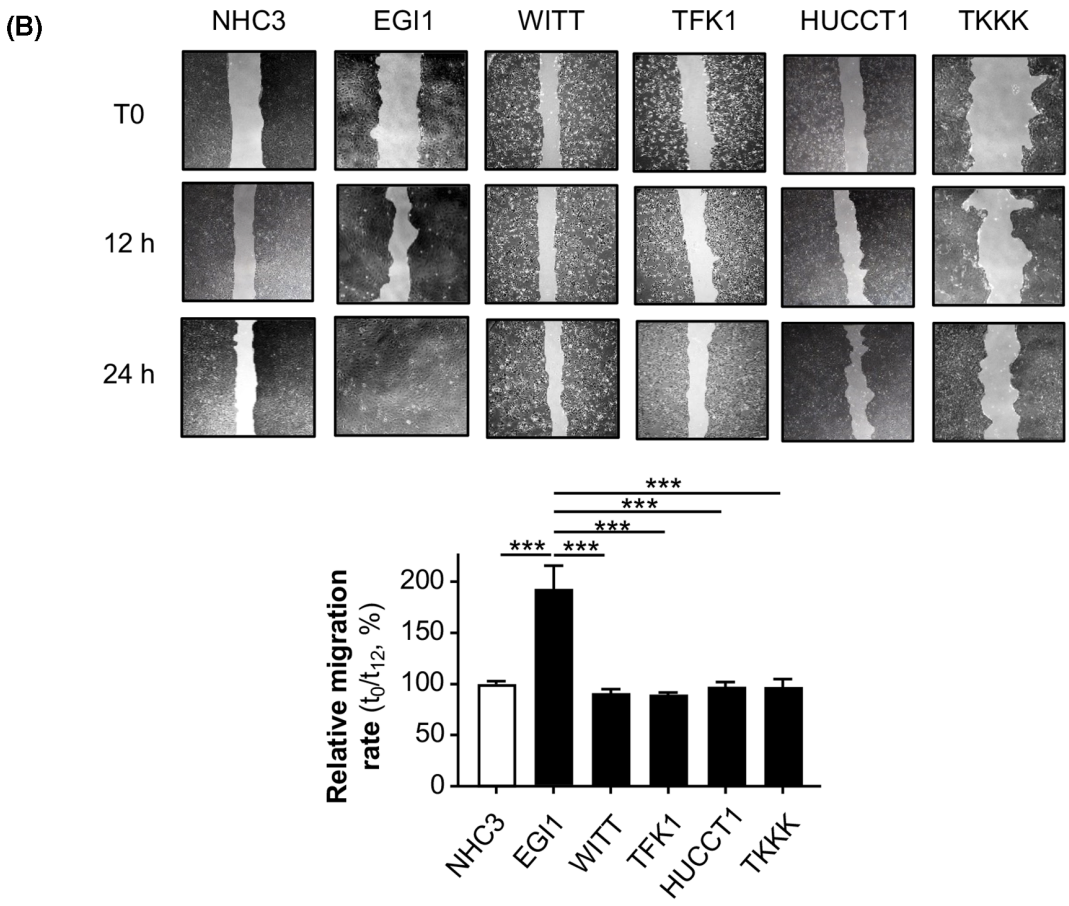
In hepatocytes, an increased uptake of lipids usually leads to increased storage of triglycerides (TGs). Given that the uptake of FAs and the TG-rich lipoprotein VLDL was increased, mainly in the EGI1 cells, the glycerolipid content and the intracellular metabolic fluxes that regulate the esterification of oleate into complex lipids, as an indicator of glycerolipid synthesis, were analyzed. The results showed that the TG content was higher in both EGI1 and HUCCT1 CCA cells than in normal cholangiocytes (NHC3) (Figure 5A). Consistent with the increased FA uptake in EGI1 compared with HUCCT1 (Figure 4A), the fluxes that regulate the synthesis of TGs were also higher in the EGI1 CCA cell line (Figure 5A). We[30] and others[31] have previously demonstrated that phosphatidylcholine (PC) might be a source of TGs; thus, the PC content was also measured. The results showed that PC levels were decreased in both CCA cell lines (Figure 5B), whereas PC synthesis was unchanged (Figure 5B).

The analysis of liver TG and PC content and the metabolic fluxes that regulate their synthesis in the *Akt1/Nicd1* CCA mouse model (Figure 6A,B) showed there was a faster clearance of circulating FAs in mice with iCCA lesions, compared with control mice (Figure 6C). In concordance, FA uptake by the liver was increased in the *Akt1*/*Nicd1* mice compared with healthy mice (Figure 6D). According to the results obtained in the cell lines (Figure 5A), TG levels and TG synthesis were also increased in the mouse CCA lesions when compared with the control mice (Figure 6E). Metabolomic analysis revealed an evident increase in a high number of TG species (Figure 6E). This was linked again with decreased PC content (Figure 6F). Furthermore, in this in vivo iCCA model, liver PC species were decreased, whereas liver PC synthesis was found increased compared with their normal controls (Figure 6D), which suggests the activation of mechanisms involved in PC catabolism, such as phospholipases or SM synthases, that might contribute to the increased FA pool,[30,31] and/or the generation of substrates for prostaglandin (PG) synthesis, altogether promoting proliferation. PC is required for the synthesis of sphingomyelin (SM) through the action of the SM synthase (SMS) using ceramide as a substrate. SM, at the same time, can be hydrolyzed by sphingomyelinases (SMases), yielding a ceramide and a phosphorylcholine (Figure S4A). Levels of ceramides were significantly decreased in both CCA cell lines compared with NHC3, with more prominent alterations in HUCCT1 cells (Figure S4B). On the contrary, SM levels were increased only in HUCCT1 compared with both NHC3 and EGI1 (Figure S4C). These changes in the lipidome were in concordance with the marked decrease in the acid SMase activity (the most abundant SMase in cholangiocytes[32] in both EGI1 and HUCCT1, and the increase in the SMS activity only in the HUCCT1 cell line (Figure S4D). The results here suggest that this remodeling in SM metabolism might not be the major metabolic pathway responsible for the observed decreased PC content in both EGI1 and HUCCT1 CCA cell lines (Figure 5B), pointing out the potential involvement of other phospholipases.[30,31]

FAO promotes the hyperproliferation of specific CCA cell subclasses

So far, the data showed that the increased uptake of extracellular free FAs, VLDLs and HDLs results in the accumulation of neutral lipids (i.e., TGs and CEs), likely leading to an intracellular pool of FAs that can be used





as an energy source through FAO. These results are also supported by our data (Figure S5A,B) and previous reports[12–16] that showed that *de novo* lipogenesis is not activated in CCA, thus confirming a more prominent role of FA uptake in promoting the accumulation of FAs.

FIGURE 3 In vitro proliferative and migration capacity of human CCA cell lines. (A) In vitro proliferation rates of NHC3, EGI1, WITT, TFK1, HUCCT1, and TKKK CCA cell lines were determined at 48 h by flow cytometry measuring loss of fluorescence of CFSE-labeled cells. Representative histograms (light-colored histograms, at 24 h; dark-colored histograms, at 72 h) (left) and quantification (right) are shown. Results are expressed as relative to proliferation at 24 h of each cell line. (B) In vitro migration rates of NHC3, EGI1, WITT, TFK1, HUCCT1, and TKKK CCA cell lines were measured at 12 h by a wound healing assay. Results are expressed as relative to time 0. Representative images of wound healing areas (up) and quantification (down) are shown. Values are means ± SEM. Statistical analysis was determined by Tukey's multiple comparison test. Significant differences are denoted as ***$p < 0.001$

To ascertain whether the catabolism of FAs was altered in the human CCA cell lines, complete oxidation of [^{14}C]-palmitate into CO_2 was measured. We found that the FAO rate was markedly increased in the EGI1 CCA cells as compared with the rest of the CCA cell lines or to the NHC (Figure 7A). Curiously, FAO rate decreased in HUCCT1 cells as compared with NHC3 cells suggesting other sources, different from lipids, as major energy substrates (Figure 7A). Thus, both glucose and glutamine uptake and complete oxidation into CO_2 were measured. The results showed that only HUCCT1 presented a significant upregulation of glucose uptake compared with NHC3 (Figure S6A). However, complete glucose oxidation into CO_2 did not change in the tumor cell lines (Figure S6B). Regarding glutamine, the results showed that its uptake remained unaltered in the CCA cell lines (Figure S6C), whereas the complete oxidation into CO_2 was up-regulated in HUCCT1 compared with NHC3 and EGI1, pointing glutamine as a relevant source to fuel the tricarboxylic acid (TCA) cycle and energy production in HUCCT1 CCA cells.

Considering the differences in FAO rates between EGI1 and HUCCT1 cells come along with differential proliferation rates (Figure 3A), we decided to investigate the contribution of FAO to the proliferation capacity of EGI1 and HUCCT1 CCA cell lines. As predicted, etomoxir, a recognized inhibitor of FAO,[33–35] effectively blocked FAO in both EGI1 and HUCCT1 cells, reaching similar oxidation values (Figure S7A). Noteworthy, FAO blockage with etomoxir induced a more pronounced inhibition of proliferation in the highly proliferative CCA cell line EGI1 than in the less proliferative HUCCT1 (Figure 7B), suggesting that EGI1 relies more on FAO to proliferate and grow. Similarly, in a subcutaneous xenograft mouse model, etomoxir administration decreased tumor growth in both EGI1 and HUCCT1 CCA cell lines (Figure 7C), more remarkably in the most proliferative one (EGI1).

CCA cells with a stem cell-like phenotype are more reliant on oxidative phosphorylation.[36] Accordingly, our results showed that the mRNA levels of the stemness markers *EPCAM*, *ITGA6*, *CD133* and *CD44* were markedly increased in the most proliferative, highly lipid-dependent EGI1 cells compared with both NHC3 and HUCCT1 (Figure S7B). Thus, these results suggest that increased lipid uptake fuels the increased mitochondrial oxidation observed in the CCA stem-like cells. Lipophagy or autophagy of cellular lipid droplets can also have a role in cancer metabolic reprogramming.[37] Given the increased FAO in the most proliferative CCA cell line, protein levels of the autophagy markers ATG5 and ATG7, as well as LC3B lipidation, were mainly increased in the most proliferative EGI1 CCA cell line compared with both HUCCT1 and NHC3 (Figure S7C), which suggests increased autophagy fluxes in CCA cells, but particularly in the EGI1 CCA cell line.

Next, we compared the proteomic profile of both CCA cell lines (EGI1 and HUCCT1) and 1409 proteins were differentially expressed. Among them, 1026 were up-regulated in EGI1 compared with HUCCT1 and 383 in HUCCT1 compared with EGI1 (Figure S8). Enrichment analysis on biological pathways was performed with the 211 differentially expressed proteins categorized into the "metabolism" biological process. From those, 145 showed higher expression in EGI1 than in HUCCT1, and 66 in HUCCT1 than in EGI1. The enrichment analysis considering only pathways with a *p* value smaller than 0.0001 indicated that differentially expressed proteins were highly enriched in processes such as "metabolism of lipids and lipoproteins," "mitochondrial FA β-oxidation," "the citric acid (TCA) cycle," and "respiratory electron transport, ATP synthesis and heat production," which usually up-regulate when FAO rate increases (Figure 7D). Most of the proteins inside these pathways showed higher expression in EGI1 than in HUCCT1 (Figure 7D).

As mentioned, EGI1 cells are more dependent on FAO for proliferation than HUCCT1, potentially explaining their higher proliferative capacity. Among the proteins involved in FAO showcasing increased levels in EGI1 when compared with HUCCT1, acyl-CoA dehydrogenase medium chain (ACADM), the first enzyme involved in FAO stood out. Validation by immunoblotting confirmed a higher expression of ACADM in CCA cell lines compared with NHC, particularly in EGI1 (Figure 7E). Accordingly, the immunostaining analysis of ACADM in 182 iCCA samples (Figure 8A) showed that the nontumor liver tissue presented a strong cytoplasmic ACADM staining in hepatocytes, whereas biliary epithelial cells exhibited faint or absent immunoreactivity for ACADM (Figure 8A, upper panels). On the contrary, 168 out of 182 (92.31%) iCCA samples showed robust immunostaining for ACADM in tumor (T) areas (Figure 8A, lower panels). In addition, *ACADM* expression was up-regulated in the tumor epithelium compared with matched tumor stroma

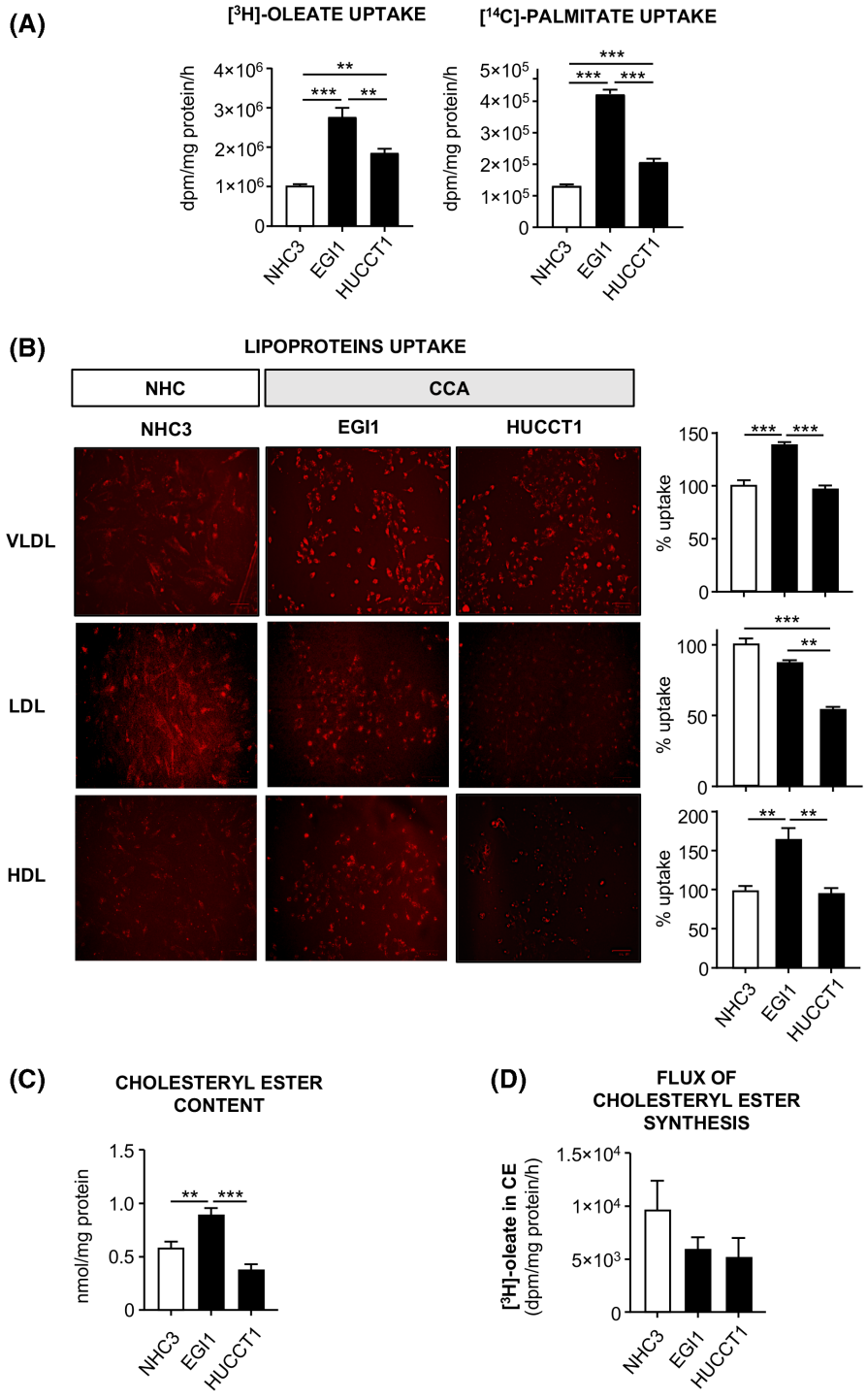


FIGURE 4 EGI1 CCA cells show markedly increased uptake of extracellular lipids. (A) [^{3}H]-oleate (left) and [^{14}C]-palmitate (right) uptake by NHC3, EGI1, and HUCCT1 cells was measured after 4 h of incubation with 400 μM oleate and 5 μCi/ml [^{3}H]-oleate, or 200 μM palmitate and 0.5 μCi/ml [^{3}H]-palmitate. (B) Fluorescein isothiocyanate (FITC)-labeled lipoprotein uptake by NHC3, EGI1, and HUCCT1 was measured after 4 h of incubation with 5 μg/ml VLDL, 10 μg/ml HDL, or 20 μg/ml LDL. Representative images of fluorescence microscopy (left) and quantification (right). (C) Lipids were extracted from NHC3, EGI1, and HUCCT1 cells, and CE levels were quantified. (D) Oleate esterification into CE in cells, as a measure of the flux of CE synthesis, was estimated after incubation of 4 h with 400 μM oleate and 5 μCi/ml [^{3}H]-oleate. Values are means ± SEM. Statistical analysis was determined by Tukey's multiple comparison test. Significant differences are denoted as $^{**}p < 0.01$ and $^{***}p < 0.001$

obtained by microdissection from the Copenhagen cohort (Figure 8B). Besides, tumor expression of *ACADM* positively correlates with the proliferation marker proliferating cell nuclear antigen (*PCNA*) in the Copenhagen cohort[17] and in the Jusakul cohort,[18] thus suggesting once more a potential connection between increased FAO and the higher proliferative CCA phenotype (Figure 8B).

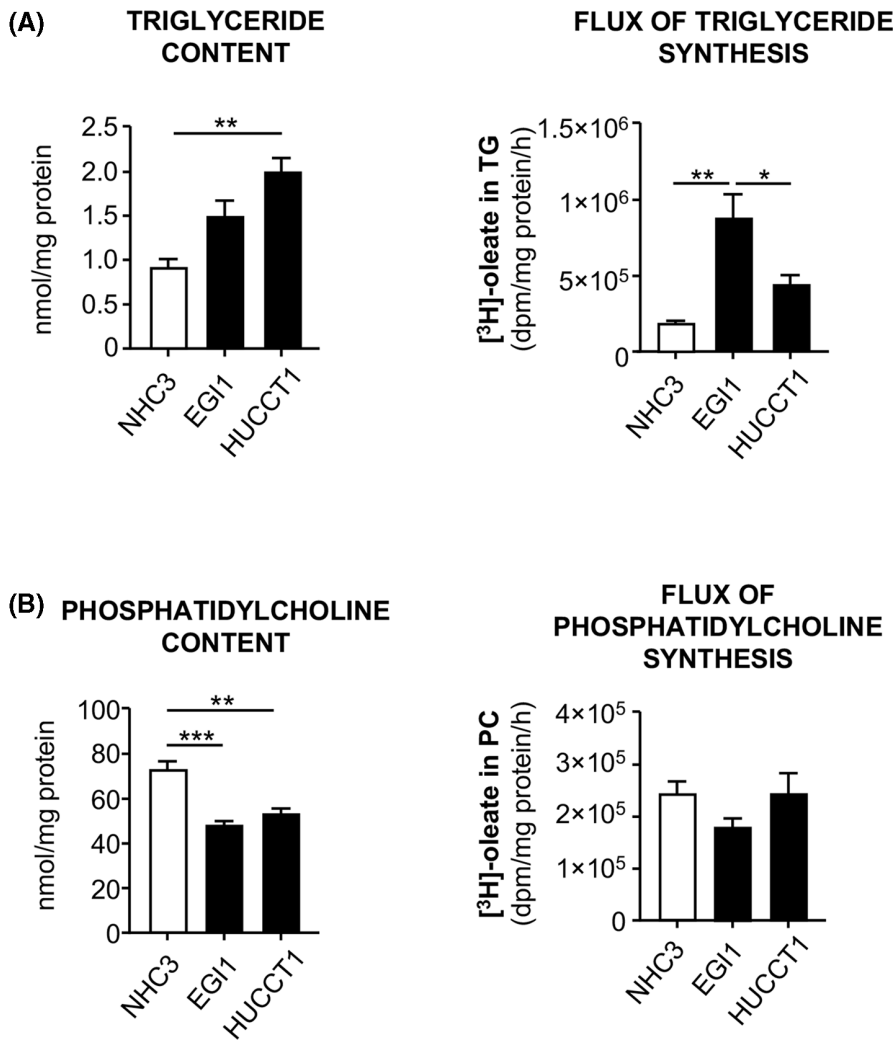


FIGURE 5 Rewiring of glycerolipid metabolism in EGI1 and HUCCT1 CCA cell lines. (A) Lipids were extracted from NHC3, EGI1, and HUCCT1 cells, and TG content was quantified (left). Oleate esterification into TG in cells, as a measure of the flux of TG synthesis, was determined after incubation of 4 h with 400 μM oleate and 5 μCi/ml [^{3}H]-oleate (right). (B) Lipids were extracted from NHC3, EGI1, and HUCCT1 cells, and PC content was quantified (left). Oleate esterification into PC in cells, as a measure of the flux of PC synthesis, was determined after incubation of 4 h with 400 μM oleate and 5 μCi/ml [^{3}H]-oleate (right). Values are means ± SEM. Statistical analysis was determined by Tukey's multiple comparison test. Significant differences are denoted as *p < 0.05, **p < 0.01, and ***p < 0.001

DISCUSSION

Cancer cells require metabolic rearrangements to survive and proliferate. The diverse background of driving oncogenes, as well as epigenetic and molecular changes, together with the tumor-induced modifications in the microenvironment (i.e., the supply of nutrients and oxygen), promote the metabolic rewiring that drives the cancer-specific metabolic hallmark. The high heterogeneity of tumors together with the continuous metabolic adaptability has been frequently related to frustrated therapeutic interventions.[38] Thus, the determination of specific metabolic reprograming will bring therapeutic options.

CCA, which includes a diverse group of malignant biliary tumors, exhibits high genomic, epigenetic, and molecular heterogeneity that hampers the possibility to find common effective treatments for patients. Therefore, the field of CCA research is rapidly moving toward the search for personalized and precision-targeted therapies. Here, we found that the proteome of human CCA cells is enriched, among others, in proteins involved in lipid and lipoprotein metabolism when compared with normal cholangiocytes. Targeting lipid metabolism has been proposed as a promising subject of research for anticancer therapies. Several drugs, in preclinical or clinical stages, modulate lipid metabolic pathways of cancer cells, including FA and CL synthesis, FA uptake, or FAO.[12,39] Concerning CCA, several statins, i.e., drugs that target the *de novo* CL synthesis in the liver, have been shown to inhibit proliferation and/or induce apoptosis.[40] Of note, statin use has even been associated with lower risk for CCA development.[41]

The present results demonstrate that the EGI1 CCA cell line is highly proliferative and exhibits increased lipid

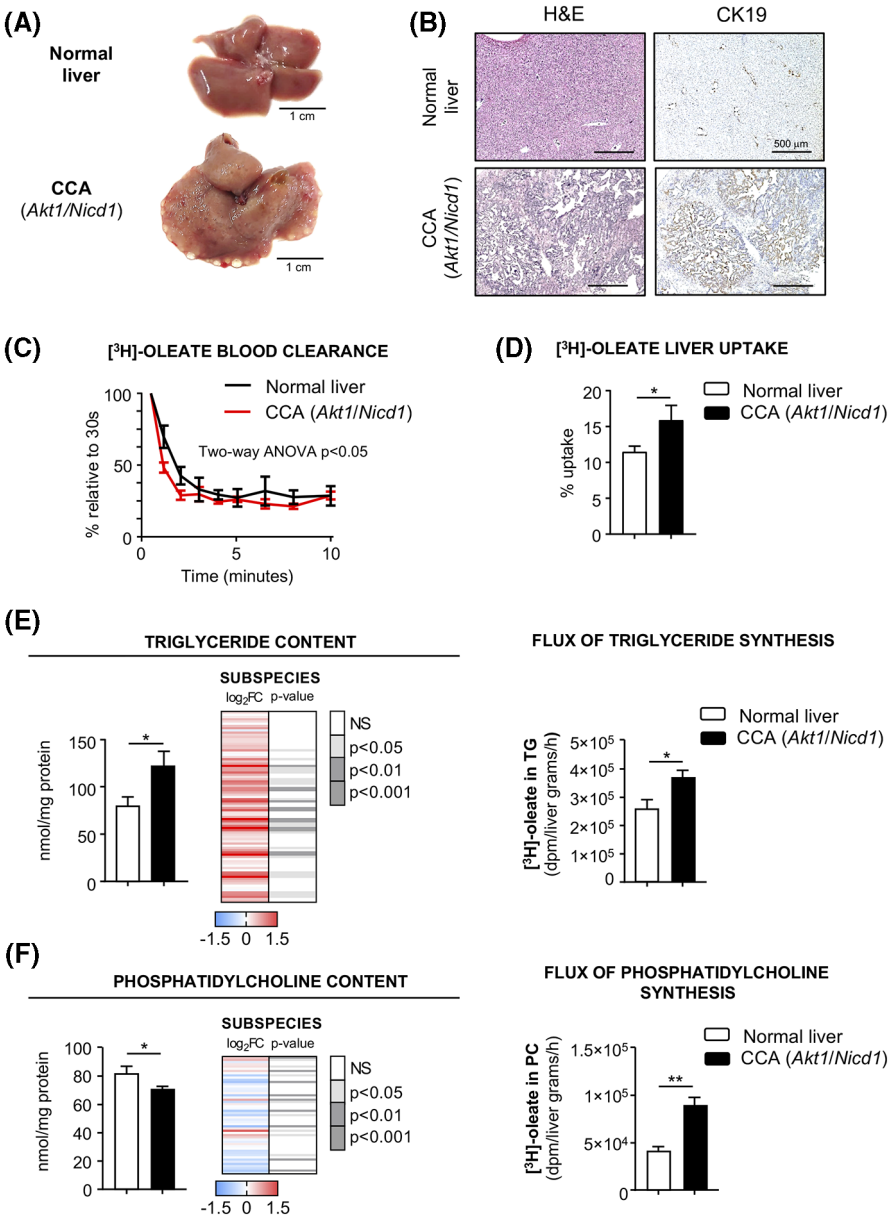


FIGURE 6 Rewiring of glycerolipid metabolism in the *Akt1*/*Nicd1* CCA mouse model. (A) Representative images of untreated livers (up) or 4 weeks after the administration of the *Akt1*/*Nicd1* plasmid combination (down). (B) Representative images of hematoxylin and eosin (H&E) and cytokeratin 19 (CK19) staining on control livers or administered with the *Akt1*/*Nicd1* plasmids. (C) FA blood clearance was measured in *Akt1*/*Nicd1* and control mice by intravenously injecting 0.5 mg/ml oleate and 1 µCi [^{3}H]-oleate. Blood was taken at each time point and its radioactivity was measured (n = 6-8). (D) FA uptake by the liver was measured in *Akt1*/*Nicd1* and control mice by an intravenous injection of 0.5 mg/ml oleate and 1 µCi [^{3}H]-oleate. After sacrificing the mice at 10 min, incorporated radioactivity was measured in liver homogenates (n = 6-8). (E) Lipids were extracted from liver samples of the *Akt1*/*Nicd1* mouse model and control mice, and TG content was quantified (n = 9-10). TG subspecies were determined by ultrahigh performance liquid chromatography-mass spectrometry (UHPLC-MS) (n = 8-9). Oleate esterification into TGs in liver samples of the *Akt1*/*Nicd1* mouse model and control mice, as a measure of the flux of TG synthesis, was determined after incubation of 4 h with 800 µM oleate and 20 µCi/ml [^{3}H]-oleate (n = 4-5). (F) Lipids were extracted from liver samples of the *Akt1*/*Nicd1* mouse model and control mice, and PC content was quantified (n = 9-11). PC subspecies were determined by UHPLC-MS and different subspecies of PCs were quantified (n = 8-9). Oleate esterification into PC in liver samples of the *Akt1*/*Nicd1* mouse model and control mice, as a measure of the flux of PC synthesis, was determined after incubation of 4 h with 800 µM oleate and 20 µCi/ml [^{3}H]-oleate (n = 4-5). Values are means ± SEM. Statistical analysis was determined by Student's two-tailed t test and by two-way ANOVA test. Significant differences are denoted as $^*p < 0.05$ and $^{**}p < 0.01$

consumption compared with normal cholangiocytes or to the rest of the analyzed CCA cell lines (Figure 8). Pharmacological inhibition of FAO with etomoxir decreased CCA cell proliferation and halted tumor growth in vivo, suggesting that in the subset of CCA tumors bearing an up-regulated FAO, FA catabolism-impairing drugs could be useful. In this sense, etomoxir has been extensively studied for the treatment of a wide range

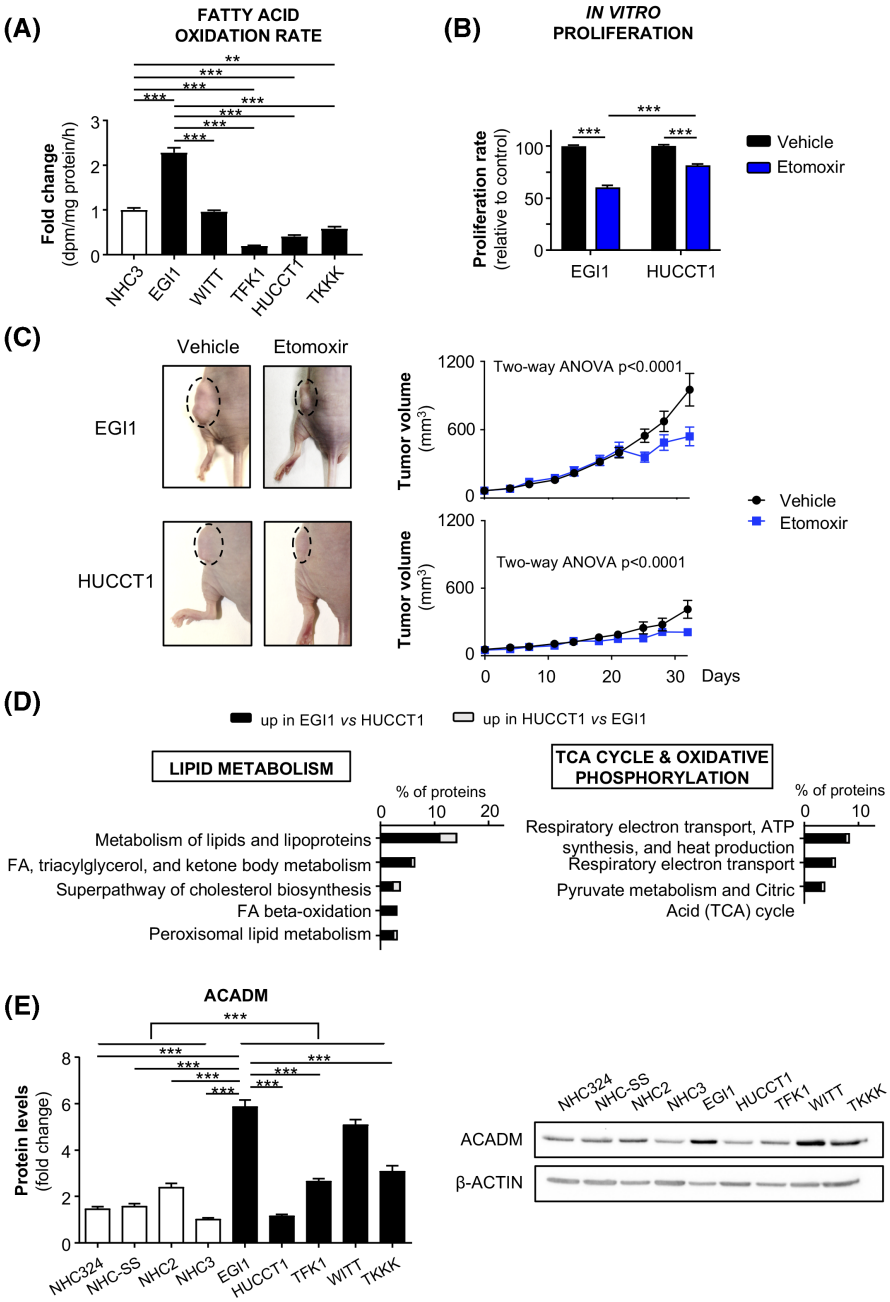


FIGURE 7 Highly proliferative CCA cells rely on FAO for proliferation. (A) FAO rate in NHC3, EGI1, WITT, TFK1, HUCCT1, and TKKK cells was assessed by [^{14}C]-palmitate oxidation into CO_2. (B) Proliferation rates of EGI1 and HUCCT1 cell lines in vitro (for 48 h) were determined by flow cytometry measuring loss of fluorescence of CFSE-labeled cells in the presence or absence of 50 μM etomoxir. Results are expressed as relative to the proliferation of control of each cell line. (C) EGI1 (left) or HUCCT1 (right) cells were subcutaneously injected into immunodeficient mice (n = 8-12 mice). Once tumors were well established, etomoxir (30 mg/kg) or vehicle were administered intraperitoneally every two days, and tumor size was measured twice a week. (D) Enrichment analysis of biological pathways was performed with metabolism-related, differentially expressed proteins between EGI1 and HUCCT1. (E) Protein expression levels of ACADM were determined by immunoblotting in NHC324, NHC-SS, NHC2 and NHC3 primary cultures of NHCs and EGI1, HUCCT1, TFK1, WITT, and TKKK CCA cell lines (n = 6). Quantification (left) and representative blot (right) are shown. Values are means ± SEM. Statistical analysis was determined by Tukey's multiple comparison test or two-way ANOVA test. Significant differences are denoted as $^{**}p < 0.01$ and $^{***}p < 0.001$

of cancers, including colon carcinoma,[33] gastric cancer,[34] and HCC,[35] among others. Other FAO inhibitors have also been investigated as anticancer drugs (i.e., trimetazidine, which targets 3-ketoacyl-coenzyme A thiolase),[42] and are being tested in phase III clinical trials for the treatment of intermediate-stage (NCT03274427) and advanced (NCT03278444) HCC in combination with arginine hydrochloride. Another strategy to impair FAO is the inhibition of malonyl-CoA decarboxylase (MCD), which converts malonyl-CoA to acetyl-CoA and

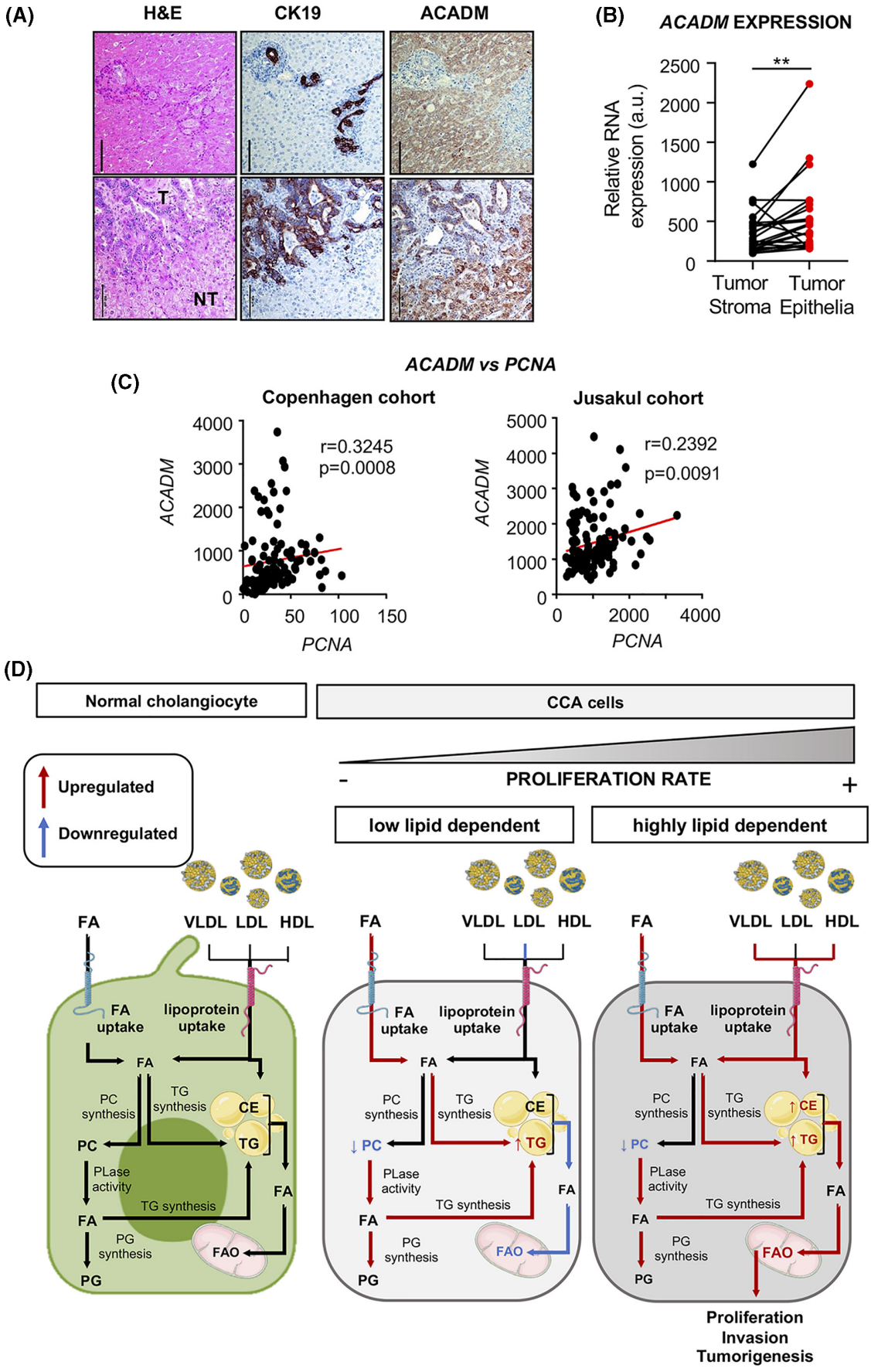

(A)
H&E
CK19
ACADM
T
NT
(B)
ACADM EXPRESSION
**
Relative RNA expression (a.u.)
2500
2000
1500
1000
500
0
Tumor Stroma
Tumor Epithelia
(C)
ACADM vs PCNA
Copenhagen cohort
r=0.3245
p=0.0008
ACADM
PCNA
Jusakul cohort
r=0.2392
p=0.0091
ACADM
PCNA
(D)
Normal cholangiocyte
CCA cells
Upregulated
Downregulated
PROLIFERATION RATE
low lipid dependent
highly lipid dependent
FA
VLDL LDL HDL
FA uptake
lipoprotein uptake
PC synthesis
TG synthesis
CE
TG
PC
PLase activity
PG synthesis
PG
FAO
Proliferation
Invasion
Tumorigenesis

FIGURE 8 Increased ACADM levels in CCA. (A) Representative images of hematoxylin and eosin (H&E), cytokeratin 19 (CK19), and ACADM staining on surrounding nontumor liver tissue (upper panels) and iCCA human samples (lower panels). (B) Expression of ACADM was measured in tumor stroma and matched tumor epithelium obtained by microdissection (n = 23). (C) The correlation between *ACADM* and *PCNA* expression was analyzed in CCA tumor tissue from the Copenhagen cohort (left) and the Jusakul cohort (right). (D) Summary chart: highly proliferative CCA cells are greatly lipid-dependent, showing increased extracellular FA uptake, increased lipoprotein uptake (VLDLs and HDLs), which promote the storage of TGs and CEs. Increased catabolism of PC promotes the release of precursors for PG and for the synthesis of other lipids. The increased lipid availability sustains the FAO rate contributing to proliferation, tumorigenicity, and invasiveness. In less proliferative, low lipid-dependent CCA cells, FA uptake and TG storage still increased while PC decreased. However, lipoprotein uptake or FAO is not increased, which indicates other energy sources for proliferation. Values are means ± SEM. Statistical analysis was determined by Wilcoxon test or two-way ANOVA test. Significant differences are denoted as $^{**}p < 0.01$

CO_2. The accumulation of malonyl-CoA inhibits carnitine palmitoyltransferase 1 and the transport of FAs to mitochondria, and therefore, its targeting inhibits FAO. MCD inhibitors are considered promising treatments of ischemia-reperfusion injury as they favor glucose oxidation over FAO, thereby improving cardiac function.[43] Moreover, their use as anticancer therapies has been suggested.[44] Here, the enrichment analysis of the differentially expressed proteins in the highly proliferative EGI1 versus low-proliferative HUCCT1 cells showed that, in concordance with the increased FAO rate, proteins involved in FAO were up-regulated in EGI1 cells. Among those, high expression of *ACADM*, which participates in the first reaction of FAO, positively correlated with the expression of the proliferation marker *PCNA* in CCA human tumors.

Up-regulated FAO leads to increased NADH and $FADH_2$, which are channeled to the electron transport chain for ATP production. As a result, an increased FAO is generally coupled to an up-regulated oxidative phosphorylation. In this sense, it has been described that cancer stem cells present an up-regulated oxidative phosphorylation in CCA; in contrast, cells with a more differentiated phenotype rely on glycolysis for energy production.[36] Considering the high self-renewal capacity that characterizes cancer stem cells, this points toward FAO as the preferred energy source in highly proliferative CCA cells. The fact that several stemness markers were found particularly increased in the EGI1 CCA cell line supports this hypothesis.

According to our data, the highly proliferative EGI1 CCA cells exhibit up-regulated uptake of FAs but more specifically of extracellular lipoproteins (VLDLs and HDLs), recognized as a critical lipid source for several cancer cells, either by extracellular hydrolysis of TGs and subsequent entry of FAs, or by receptor-mediated endocytosis.[45] In this sense, lipoprotein-derived lipid entry into CCA cells could be another target of treatment that has already been studied in other forms of cancer.[46] The results here showed that with the exception of EGI1, CCA cell lines exhibited decreased LPL and CD36 levels when compared with the NHC. However, levels of FABP5, known to regulate FA uptake, intracellular transport, and intracellular metabolism and those of ACSL5, involved in the activation of FAs to acyl-CoA were increased in CCA cells and more markedly in EGI1, when compared with NHC. Interestingly, FABP5 has been described as key in the metabolic reprogramming of HCC[47] and also as a prominent target during CCA malignant progression.[26] Overall, the upregulation of ACADM, ACSL5, CD36 and LPL particularly in the EGI1 CCA cell line might pinpoint that both the intracellular metabolism and the uptake of extracellular lipids are highly coordinated processes in CCA, being up-regulated in the most proliferative, lipid-dependent tumors.

PC is a membrane lipid required for the generation of cellular membranes, assembly of lipoproteins, correct biliary secretion, and membrane fluidity control. When PC content is decreased, its proportion with other lipids changes resulting in alterations in membrane fluidity. For instance, in nonalcoholic steatohepatitis, an altered PC/PE ratio leads to membrane leakage and worsens disease progression.[48] Furthermore, reduced PC levels in the bile of patients with CCA has also already been described.[49] The data presented here show that in the different CCA models (i.e., cell lines and livers from the *Akt1*/*Nicd1* CCA mouse model), the PC content is diminished compared with their respective normal controls. Although this decrease was not linked to less esterification of exogenous FAs into PC in the CCA cell lines, the esterification of oleate into PC was increased, whereas the *de novo* lipogenesis remained unaltered in the CCA mouse model. Therefore, this decrease in PC levels is unlikely associated with a decreased synthesis. Here, we propose that the observed decrease of PC in liver or the bile of patients with CCA could result from the up-regulated catabolism through more active phospholipases or SM synthase. In turn, this could be fueling TG synthesis for energy storage[31] and contributing to the release of arachidonic acid for PG synthesis. In fact, the role of cyclooxygenase-derived PG signaling in CCA development and progression has been studied extensively.[50] The results suggested that SM synthase was not involved in the decreased PC levels. However, in CCA cell lines there is an import remodeling in SM metabolism, in which ceramide and SM content were heterogeneously changed in the two analyzed CCA cell lines.

Our results are indicative of the heterogeneity that characterizes CCA. Accordingly, previous studies have categorized CCA tumors into different subclasses

according to their mutational and genetic expression profile.[7,8]. A "proliferation class," present in both iCCA and eCCA, has been defined. Our results indicate that highly proliferative CCA cells are characterized by higher lipid consumption, and treatments that affect the entry or consumption of lipids could have therapeutic value for CCA tumors with these characteristics.

In summary, highly proliferative CCA cells are strongly lipid-dependent, as shown by their up-regulated lipid and lipoprotein uptake and catabolism for proliferation (Figure 8E). This observation underscores the potential relevance of targeting lipid metabolism for the treatment of specific subtypes of CCA.

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CONFLICT OF INTEREST

Authors disclose no conflicts related to this study.

AUTHOR CONTRIBUTIONS

Mikel Ruiz de Gauna, Francesca Biancaniello, Jesús M. Banales, and Patricia Aspichueta designed the study. Mikel Ruiz de Gauna, Francesca Biancaniello, Francisco González-Romero, Pedro M. Rodrigues, Ainhoa Lapitz, Sabina Di Matteo, Igor Aurrekoetxea, Ibone Labiano, Ane Nieva-Zuluaga, Asier Benito-Vicente, María J. Perugorria, Maider Apodaka-Biguri, Diego Sáenz de Urturi, Beatriz Gómez-Santos, Xabier Buqué, Igotz Delgado, Mikel Azkargorta, Felix Elortza, Jesper B. Andersen, Luis Bujanda, Jesús M. Banales, and Patricia Aspichueta performed experiments and investigations. César Martín, Diego F. Calvisi, Jesper B. Andersen, Domenico Alvaro, Vincenzo Cardinale, Luis Bujanda, Jesús M. Banales, and Patricia Aspichueta designed experimental protocols. Mikel Ruiz de Gauna, Jesús M. Banales, and Patricia Aspichueta wrote the paper, and all authors contributed to editing.

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