ATP Citrate Lyase Knockdown Induces Growth Arrest and Apoptosis through Different Cell- and Environment-Dependent Mechanisms

Nousheen Zaidi, Ines Royaux, Johannes V. Swinnen, and Karine Smans

Abstract

ATP citrate lyase (ACLY) is a cytosolic enzyme that catalyzes generation of acetyl-CoA, which is a vital building block for fatty acid, cholesterol, and isoprenoid biosynthesis. ACLY is upregulated in several types of cancer, and its inhibition induces proliferation arrest in certain cancer cells. As ACLY is involved in several pathways, its downregulation may affect multiple processes. Here, we have shown that short hairpin RNA-mediated ACLY silencing in cell lines derived from different types of cancers induces proliferation, cell-cycle arrest, and apoptosis. However, this antiproliferative effect of ACLY knockdown was observed only when cells were cultivated under lipid-reduced growth conditions. Proliferation arrest induced by ACLY silencing was partially rescued by supplementing the media with fatty acids and/or cholesterol. This indicates that the ACLY knockdown-mediated growth arrest might be the result of either fatty acid or cholesterol starvation or both. In the absence of ACLY, the cancer cells displayed elevated expression of sterol regulatory element binding protein–regulated downstream genes involved in de novo fatty acid and cholesterol biosynthesis. Furthermore, ACLY suppression resulted in elevated expression of acyl-CoA synthetase short-chain family member 2 (ACSS2), an enzyme that also produces acetyl-CoA using acetate as a substrate. Acetate supplementation partially rescued the cancer cells from ACLY suppression–induced proliferation arrest. We also observed that the absence of ACLY enhanced ACSS2-dependent lipid synthesis. These findings provide new insights into the role of ACLY in cancer cell growth and give critical information about the effects of ACLY silencing on different pathways. This information is crucial in understanding the possible application of ACLY inhibition in cancer therapeutics.

Introduction

ATP citrate lyase (ACLY) is a cytosolic enzyme that connects glucose/glutamine metabolism to de novo lipid synthesis (1–5). ACLY converts citrate into acetyl-CoA, a precursor for fatty acid and mevalonate synthesis pathways (6–8). Both of these pathways are associated with cancer cell growth and transformation (9–11).

In the fatty acid synthesis pathway, acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA-carboxylase (ACACA). Both acetyl-CoA and malonyl-CoA are then used in a condensation reaction by the main lipogenic enzyme fatty acid synthase (FASN) to produce long-chain fatty acids (9; Fig. 1). The fatty acid synthesis pathway is upregulated in many cancer types. This upregulated fatty acid synthesis fuels membrane biogenesis in rapidly proliferating cancer cells and renders membrane lipids more saturated (12). This affects fundamental cellular processes, including signal transduction, gene expression, ciliogenesis, and therapy response (12–14).

Another pathway that uses acetyl-CoA as a substrate is the mevalonate pathway. As shown in Fig. 1, this pathway leads to the synthesis of farnesyl pyrophosphate (FPP), which is the branch point for several pathways leading to various end products. One of these end products is cholesterol. De novo cholesterol synthesis is also shown to be elevated in cancer cells (10). Cholesterol is reported to be required for the growth of cancer cells (10, 15). In addition, FPP can also be converted into geranylgeranyl pyrophosphate (GGPP; ref. 16). Both FPP and GGPP are respectively involved in farnesylation and geranylgeranylation of a variety of proteins (Fig. 1; ref. 16). Farnesylation and geranylgeranylation are required for the ability of Ras and Rho proteins to induce malignant transformation, invasion, and metastasis (11, 17). Hence, ACLY is involved in multiple pathways that are known to be relevant in several types of cancers.
Distinctive elevation of ACLY expression and activity has been reported in lung, prostate, bladder, breast, liver, stomach, and colon tumors (18–24). Moreover, inhibition of ACLY by either RNAi or pharmacologic inhibitors results in growth arrest in tumor cells, both in vitro and in vivo (5, 18, 25, 26). However, the exact mechanism by which this growth arrest is implemented is not completely understood.

Lipogenesis is a highly coordinated process, and perturbation at different steps of the lipogenic pathway can bring about major changes in expression and activity of other metabolic genes. Therefore, for therapeutic exploitation of this pathway, it is very important to understand the effects of blocking one metabolic enzyme on expression and function of other metabolic genes. In addition, in drug discovery, it is essential to consider the alternate mechanisms/pathways that may compensate for the inhibition of any enzyme/pathway.

It is known that ACLY is not the only source of acetyl-CoA production in mammalian cells. The cytosolic enzyme acyl-CoA synthetase short-chain family member 2 (ACSS2) also produces acetyl-CoA using acetate as a substrate (ref. 27; Fig. 1). Previous studies have speculated that in the absence of ACLY, ACSS2 becomes more relevant (5). It was also suggested that ACSS2 may help the cells in escaping ACLY silencing–induced effects provided its substrate is available (5, 28). However, the effect of ACLY knockdown on the expression of ACSS2 has not been studied in detail.

Here, we investigated the effects of short hairpin RNA (shRNA)-mediated ACLY silencing in cell lines derived from different types of cancer. Our data clearly shows that ACLY suppression induced proliferation arrest in multiple cancer cell lines. However, this effect was mainly observed when cancer cells were cultivated under lipid-reduced growth conditions. We observed that supplementation of oleic acid or cholesterol partially rescued the cancer cells from antiproliferative effects induced by ACLY knockdown. It indicates that the antiproliferative effects of ACLY silencing are mediated through fatty acid or cholesterol synthesis pathways. It was also observed that ACLY suppression induces upregulation of the genes involved in both of these pathways, which could be a compensatory mechanism adopted by the cancer cells to overcome ACLY deficiency. We found that upon ACLY silencing, the expression level of ACSS2 was markedly enhanced. In addition, acetate supplementation partially rescued the
cancer cells from ACLY knockdown–induced growth arrest, by increasing ACSS2-dependent lipid synthesis. Collectively, our data provide new understanding of the involvement of ACLY in cancer cell growth, and it shows the impact of ACLY silencing on different pathways that are cross-linked through ACLY.

Materials and Methods

Cell lines and treatments

All the cell lines were obtained from the American Type Culture Collection and authenticated. FPP, GGPP, oleic acid, and cholesterol were purchased from Sigma. Simvastatin was purchased from Merck Sharp. Soraphen A was received from Dr. R. Jansen, Helmholtz-Zentrum f. Infektionsforschung, Mikrobielle Wirkstoffe, Braunschweig (29, 30). siRNAs were purchased from Ambion. For lipid-reduced conditions, the media were supplemented with HyClone lipid-reduced FBS (Thermo Scientific).

Cloning of shRNA cassettes into an inducible expression vector

The TRC has developed a tightly regulated inducible shRNA expression system based on the lac operon, using isopropyl-β-D-thio-galactoside (IPTG) as inducer. The optimal IPTG concentration needed for maximal induction was found to be between 0.5 and 1.0 mmol/L for most cell lines (manuscript in preparation). The inducible vector is available through the consortium (vector number TRC904) or at Sigma-Aldrich under the name pLKO-puro-IPTG-1xLacO. The sequences for the hairpins were selected from the TRC library and cloned into TRC904. For ACLY silencing CGTGAGAGCAATTCGAGATTA (shACLY-86) were used. As a control a nontargeting (shACLY-17) and CACCGAGTG-AAGTCGATAAAC (shMock) was used; recombinant lentiviruses were produced by the transfection of 293FT cells.

Lentiviral transduction and selection

Cells were plated in 24-well plates. Next day, cells were infected at a 1:25 dilution of virus in the presence of 8 μg/mL polybrene. Virus-containing medium was then replaced by normal growth medium. Twenty-four hours after infection, puromycin selection was started to obtain stable cell populations.

Immunoblotting analysis

Following antibodies were used for immunoblots ACLY (monoclonal rabbit, ab40793; Abcam), β-actin (monoclonal mouse; Sigma), SREBP-1 (Active Motif, monoclonal mouse, 39940), or SREBP2 (Active Motif, monoclonal mouse, 39942).

Proliferation assay

The cell lines transduced with IPTG-inducible ACLY-shRNA (shACLY) constructs were cultivated for 48 hours in normal medium ± 0.5 mmol/L IPTG (Sigma catalog no. l6758). The cells were seeded in normal or lipid-reduced medium ± IPTG. Growth curves were constructed by imaging plates using the IncuCyte system (Essen Instruments).

RNA isolation and quantitative real-time PCR

Total RNA from cultured cells was extracted and 3 μg of total RNA served as template for cDNA synthesis using Oligo dT primers and Superscript III reverse transcriptase. Quantitative real-time PCR (qRT-PCR) was carried out on an ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using a qPCR core kit w/o dUTP (Eurogentec). Validated predesigned TaqMan Gene Expression Assays (Applied Biosystems) corresponding to the housekeeping genes TRFC (Hs00951083_m1) and PGK1 (Hs00943178_g1) were used to generate standard curves on serial dilutions of cDNA. After normalization by TRFC or PGK1, the relative expression values were calculated. The other TaqMan Gene Expression assays used in this study are ACLY (Hs00982738_m1), ACSS2 (Hs00218766_m1), FASN (Hs00105622_m1), ACACA (Hs01046047_m1), and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR; Hs00168352_m1).

Examination of cell-cycle regulation by flow cytometry

Corresponding cell lines were preinduced for 48 hours with IPTG in normal growth conditions to ensure silencing of ACLY. The cells were then seeded in lipid-reduced growth conditions and were incubated for 24-hours ± IPTG. Cell-cycle analysis was carried out by using cell-cycle kit according to the manufacturer’s protocol (Guava Technologies).

Apoptosis assay

Corresponding cell lines were preinduced for 48 hours with IPTG in normal growth conditions to ensure silencing of ACLY. The cells were then seeded in lipid-reduced growth conditions and were incubated for 72 hours ± IPTG. Apoptosis was assessed by using the Guava Nexin kit and the Guava PCA system (Guava Technologies) according to the manufacturer’s protocol.

siRNA transfections

Cells were transfected with individual siRNA duplexes at a final concentration of 25 nmol/L using HiPerFect (Qiagen) transfection reagent, following the manufacturer’s reverse transfection protocol.

Lipid synthesis

PC3M cells were seeded in 24-well plates and incubated for 72 hours. A total of 2 μCi/mL of [1-14C] acetate (36 μmol/L final concentration) was added to the cells, and cells were incubated at 37°C for 2 hours. Lipids were extracted using a methanol/chloroform method. Lipids were dissolved in 500 μL hexane. Samples were counted for 14C using a Tri-Carb liquid scintillation counter (PerkinElmer).
Statistical analysis

The results were analyzed by Students t test where applicable. P values less than 0.05 were considered statistically significant. The data presented represent means ± SD, as indicated in the corresponding figure legends.

Results

ACLY knockdown induces proliferation arrest by affecting cell-cycle progression and induction of apoptosis in various cancer cell lines

To assess the effects of ACLY knockdown on tumor cells, we generated 2 independent expression constructs containing IPTG-inducible shRNAs against ACLY (shACLY-17 and 86), as described in Materials and Methods. PC3M, HOP62, and HepG2 cell lines were transduced with these constructs. qPCR analysis revealed that IPTG induction (48 hours) in PC3M-shACLY-17 and 86 cells reduced ACLY expression by 5- and 6-fold, respectively (Fig. 2A). This ACLY silencing was confirmed by Western blot analysis carried out after 72 hours of IPTG induction (Fig. 2B). There was negligible effect on ACLY expression on mRNA and protein level in PC3M-shMock control cell line (Fig. 2A and B). IPTG induction in HepG2- and HOP62-derived shACLY-17 cell lines also resulted in decrease in expression of ACLY on mRNA and protein levels (Supplementary Figs. S1 and S2).

The shRNA-mediated silencing of ACLY showed no effects on growth rates of PC3M-shACLY-17 and 86 cells when cultivated under normal growth conditions (Fig. 2C, Supplementary Fig. S3A–C). However, these cell lines exhibited reduced growth rates upon ACLY-suppression in lipid-reduced growth conditions (Fig. 2C, Supplementary Fig. S3B and C). IPTG induction of mock shRNA in PC3M-shMock cells did not display any effect on proliferation in either normal or lipid-reduced growth conditions (Fig. 2C, Supplementary Fig. S3A and D). The observed reduction in growth rates induced by ACLY-KD was not limited to PC3M-derived cell lines. Similar effects were also observed in shACLY-17 cell lines derived from HepG2 and HOP62 cells (Fig. 3) in lipid-reduced growth conditions. HOP62-shACLY-17 cells also displayed reduced rate of proliferation upon ACLY silencing in normal growth conditions (Fig. 3). However, these effects were much more enhanced in lipid-reduced conditions and were followed by cell death after 48 hours of ACLY silencing in HOP62-shACLY-17 cells (Fig. 3).

It has been previously reported that ACLY deficiency induces apoptosis in cancer cells (31). To quantify the

Figure 2. ACLY silencing induces decreased growth rates in PC3M cells in lipid-reduced growth conditions. Two independent IPTG-inducible expression vectors were constructed for conditional expression of shRNA targeting ACLY and stably integrated into the genome of PC3M cells. After transduction, the independent clones (shACLY-17 and 86) as well as the clone transfected with mock shRNA were cultivated + IPTG (0.5 mmol/L). A, ACLY expression at mRNA level was analyzed by qRT-PCR and normalized to PGK1 (mean ± SD). B, ACLY expression at protein level was analyzed by Western blot analysis. C, proliferation curves for PC3M-shMock, shACLY-17, and 86 clones. Cells were cultivated ± IPTG (0.5 mmol/L), and cell proliferation was monitored by IncuCyte real-time imaging.
percentage of apoptotic cells in ACLY-KD cell population, we carried out Annexin-V and 7-amino actinomycin D (7-AAD) staining. It was observed that in lipid-reduced growth conditions, ACLY downregulation induces apoptosis in PC3M-derived shACLY cells, increasing the number of cells in early- and late-apoptotic stages (Fig. 4A). Similar trends in induction of apoptosis were observed upon ACLY silencing in shACLY cells derived from HOP62 and HepG2 cells (Supplementary Fig. S4). The induction of apoptosis was observed after 72 hours of ACLY knockdown.

The effects of ACLY knockdown on cell-cycle progression were also investigated. ACLY silencing resulted in a decrease in the percentage of cells in the S-phase accompanied by an increase in the percentage of cells in G0–G1 phase (Fig. 4B). The cell-cycle arrest was observed after 24 hours of ACLY knockdown.

ACLY knockdown induces proliferation arrest in cancer cells by targeting multiple downstream pathways

ACLY is involved in fatty acid and mevalonate synthesis pathways, hence its knockdown may affect multiple pathways. Therefore, to elucidate which pathways are most affected by ACLY silencing, different cell populations undergoing ACLY silencing-induced proliferation arrest were exposed to fatty acids (oleic acid), cholesterol, or the isoprenoid intermediates; FPP/GGPP.

Exposure to exogenous cholesterol partially rescued PC3M-shACLY-17 cells from proliferation/cell-cycle arrest and apoptosis induced by ACLY knockdown (Fig. 5A–C). Oleic acid also slightly improved the proliferation rate and helped the cells escape from induction of apoptosis (Fig. 5A–C). FPP supplementation did not recover the cells from antiproliferative effects of ACLY suppression in PC3M-shACLY-17 cells (Fig. 5A–C). GGPP was also ineffective (Supplementary Fig. S5). Treatment of PC3M-shACLY-17 cells with oleic acid (100 μmol/L), cholesterol, GGPP, or FPP (20 μmol/L) in absence of IPTG has negligible effect on proliferation (Supplementary Fig. S6).

Interestingly, different cell lines depict different rescue profiles. In contrast to PC3M cells, HepG2-shACLY-17 cells completely recovered from ACLY silencing-induced growth arrest by oleic acid supplementation, whereas cholesterol supplementation had only a slight effect.

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**Figure 3.** ACLY silencing induces decreased growth rates in HepG2 and HOP62 cells in lipid-reduced growth conditions. Proliferation curves for HOP62 and HepG2-derived shACLY-17 cell lines. Cells were cultivated ± IPTG (0.5 mmol/L), and cell proliferation was monitored.
It suggests that fatty acid supplementation is more effective in HepG2-shACLY-17 cells. Oleic acid or cholesterol supplementation prevented cell death that follows ACLY-KD after 48 hours in HOP62 cells but did not reverse the proliferation arrest induced by ACLY silencing (Fig. 5A). The effect of cholesterol supplementation on cell viability is more long lasting than that of oleic acid supplementation in HOP62-shACLY-17 cells.

These results confirmed our hypothesis that ACLY silencing affects both the cholesterol and the fatty acid synthesis pathways. Although, the extent to which a certain pathway is affected may vary among different cell lines.

Next we sought to determine the rescue potential of oleic acid and cholesterol when their respective pathways were specifically blocked. Soraphen A is a highly potent inhibitor of ACACA, hence it blocks de novo lipid synthesis and induces apoptosis in cancer cells (32). The effects of soraphen A are largely reversed by supplementation of medium with fatty acids (32). In line with these findings, treatment of PC3M cells with soraphen A resulted in cell death (Fig. 5D), and these effects were counteracted by exogenous oleic acid (Fig. 5D). As expected, cholesterol addition had no effect on soraphen A–induced cell death in PC3M cells (Supplementary Fig. S7). Specific blockade of the mevalonate pathway by statin, namely simvastatin, also induced cell death in PC3M cells that was rescued by incubation of cells with cholesterol (Fig. 5E) but was unaffected by oleic acid supplementation (Supplementary Fig. S8). It shows that the blockade of other lipogenic enzymes lying downstream of ACLY in cholesterol or fatty acid synthesis pathways may affect their respective pathways.

ACLY silencing influences the expression profile of downstream lipogenic genes regulated by sterol regulatory element binding proteins

We examined the effect of ACLY silencing on expression levels of downstream lipogenic genes in different cancer cells cultivated in lipid-reduced growth conditions. We found that upon ACLY suppression, the expression of FASN that is one of the main players in the fatty acid synthesis pathway was significantly increased.
by 1.4-, 1.3-, and 1.3-fold in PC3M, HepG2, and HOP62 cells, respectively (Fig. 6A).

The expression profile of HMGCR that is the rate-controlling enzyme of mevalonate pathway is also affected by ACLY depletion. We found that shRNA-mediated silencing of ACLY significantly enhanced the expression of HMGCR by 1.4-fold in PC3M-shACLY-17 cells and by 2-fold in HepG2- and HOP62-derived cells (Fig. 6B).

Expression of FASN and HMGCR is regulated by common family of transcription factors designated as sterol regulatory element binding proteins (SREBP; ref. 33–35). To evaluate the involvement of SREBPs in ACLY silencing–induced upregulation of these genes, we examined the protein expression of mature nuclear form of SREBP-1 and 2 that indicates activation of these proteins. We observed that ACLY silencing resulted in increase in mature nuclear form of SREBP-1 and 2 proteins in PC3M-, HepG2-, and HOP62-derived cell lines (Fig. 6C).

ACSS2, another enzyme that is known to be upregulated in ACLY-deficient hepatocytes (36), is also regulated by SREBPs (37). We examined the expression of ACSS2 in cancer cells in which ACLY was knocked down. It was observed that upon ACLY silencing, ACSS2 expression was also significantly increased by 1.4-fold in PC3M-shACLY-17 cells and by 2.8-fold in HepG2- and HOP62-derived cells (Fig. 6D).

In ACLY-deficient cancer cells, ACSS2 may compensate for the loss of ACLY

The enhanced expression of ACSS2 upon ACLY silencing suggests that in case of ACLY deficiency ACSS2 becomes more relevant. It has been recently reported that in the absence of physiologic ACLY levels, incubation of
cells with supraphysiologic levels of acetate, the substrate used by ACSS2, rescues cells from proliferation arrest or decreased histone acetylation caused by ACLY knockdown (28, 31). We observed that acetate supplementation clearly reverses the growth arrest induced by shRNA-dependent ACLY silencing in PC3M-derived cells (Fig. 7A and B).

To further evaluate the dependence of cancer cells on either of these 2 pathways, we studied the effect of silencing of ACLY, ACSS2, or both on cell viability. In contrast to ACLY silencing that caused significant induction of apoptosis in PC3M cells, ACSS2 knockdown by 2 independent siRNAs had less of an effect on cell viability. However, coinhibition of ACLY and ACSS2 caused a dramatic increase in the number of cells in early-apoptotic cell fraction (Fig. 7B). It shows that coinhibition of the 2 pathways known to be involved in cytosolic acetyl-CoA production in cancer cells (5, 27, 38, 39) is highly cytotoxic for the cells.

To confirm the effect of ACLY-KD on acetate-dependent lipid biosynthesis, cells were exposed to stable isotope-labeled acetate, and its incorporation into cellular lipids was measured. As expected acetate-dependent lipid synthesis was significantly increased in PC3M upon ACLY silencing (Fig. 7C).

Discussion
The fact that ACLY is upregulated in several types of cancer cells and that upon inhibition of ACLY, cancer cells undergo proliferation arrest indicate that this enzyme plays an important role in cancer cell progression (5, 18). ACLY converts citrate into acetyl-CoA (6–8) that is an essential substrate for fatty acids synthesis pathway and mevalonate pathway that leads to cholesterol biosynthesis and isoprenylation of various proteins. Hence, ACLY affects several pathways and consequently its suppression will also influence multiple downstream pathways.

In this study, we investigated the effects of shRNA-mediated ACLY silencing in different cell lines derived from different types of tumors. These cell lines were selected on the basis of previous reports that indicated overexpression or increased activity of ACLY in these cell lines (5, 18) and also on our preliminary studies using
ACLY is involved in several pathways; hence, the tumoricidal effects that occur after ACLY blockade may relate to the cellular starvation of the end products of any of these aforementioned pathways. In one of the previous studies, it was reported that fatty acid supplementation does not rescue lung cancer–derived A549 cells from the proliferation arrest mediated by ACLY knockdown (18). We observed that exposure to exogenous oleic acid, a monosaturated fatty acid, completely rescues HepG2 cells from ACLY silencing–mediated growth arrest. However, in PC3M and HOP62 cells, oleic acid supplementation does not completely reverse but partially suppress these antiproliferative effects induced by ACLY deficiency.

Recently, it was reported that the antitumor effects of ACLY inhibition are dramatically enhanced in combination with statins, the cholesterol-lowering drugs that inhibit HMGCR (31). It was speculated that this effect might be due to cholesterol starvation that may interfere with cell growth via the impairment of cell membrane synthesis. To study whether the antiproliferative effects of ACLY suppression were mediated by cholesterol starvation, we supplemented the cells undergoing growth arrest after ACLY silencing with cholesterol. Cholesterol supplementation markedly rescued PC3M and HOP62 cells from ACLY knockdown–mediated growth arrest, whereas HepG2 cells showed only slight effect on cholesterol administration. It shows that multiple downstream pathways could be affected by ACLY suppression in different cell lines. However, the extent to which certain pathway is affected varies from one cell line to another. The
underlying mechanisms that regulate these effects require further elucidation.

Blockade of de novo fatty acid synthesis by inhibiting ACACA, which is one of the key players in this pathway, induces apoptosis in cancer cells. But this effect could be rescued completely by fatty acid supplementation (32). We also observed that cell death induced by soraphen A that is a highly potent inhibitor of ACACA could be rescued by oleic acid supplementation. Cholesterol supplementation has no effect on the cells undergoing apoptosis because of ACACA blockade. On the other hand, inhibition of mevalonate pathway by simvastatin could be rescued by cholesterol but not by fatty acid supplementation. However, as we discussed earlier, the antiproliferative effects of ACLY could be reverted back by cholesterol and/or oleic acid supplementation depending on the cell line, indicating that these effects are mediated by fatty acid synthesis or/and mevalonate pathways.

Perturbation at different steps of the lipogenic pathway could bring about major changes in expression profile of other metabolic genes (36). Here, we found that the expression of FASN, a key player in fatty acid pathways, was upregulated upon ACLY silencing in different cancer cell lines. In addition, the expression of HMGCR that is the rate-limiting enzyme in mevalonate pathway is also significantly enhanced after ACLY knockdown. These transcriptional effects indicate that ACLY deficiency compels the cells to upregulate the expression of downstream genes in fatty acid or cholesterol synthesis pathways to compensate for the loss of ACLY.

Fatty acid and cholesterol biosynthesis pathways are influenced by a single family of transcription factors, namely SREBPs (33). Specific analyses of individual isoforms suggest that SREBP-1 may be selectively involved in activation of genes involved in fatty acid synthesis such as FASN and ACACA (34). SREBP-2 is more selective for genes involved in cholesterol homeostasis such as HMGCR (34, 35). SREBP proteins (125 kDa) are anchored to the endoplasmic reticulum membrane (33). Through proteolytic cleavage, the activated amino-terminus (68 kDa) of SREBP translocates into the nucleus to bind SRE (sterol regulatory element) cis-acting elements and trigger gene expression (40). SRE is found in the promoter regions of genes encoding enzymes for fatty acid and cholesterol biosynthesis (41). We observed that upon ACLY knockdown, there was an increase in the expression of mature nuclear form of SREBP-1 and 2 proteins in PC3M-, HepG2-, and HOP62-derived cells. This indicates that ACLY-induced upregulation of FASN and HMGCR may also be controlled by SREBPs.

The upregulation of downstream metabolic genes after ACLY silencing may partially compensate for the loss of acetyl-CoA production by ACLY. However, to keep fatty acid and mevalonate synthesis pathways running, it is essential to have at least some supply of the precursor acetyl-CoA. Mammalian cells also express another enzyme, namely ACSS2, that catalyses synthesis of acetyl-CoA from acetate (Fig. 1). However, mammalian cells mainly use glucose as their major carbon source and have exposure to only low concentration of extracellular acetate. Moreover, conversion of acetate to acetyl-CoA is an energy-dependent process, whereas ACLY-dependent production of acetyl-CoA from glucose is an energy-producing reaction. It makes ACLY-dependent acetyl-CoA production pathway more preferable in mammalian cells, but the absence of ACLY may derive the cells toward ACSS2-dependent pathway. Our data clearly show that the expression of ACSS2 is highly elevated upon ACLY suppression in all the cell lines tested. We further clarified the importance of ACSS2 upon ACLY silencing by exposing the ACLY-deficient cells to acetate. As expected, the antiproliferative effects of ACLY knockdown were partially recovered by acetate supplementation. The effects of ACLY silencing on the activity of acetate-dependent de novo lipid synthesis was evaluated by determination of 14C-acetate into newly synthesized cellular lipids. As expected, upon ACLY silencing, PC3M cells showed upregulated acetate-dependent de novo lipid synthesis.

In this study, we showed that ACLY silencing clearly induces proliferation arrest and apoptosis in variety of cancer cell lines by affecting multiple downstream pathways. Our data clearly shows that ACLY knockdown also influences the expression of other lipogenic genes. This may indicate the attempts made by the cancer cells to counterbalance ACLY deficiency. In addition, this work also shows the importance of ACSS2 in the absence of ACLY. This study provides a new outlook on the role of ACLY in cancer cell growth. Lipogenesis is an extremely synchronized mechanism, and perturbation at different steps of this pathway cause alterations in expression and activity of related enzymes. Our study indicates that therapeutic targeting of ACLY may not be enough for cancer treatment. However, combinatorial approaches targeting several metabolic enzymes simultaneously may have more beneficial effects. Low-lipid diet may also have synergistic effects together with treatments targeting lipogenesis.

Disclosure of Potential Conflicts of Interest
J.V. Swinnen is a recipient of commercial research grant from Janssen Pharmaceutica and is also a consultant and an advisory board member of Janssen Pharmaceutica.

Authors’ Contributions
Conception and design: N. Zaidi, J.V. Swinnen, K. Smans
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Zaidi
Study supervision: K. Smans
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