Translational Oncology 19 (2022) 101386

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

Acetyl-CoA synthetases ACSS1 and ACSS2 are 4-hydroxytamoxifen responsive factors that promote survival in tamoxifen treated and estrogen deprived cells

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ARTICLE INFO

Keywords:
Breast cancer
Tamoxifen
Acetyl-CoA synthetases
ACSS1
ACSS2
Estrogen receptor-α

ABSTRACT

Acetyl-CoA synthetases ACSS1 and ACSS2 promote conversion of acetate to acetyl-CoA for use in lipid synthesis, protein acetylation, and energy production. These enzymes are elevated in some cancers and important for cell survival under hypoxia and nutrient stress. 4-hydroxytamoxifen (4-OHT) can induce metabolic changes that increase cancer cell survival. An effect of 4-OHT on expression of ACSS1 or ACSS2 has not been reported. We found ACSS1 and ACSS2 are increased by 4-OHT in estrogen receptor-α positive (ER+) breast cancer cells and 4-OHT resistant derivative cells. ERα knockdown blocked ACSS1 induction by 4-OHT but not ACSS2. 4-OHT also induced ACSS2 but not ACSS1 expression in triple negative breast cancer cells. Long-term estrogen deprivation (LTED) is a model for acquired resistance to aromatase inhibitors. We found LTED cells and tumors express elevated levels of ACSS1 and/or ACSS2 and are especially sensitive to viability loss caused by depletion of ACSS1 and ACSS2 or treatment with an ACSS2-specific inhibitor. ACSS2 inhibitor also increased toxicity in cells treated with 4-OHT. We conclude ACSS1 and ACSS2 are 4-OHT regulated factors important for breast cancer cell survival in 4-OHT-treated and long-term estrogen deprived cells.

Introduction

Acetate metabolism has emerged as an important mechanism for cancer cell survival and a potential therapeutic target [1–3]. Cancer cells can alter their metabolism in response to metabolic stress in ways that include increased uptake and utilization of acetate. Acetyl-CoA synthetases ACSS1 and ACSS2 convert acetate to acetyl-CoA which can then be used in various processes including lipid synthesis, histone acetylation, and ATP generation in the mitochondria [3–10]. Schug et al. reported that metabolic stress caused by hypoxia and low serum caused an increase in acetate uptake and de novo lipogenesis in various cancer cells and xenograft tumors and increased dependency on this lipogenesis for survival [4]. They further showed that ACSS2 expression was increased by hypoxia/low serum and was required for the altered lipid metabolism and survival [4]. In another study, Lakhter et al. reported that a subset of glucose deprived melanoma cell lines depends on acetate metabolism to maintain ATP production and survival [8]. They further showed glucose deprivation increased ACSS1 and ACSS2 expression in these cells and that ACSS1 or 2 depletion reduced melanoma tumor growth [8]. These findings highlight that cancer cell survival in response to metabolic stress includes a switch to acetate metabolism and that this switch is dependent on ACSS1 and/or ACSS2.

Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) and front-line therapy for estrogen receptor-α positive (ER+) breast cancers [11]. TAM is a prodrug that is activated to 4-hydroxytamoxifen (4-OHT) by the CYP2D6 enzyme system. 4-OHT competitively inhibits ERα binding to estrogen and in this way inhibits ERα and estrogen-mediated pathways needed for proliferation and cell survival [11]. Notably however, TAM and 4-OHT can also cause changes in metabolism that can impact cancer cell survival and that can occur independent of ERα [12–14]. For example, several studies have reported that TAM and 4-OHT treatment induces pro-survival autophagy, including in TNBC cells that lack ERα expression [12,15–18]. Daurio found that TAM treatment inhibited mitochondria complex I and oxygen

Abbreviations: tamoxifen, (TAM); 4-hydroxytamoxifen, (4-OHT); long term estrogen deprived, (LTED); acetyl-CoA synthetase, (ACSS); triple negative breast cancer, (TNBC); selective estrogen modulator, (SERM); sterol regulatory binding protein, (SREBP); estrogen receptor-α positive, (ER+); Patient derived xenograft, (PDX); VY-3–249, (ACSS2iA2i); NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, (Nod-scid-GAMMANSG).

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https://doi.org/10.1016/j.tranon.2022.101386
Received 21 February 2022; Accepted 22 February 2022

consumption in TNBC cells and this, in turn, activated AMPK and increased glycolysis and autophagy [12]. Glycolysis inhibitors sensitized TNBC cells to killing by TAM [12]. These findings and others indicate TAM and 4-OHT can induce adaptive alterations in metabolism that increase survival. To date, an effect of TAM treatment on ACSS1/ACSS2 expression or acetate metabolism has not been reported.

In the current report we found ACSS1 and ACSS2 expression are induced in response to 4-OHT in ER+ breast cancer cells and expressed at heightened levels in 4-OHT resistant derivatives of these cells. The induction of ACSS1 expression by 4-OHT appears to be ERα dependent, while the induction of ACSS2 occurs independent of ERα. Long-term estrogen deprivation (LTED) in breast cancer cells is a model for acquired resistance to aromatase inhibitors [22,19]. We found LTED cells and tumors expressed elevated levels of ACSS1 and/or ACSS2 and were especially sensitive to viability loss caused by depletion of ACSS1 and ACSS2 or treatment with an ACSS2-specific inhibitor. We conclude ACSS1 and ACSS2 are 4-OHT regulated factors important for breast cancer cell survival under long-term estrogen deprivation.

Materials and methods

Cells and reagents

MCF7, MDA-MB-231, T47D, and SUM159PT cells were obtained from ATCC. MCF7, TRC, and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). SUM159PT cells were grown Ham's F-12 nutrient mix supplemented with 5% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), HEPES (10 mM), insulin (5 μg/mL), and hydrocortisone (2 mg/mL). T47D cells were grown in RPMI medium with 10% FBS, penicillin, and streptomycin. Cells were plated for 24 h before treatment with vehicle (VE) or 4-hydroxytamoxifen (4-OHT; active metabolite of TAM; Sigma) or ACSS2 inhibitor (VY-3–249, from Selleckchem, referred to as A2i) at the indicated concentrations. For autophagic flux experiments, cells were treated with vehicle, 4-OHT, and A2i alone or in combination for 24 h, followed by 10 nM bafilomycin-A1 (Sigma) treatment for 6 h. MCF7 derived 4-OHT resistant cells (TRC) and MCF7 4-OHT resistant clones were described in Duan et al. [18]. MCF7 LTED and T47D LTED cells were derived by growing parental MCF7 and T47D cell lines in phenol-red free DMEM with 10% charcoal stripped FBS (CSS) for 13 and 17 months, respectively, with daily media change. MCF7 LTED and T47D LTED cells were maintained in phenol-red free DMEM with 10% charcoal stripped FBS.

Immunoblotting

Whole cell extracts were prepared by scraping cells in lysis buffer (150 mM NaCl, 5 mM EDTA, 0.5% NP40, 50 mM Tris, pH 7.5). The extracts were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific). Antibodies to β-Actin (sc-47,778) and ERα (D12; sc-8002) were from Santa Cruz. Antibody for ACSS2 was from Cell Signaling Technology (#3658). Antibody for ACSS1 was from ProteinTech (17,138–1-AP). Antibody for LC3B was from AbCam (ab51520). Primary antibodies were detected via goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Life Technologies), using Clarity chemiluminescence (BIO-RAD). A representative immunoblot of 3 experiments is shown for each experiment. Relative density of proteins was quantified using ImageJ.

Flow cytometry

Cells were harvested and fixed in 25% ethanol overnight for cell cycle analysis. Cells were then stained with propidium iodide (25 μg/mL, Calbiochem) and 8 μg RNAse (Sigma). Flow cytometry was performed on BD FACSCantos II and analyzed using FloJo10 (Treestar Inc.) for percent sub-G1 content. 10,000 events were collected per sample.

Cell viability and proliferation

For drug treatments, cells were plated for 24 h and then treated with vehicle or the indicated drug for 4 days. For siRNA transfections, cells were collected after 5 days. Drug treated and siRNA transfected cells were trypsinized and resuspended. The cells were then diluted 1:2 in trypan blue and then counted on a hemocytometer. Cell number was determined by calculating the total number of viable cells per mL. Percent dead cells was calculated by dividing the number of non-viable by the total number of cells.

siRNA-mediated transient knockdown

Pooled siRNA for ERα (ESR1), ACSS1, and (On-target plus smart pool) and control siRNA (On-target plus siControl non-targeting pool) were purchased from Dharmacon. Single siRNA for ACSS2 and non-targeting siRNA Universal Negative Control #1 (Mission siRNA) were purchased from Millipore Sigma. Cells were plated at ~60% confluency and then transfected according to the manufacturer's guidelines with the indicated siRNA using DharmaFECT I Reagent.

RNA isolation and real-time qPCR analysis

Total RNA was prepared using Total RNA Mini Kit (IBI Scientific). The first DNA strand was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The protocol was performed according to the manufacturer's protocol for both kits. PCR primers for ACSS1, ACSS2, ESR1, and β-Actin (ACTB) are listed in Supplementary Table 1. SYBR Green PCR Kit (Applied Biosystems) was used according to manufacturer guidelines. Real-time quantative PCR was performed on QuantStudio3 (Applied Biosystems) according to manufacturer conditions using the Comparative CT method (melt curve step performed at the end). Relative gene expression was calculated by $\Delta\Delta C_t$ method using β-Actin as the housekeeping gene for normalization. The gene microarray was performed using an Affymetrix transcriptome analysis array (Applied Biosystems).

Patient derived tumor xenograft study

Female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (Nod-Scid-Gamma; NSG) mice purchased from The Jackson Laboratory carrying fragments of TM00386 PDX ER+PR+HER2- PDX (The Jackson Laboratory) were grown with and without supplemental 17β-estrogen pellets (1.7 mg/pellet 90-day release; Innovative Research). When tumors reached a volume of ~200 mm^3, measurements were recorded using a caliper. Once tumors reached a volume greater than 2000 mm^3, mice were sacrificed and tumors were collected in RIPA buffer, homogenized, and sonicated for immunoblotting. These animal experiments were carried out under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Rush University's Comparative Research Center in accordance with IACUC standards for the welfare of animals.

Statistical analysis

One-way analysis of variance with Tukey's post-hoc analysis was used to determine the statistical significance of differences among experimental groups. Student's *t*-test was used to determine the statistical significance between control and experimental groups. An average of three experiments with standard error of the mean (SEM) are shown for all cell cycle analysis, cell count, and cell viability assays. An average of three experiments with standard error of the mean (SEM) are shown for qPCR analysis. A representative immunoblot of 3 experiments is

shown for each experiment.

Results

ACSS1 and ACSS2 are 4-OHT responsive genes

Our lab has a keen interest in gene expression and metabolic changes induced by endocrine therapy (e.g., TAM) and the potential impact of these changes on endocrine therapy sensitivity. MCF7 is a 4-OHT-sensitive and ER+ breast cancer cell line. In previous studies, Duan et al. introduced a cDNA library into MCF7 cells and selected 4-OHT resistant clones [18]. Several genes that conferred 4-OHT resistance were identified. In the current report, we compared gene expression in untreated and 4-OHT treated MCF7 cells and several of the 4-OHT resistant MCF7 clones from the Duan study using a gene expression microarray. Acetyl-CoA synthetase 1 (ACSS1) is an enzyme that localizes in the mitochondria and regulates acetate metabolism by converting acetate to acetyl-CoA [10]. We found ACSS1 mRNA is expressed at elevated levels in 4-OHT resistant clones compared to parental and control (GFP expressing) MCF7 cells and is induced to higher levels by 4-OHT in the 4-OHT resistant clones compared to parental and control MCF7 cells (Fig. 1A).

ACSS2 is a cytoplasmic ACSS isoform responsible for converting acetate to acetyl-CoA in the cytoplasm [10]. We wished to know if ACSS2 was also induced by 4-OHT and expressed at higher levels in 4-OHT resistant cells (the ACSS2 gene was not included in the original microarray). TRC are 4-OHT resistant MCF7 cells that were generated by prolonged treatment of MCF7 cells with low dose 4-OHT [18]. As shown in Fig. 1B–D, both ACSS1 and ACSS2 mRNA and protein were expressed at higher levels basally in TRC compared to MCF7 and were induced to higher levels in TRC cells in response to 4-OHT. The results in Fig. 1 indicate ACSS1 and ACSS2 are 4-OHT responsive genes and are expressed at elevated levels in 4-OHT resistant cells.

4-OHT is an ERα antagonist that competitively inhibits ERα binding to estrogen. However, TAM can also serve as a partial agonist for ERα in certain tissues [20,21]. We took two approaches to ask if ERα is required for ACSS1 and ACSS2 induction by 4-OHT. First, we siRNA depleted ERα in MCF7 cells and measured ACSS1/2 mRNA and protein levels in response to 4-OHT. Non-targeting siRNA served as the control. Fig. 2D and C show successful depletion of ERα. As shown in Fig. 2A–C, ERα (ESR1) knockdown blocked ACSS1 induction by 4-OHT but did not block the induction of ACSS2. This suggests the induction of ACSS1 by 4-OHT is ERα dependent while the induction of ACSS2 by 4-OHT is not.

To examine this further, we next monitored the ability of 4-OHT to induce ACSS1 and ACSS2 expression in triple negative breast cancer cells (TNBC cells), that lack ERα expression, and in an additional ER+ cell line, T47D. As shown in Fig. 3A and B, ACSS1 mRNA and protein were induced by 4-OHT in ER+ MCF7 and T47D cells but not in TNBC cell lines, MDA-MB-231 and SUM159PT. In contrast, ACSS2 mRNA and protein were induced by 4-OHT treatment in both ER+ MCF7 and T47D and also in MDA-MB-231 and SUM159PT cells (Fig. 3A and C). These results are consistent with Fig. 2 and support that ACSS1 induction by 4-OHT requires ERα while ACSS2 induction by 4-OHT does not.

We noted that ACSS1 was expressed at very low levels in the two TNBC cells lines compared to the ER+ MCF7 cells (Fig. 3A and B), suggesting ACSS1 expression may vary between these two breast cancer types. Two approaches were taken to test this possibility. First, we used RNA-seq gene expression data in the Cancer Cell Line Encyclopedia (CCLE) to compare ACSS1 and ACSS2 expression levels in ER+ and ER- cell lines. The results in Fig. 4A show ACSS1 mRNA levels are significantly lower in ER- cell lines compared to ER+ cell lines. ACSS2 mRNA levels were not different between ER- and ER+ cells (Fig. 4B). Next, we compared ACSS1 and ACSS2 mRNA expression levels in ER+ and TNBC breast cancer cases in the TCGA database. As shown in Fig. 4C, ACSS1 mRNA levels were significantly higher in ER+ cases than TNBC cases in TCGA. In contrast, ACSS2 expression was not significantly different in ER+ and TNBC cases (Fig. 4D).

4-OHT antagonizes the effects estrogen by competing for the same estrogen receptors [11]. Thus, we speculated estrogen deprivation may also increase ACSS1 and ACSS2 expression. To examine this, MCF7 were

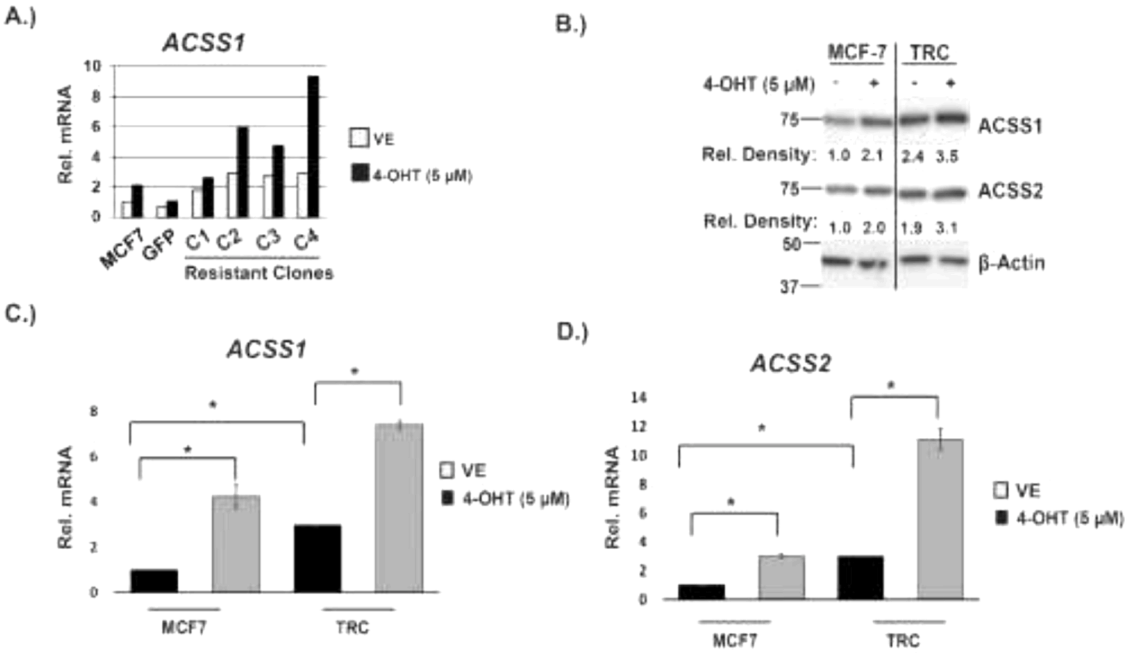

Fig. 1. ACSS1 and ACSS2 are 4-hydroxytamoxifen (4-OHT) responsive genes (A) MCF7 parental and control (GFP expressing) and 4-OHT resistant MCF7 clones (C1, C2, C3, C4; from Duan et al.) were grown for 24 h in the presence of vehicle (VE) or 5 μM 4-hydoxytamoxifen (4-OHT). ACSS1 mRNA levels were determined by quantitative polymerase chain reaction (qPCR). (B) TAM-resistant TRC cells were derived from MCF7 cells and were described previously [18]. ACSS1 and ACSS2 protein levels were determined by immunoblotting. Relative densities of each protein were determined. (C, D) ACSS1 and ACSS2 mRNA expression was determined by (qPCR). There are significant differences ($P < 0.05$) between basal expression of ACSS1 and ACSS2 between MCF7 and TRC; and ACSS1 and ACSS2 are significantly ($P < 0.05$) increased by 4-OHT in MCF7 and TRC cells.

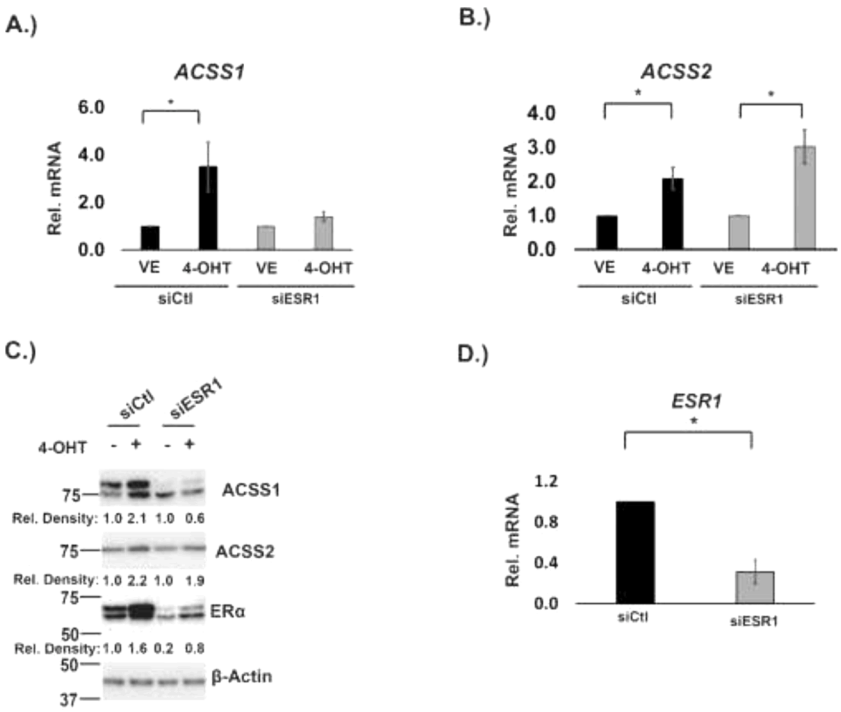

Fig. 2. ACSS1 and ACSS2 are regulated by 4-OHT in ER-dependent and ER-independent manners. (A-D) MCF7 cells were transfected with control siRNA or ERα siRNA for 24 h. Cells were then treated with vehicle (VE) or 5 μM 4-OHT for an additional 24 h. (A, B) mRNA expression was analyzed by qPCR for the indicated genes. (C) Lysates were collected for the indicated proteins. Relative densities of each protein normalized to control is indicated below its blot. (D) ERα (ESR1) mRNA expression was analyzed by qPCR. ACSS1 mRNA expression was significantly ($P < 0.05$) increased by 4-OHT compared to VE in siCtl cells. ACSS2 mRNA expression was significantly ($P < 0.05$) increased by 4-OHT compared to VE in siCtl and siESR1 cells. ESR1 gene expression was significantly ($P < 0.05$) decreased by siRNA.

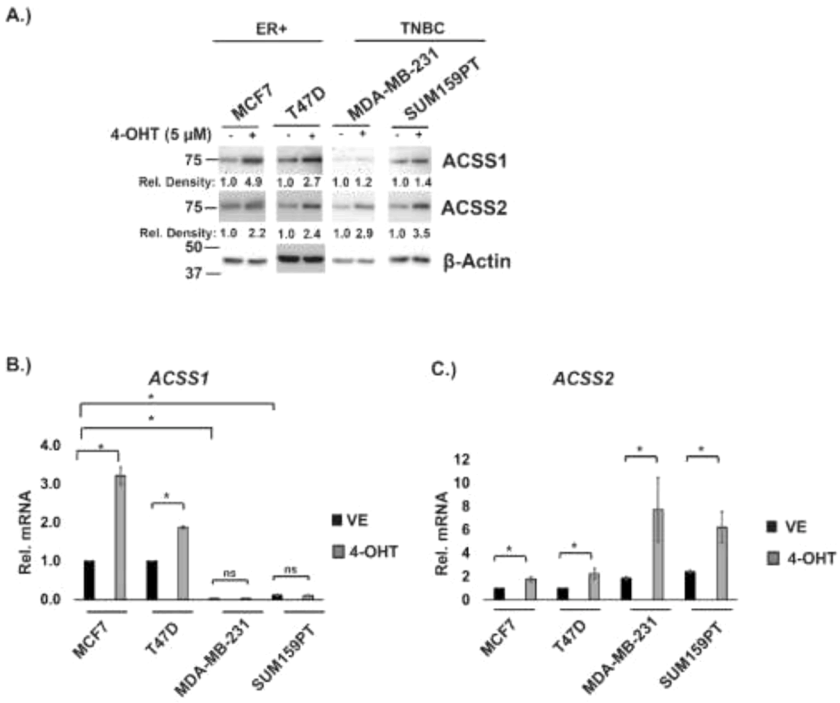

Fig. 3. ACSS2 is increased by 4-OHT in both ER+ and TNBC cells, but ACSS1 is only increased by 4-OHT in ER+ cells. (A-C) Cells were treated with vehicle or 5 μM 4-OHT for 48 h. (A) Lysates were collected for the indicated proteins. Relative densities of each protein normalized to control is indicated below its blot. (B and C) ACSS1 and ACSS2 mRNA expression was analyzed by qPCR. There were significant differences ($P < 0.05$) in induction of ACSS2 in TNBC cells, but no significant differences of induction in ACSS1 in TNBC cells. There was a significant ($P < 0.05$) difference of ACSS1 basal expression between ER+ and TNBC cells.

deprived of estrogen for 24–48 h by culturing the cells in phenol-red free growth medium containing 10% charcoal-stripped fetal bovine serum. As shown in Fig. 5A and B, ACSS1 protein and mRNA expression and to a lesser extent ACSS2 protein and mRNA expression was increased by estrogen deprivation for 24–48 h. These results indicate estrogen deprivation can also induce ACSS1 and ACSS2 expression, similar to 4-OHT treatment. Cell survival of ER+ breast cancer cells under long-term estrogen deprived conditions is a model for acquired resistance to aromatase inhibitors [19,22]. To examine the effect of estrogen deprivation on ACSS1/2 expression further, we compared ACSS1 and ACSS2 expression in parental MCF7 and T47D cells and derivatives of these cells that survived and emerged after long term estrogen deprivation (LTED) of 13–17 months. As shown in Fig. 5C and D, ACSS1 and ACSS2 expression were increased in both LTED cell derivatives compared to their parental counterparts. Thus, both short term and long-term deprivation of estrogen is associated with increased expression of ACSS1 and ACSS2.

Lastly, we wished to know if increased expression of ACSS1 and/or ACSS2 could also be observed in vivo in response to estrogen deprivation. To this end, an ER+ breast cancer patient derived xenograft (PDX)

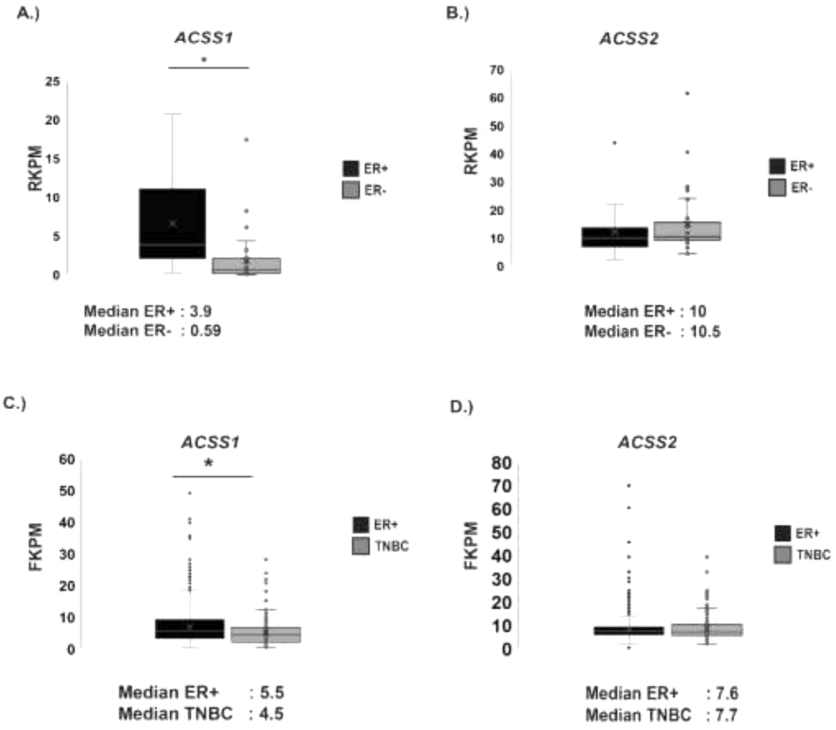

Fig. 4. ACSS1 is expressed at higher levels in ER+ breast cancers compared to ER- breast cancers. (A and B) Analysis of publicly available CCLE breast cancer cell RNA-seq data of mean Reads Per Kilobase Million (RPKM) from 51 breast cancer cell lines (18 ER+; 33 ER-) (C and D) Analysis of publicly available TCGA breast cancer data of mean Fragments Per Kilobase Million FPKM from 502 ER+ tissues and 164 TNBC tissues. There are significant differences ($P < 0.05$) between mean RPKM of ACSS1 expression between ER+ and ER- cell lines. There are significant differences ($P < 0.05$) mean FPKM ACSS1 expression of ER+ and TNBC patient tissue.

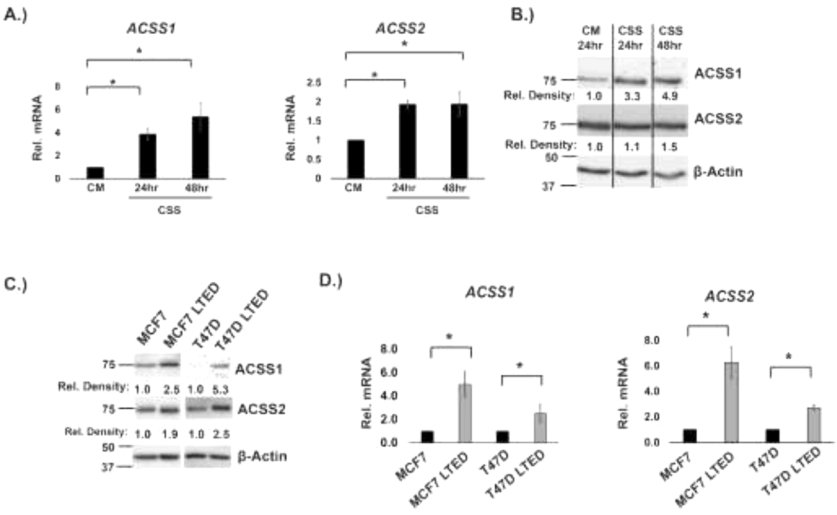

Fig. 5. ACSS1 and ACSS2 are induced by estrogen deprivation in vitro. (A) MCF7 cells were grown in DMEM containing phenol red with 10% normal FBS (CM) or phenol red free DMEM with 10% charcoal stripped FBS (CSS) for 24 or 48 h. (A) mRNA expression was analyzed for ACSS1 and ACSS2. (B) Lysates from similarly grown cells were immunoblotted for ACSS1 and ACSS2. (C and D) ER+ MCF7 and T47D were grown in phenol red-free DMEM with 10% CSS for 17 and 13 months, respectively. (C) Lysates were collected for the indicated proteins. Densities of each protein relative to control is indicated. (D) mRNA expression was analyzed with qPCR for ACSS1 and ACSS2 . Significance ($P < 0.05$) in changes of gene expression between control and estrogen deprived cells is indicated by asterisks.

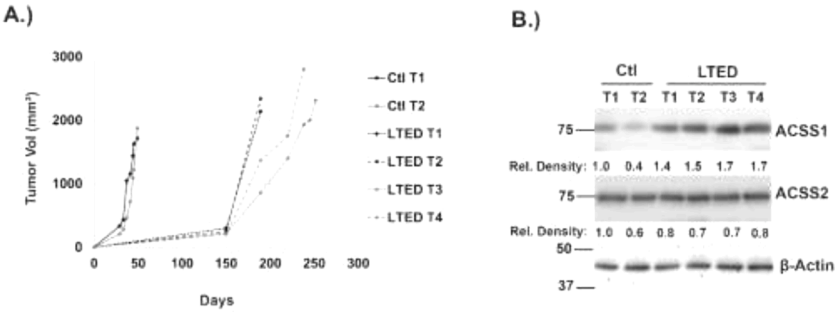

Fig. 6. ACSS1 and ACSS2 are induced by estrogen deprivation in vivo. (A) Female NSG mice were orthotopically injected in the mammary pad with an ER+ patient derived xenograft (PDX) tumor. Mice were supplemented with 17β-estradiol (E2) pellets (Ctl) or not (LTED), and tumor growth (volume) monitored until tumors reached a volume > 2000 mm^3. Mice were then sacrificed. (B) Tumor lysates were collected and analyzed by immunoblotting for the indicated proteins. Relative densities of each protein normalized to control is indicated below its blot.

tumor was first passaged in NSG mice and then tumor growth was monitored in the absence or presence of an estrogen pellet. Tumors were harvested when they reached ~2000 mm^3 or greater, at which point ACSS1 and ACSS2 tumor protein levels were determined. As shown in Fig. 6A and as expected, PDX tumors grew rapidly in the presence of the estrogen pellet (Ctl T1, T2) but slowly in the absence of the estrogen pellet (LTED T1–4). PDX tumors supplemented with estrogen took around 30 days to form a palpable mass, whereas PDX tumor cells failed to form a palpable mass in the absence of estrogen pellet until ~150 days after tumor cell injection. However, after this time tumor growth accelerated, indicating the tumor cells had adapted to grow in the absence of estrogen pellet. These tumors reached ~2000 mm^3 size after ~200 days at which time the tumor was harvested. Immunoblotting of harvested tumor cell lysates showed that ACSS1 expression, though not ACSS2 expression, was elevated these LTED tumors (Fig. 6B). Thus, ACSS1 expression is elevated by estrogen deprivation in ER+ tumors as it is in cell lines.

ACSS2 inhibitors can target LTED cells and increase breast cancer cell killing by 4-OHT

MCF7 LTED and T47D LTED cells express higher levels of ACSS1 and ACSS2 protein compared to their parental counterparts, and LTED tumors also expressed elevated levels of ACSS1. Based on these findings we wished to examine if LTED cells depend on ACSS1 and/or ACSS2 for survival. To address this, we first depleted ACSS1 or ACSS2 in MCF7 and MCF7 LTED cells and then monitored the effects of this depletion on cell proliferation and cell viability. As shown in Fig. 7, siRNA depletion of both ACSS1 and ACSS2 reduced proliferation (Fig. 7A and E) and viability (Fig. 7B and F) slightly in parental MCF7 cells, but depletion caused a more pronounced reduction in proliferation and viability in the MCF7 LTED cells. Cell viability was also assayed by measuring the percentage of cells with sub-G1 DNA content (%sub-G1). The %sub-G1 cells was increased by depletion in MCF7 and MCF7 LTED cells, and more so in MCF7 LTED cells (Fig. 7C and G). Notably, depletion of ACSS2 caused a greater reduction in proliferation, viability, and an increase in cell death than did ACSS1. The results suggest the LTED cells have a greater dependency on ACSS1 and ACSS2 for proliferation and survival than parental MCF7 cells.

Comerford et al. described a small molecule and potent ACSS2 inhibitor, VY-3–249 (A2i) [3]. Because MCF7 and MCF7 LTED cells express high levels of ACSS2 we compared sensitivity of MCF7 and MCF7 LTED cells to this ACSS2 inhibitor. First, we determined the percentage of cells with sub-G1 DNA content before and after treatment as an indicator of cell death. As shown in Fig. 8A, the LTED cells were markedly more sensitive to A2i treatment than parental cells. The MCF7 LTED cells had ~70% of cells with sub-G1 DNA content after A2i treatment compared to ~12% in parental cells, and T47D LTED cells had ~45% of cells with sub-G1 DNA content compared to ~23% in parental cells. The results indicate LTED cells are more sensitive to ACSS2-inhibitor treatment, suggesting they are more dependent on ACSS2 for survival. The LTED cells also showed greater reductions in cell proliferation and viability in response to A2i than did the parental cells (Fig. 8B and C). In total, the results indicate LTED cells express heightened levels of ACSS1 and ACSS2 and have a heightened dependency on these factors for survival.

Lastly, because the 4-OHT resistant TRC cells also expressed heightened basal levels of ACSS2 (Fig. 1), we speculated they may be targeted by A2i alone or combination 4-OHT plus A2i. In MCF7 cells, single treatments with 4-OHT or A2i reduced proliferation and viability, and combined treatment had a greater effect (Fig. 8D and E). As expected, TRC cells (4-OHT resistant) were largely unaffected by 4-OHT alone. However, single treatment with A2i reduced proliferation and viability in these cells, and combined treatment of 4-OHT plus A2i had an even greater effect. The results suggest ACSS2 inhibitors (A2i) can increase sensitivity of ER+ and 4-OHT resistant breast cancer cells to 4-OHT.

Discussion

TAM is a standard-of-care treatment for premenopausal women with ERα + breast cancer. The most well characterized anti-cancer activity of TAM is its ability to antagonize ERα and block estrogen and ERα-mediated proliferation and survival signaling pathways [11]. However, TAM treatment can also increase autophagy in cancer cells and induce metabolic changes that could potentially reduce its effectiveness [17,18,36,37]. ACSS1 and ACSS2 are metabolic enzymes that convert acetate to acetyl-CoA that can then be used for lipid synthesis, protein acetylation, and ATP production in the mitochondria [3–10]. The current report demonstrates for the first time that 4-OHT induces ACSS1 and ACSS2 mRNA and protein expression in breast cancer cells. The induction of ACSS1 by 4-OHT is ERα dependent while the induction of ACSS2 by 4-OHT is not. Estrogen deprived breast cancer cells and tumors express elevated levels of ACSS1 and/or ACSS2 and are especially sensitive ACSS1/2 depletion or treatment with an ACSS2-specific inhibitor. The ACSS2-specific inhibitor also increased cytotoxicity and proliferation loss in ER+ breast cancer cells treated with 4-OHT. We conclude ACSS1 and ACSS2 are 4-OHT regulated factors that increase survival in 4-OHT treated and estrogen deprived breast cancer cells.

TAM is a pro-drug that is metabolically activated by the liver enzyme CYP2D6 into 4-OHT and endoxifen [23]. TAM is typically given as a 20 mg oral tablet daily [24–26]. TAM metabolites 4-OHT and endoxifen reach concentrations greater than 5 μM in tissue [24–26]. Thus, we used a dose of 5 μM 4-OHT in our studies. ERα knockdown blocked ACSS1 induction by 4-OHT and ACSS1 was not induced by 4-OHT in TNBC cells, indicating the ACSS1 induction by 4-OHT is ERα dependent. Estrogen deprivation also induced ACSS1 gene expression. These findings together suggest 4-OHT induces ACSS1 by antagonizing ERα. In contrast, ACSS2 induction by 4-OHT was not blocked by knockdown of ERα, and 4-OHT was able to induce ACSS2 in TNBC cells that lack ERα expression. These results indicate ACSS2 induction by 4-OHT is independent of ERα. Sterol regulatory binding protein (SREBP) transcription factors and liver X receptors (LXRs) regulate genes involved in lipid and cholesterol synthesis and are established activators of the ACSS2 gene [27–29]. Selective estrogen receptor modulators (SERMs), such as TAM, have been shown to disrupt the release of cholesterol from low density lipoproteins (LDLs), which can activate SREBP and LXRs independent of ERα [28–30]. Therefore, we speculated SREBPs and/or LXRs may be involved in regulation of ACSS2 in response to 4-OHT. A pan-inhibitor of SREBPs (Fatostatin) failed to block the induction of ACSS2 or ACSS1 by 4-OHT in our ongoing studies, suggesting 4-OHT does not induce ACSS1/2 expression by activating SREBPs (data not shown). The possible role of LXRs in ACSS2 induction by 4-OHT is unknown. Notably, LXRs can also be regulated by estrogen [30,31]. The fact that both 4-OHT and estrogen deprivation increased ACSS2 expression suggests that 4-OHT induces ACSS2 by antagonizing an estrogen-regulated factor. In addition to ERα, TAM can also bind and/or regulate the expression or activity of other estrogen receptors including ER-β and G-protein coupled estrogen receptor (GPER) [32–34]. We speculate 4-OHT may induce ACSS2 through effects on LXR or these other estrogen regulated factors.

Both 4-OHT and estrogen deprivation increased ACSS1 expression, implying that estrogen signaling through ERα may normally repress ACSS1. Indeed, in our ongoing experiments we found estrogen treatment decreased ACSS1 mRNA and protein levels in hormone deprived MCF7 cells (Supplemental Fig. 1). Thus, a surprising finding from the current study is that ACSS1 expression is higher in ER+ breast cancers compared to TNBCs that lack ERα expression (Fig. 4). This was found to be true both in our analysis of breast cancer cell lines in the CCLE and in breast cancer cases in TCGA. Together the results suggest ACSS1 expression is maintained through one or more unknown factors in ER+ breast cancers and that its expression increases when ERα is inhibited.

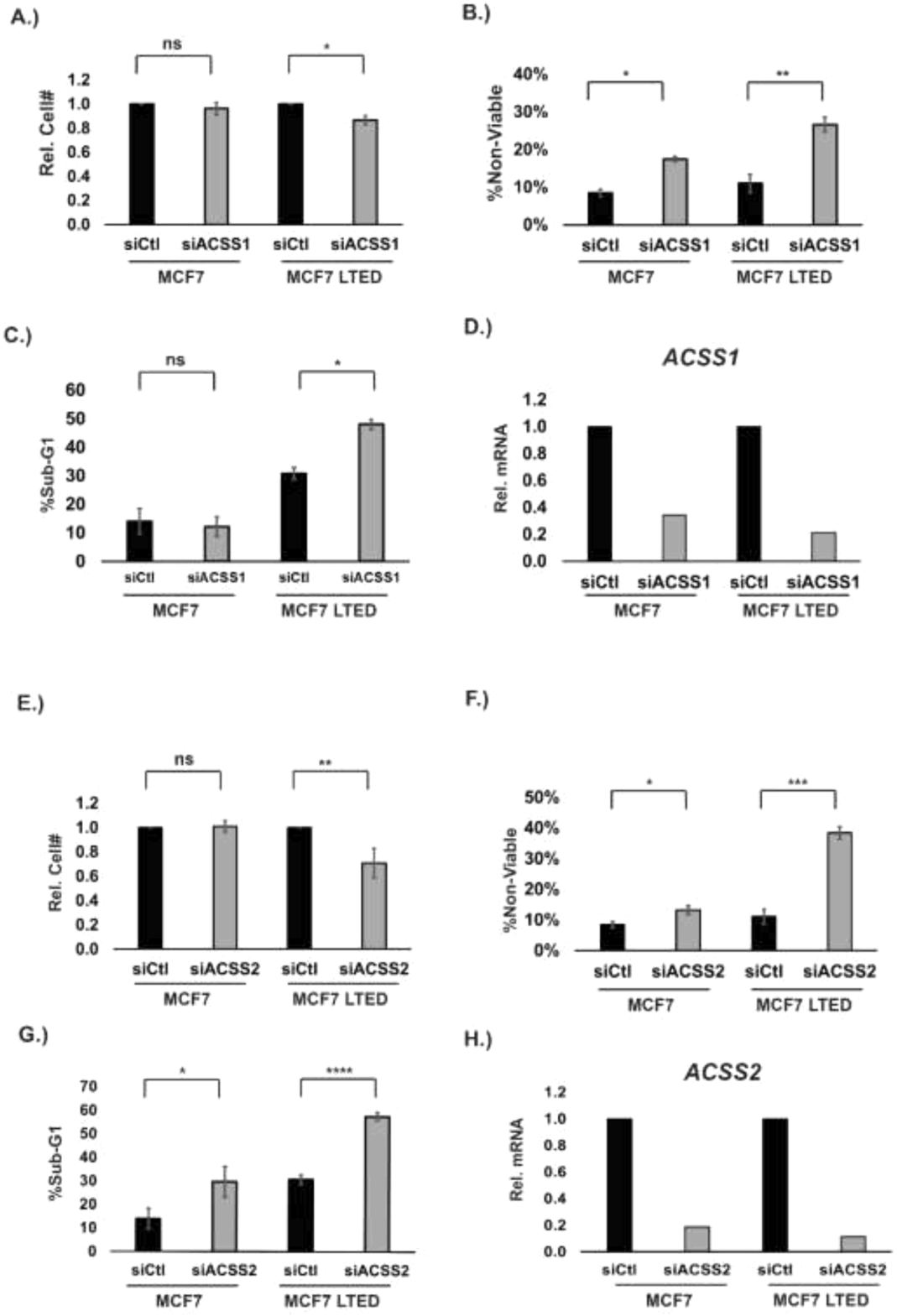

Fig. 7. Depletion of ACSS1 and ACSS2 increases cell death, reduces proliferation, and reduces cell viability. (A-H) MCF7 parental and MCF7 LTED cells were transfected with control siRNA or siRNA against ACSS1 or ACSS2 and then collected for analysis after 5 days. (A) Cells were counted, and relative cell number was plotted. (B) Cell viability was assessed using trypan blue staining to detect non-viable cells. (C) Cells were collected for cell cycle analysis and the percentage of sub-G1 cells plotted. (D) mRNA expression was analyzed by qPCR for the indicated genes. (E) Cells were counted, and relative cell number was plotted. (F) Cell viability was assessed using trypan blue staining to detect non-viable cells. (G) Cells were collected for cell cycle analysis and the percentage of sub-G1 cells plotted. (H) mRNA expression was analyzed by qPCR for the indicated genes. Averages from three separate experiments are presented with standard error of the mean for A, B, C, E, F, and G. Significance of $P < 0.05$ is indicated by one asterisk; significance of $P < 0.01$ is indicated by two asterisks; significance of $P < 0.001$ is indicated by three asterisks; and significance of $P < 0.0001$ is indicated by four asterisks.

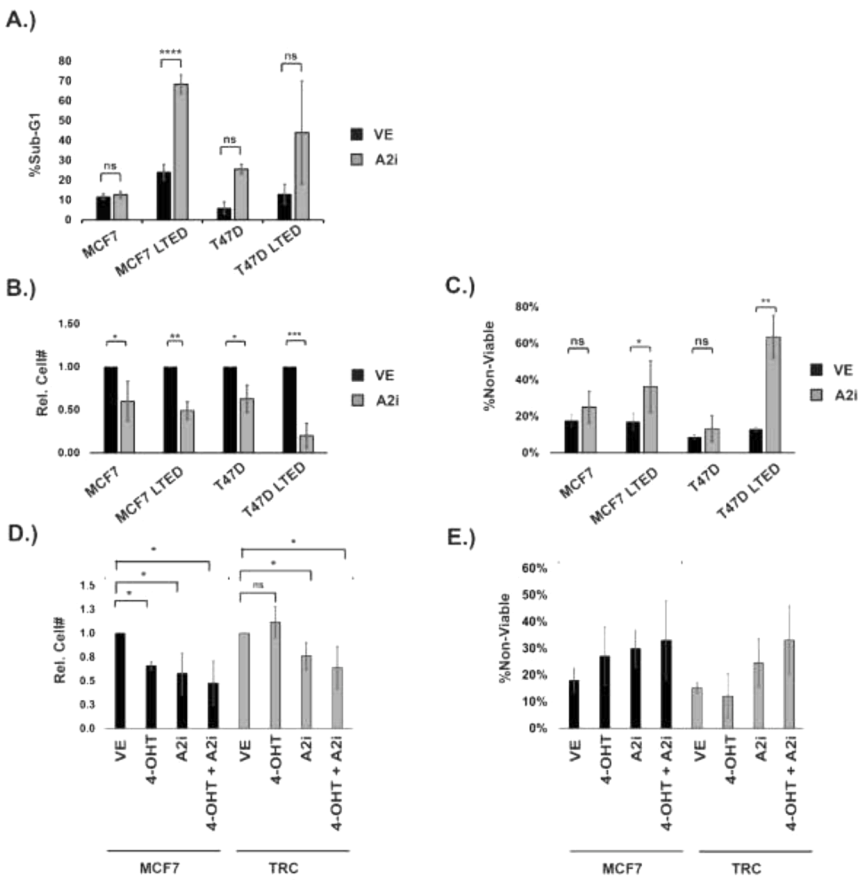

Fig. 8. An ACSS2 inhibitor can target LTED cells and increase breast cancer cell killing by 4-OHT. (A) MCF7, T47D, MCF7 LTED, and T47D LTED cells were treated with vehicle (VE) or 10 μM VY-3–249 (A2i) for 4 days. The percentage of cells with sub-G1 DNA content at this time point was determined. Results from three separate experiments are plotted plus/minus standard error. (B and C) MCF7, T47D, MCF7 LTED, and T47D LTED cells were treated with vehicle (VE) or 10 μM VY-3–249 (A2i) for 4 days. (B) Total cell number was determined, and the relative cell number is plotted. (C) The percentage of non-viable cells was determined by trypan blue staining and is plotted. Results from three separate experiments are plotted plus/minus standard error of the mean. (D and E) MCF7 and TRC cells were treated with vehicle (VE), 5 μM 4-OHT, 10 μM A2i, or both for 4 days. Total cell number was determined, and the relative cell number is plotted in (D). The percentage of non-viable cells was determined by trypan blue staining and is plotted in (E) Results from three separate experiments are plotted plus/minus standard error. Significance of $P < 0.05$ is indicated by one asterisk; significance of $P < 0.01$ is indicated by two asterisks; significance of $P < 0.001$ is indicated by three asterisks; and significance of $P < 0.0001$ is indicated by four asterisks.

The specific role ACSS1 may play in ER+ breast cancers is unknown.

Long-term estrogen deprivation is a model for acquired resistance to aromatase inhibitors in ER+ breast cancer [19,22]. We found a long-term estrogen deprived PDX tumor (ERα +) and two long-term estrogen deprived cell lines (MCF7 LTED, T47D LTED; both ER+) expressed elevated levels of ACSS1 and/or ACSS2. Notably, ACSS1 was increased in the long-term estrogen deprived PDX tumor while ACSS2 was not. This suggests ACSS1 may be more important in an estrogen-deprived in vivo setting, though experiments with more PDXs would be needed to confirm this. Overall, increased ACSS1 and/or 2 appears to be a common feature of breast cancer cells after long-term estrogen deprivation and may be a feature of cells with aromatase inhibitor resistance. We speculated LTED cells may depend on ACSS1 and/or ACSS2 for survival. Consistent with this, depletion of ACSS1/2 or treatment with an ACSS2-specific inhibitor caused a more pronounced loss of viability and proliferation block in LTED cells compared to their parental counterparts. We also found the ACSS2 inhibitor reduced viability and proliferation when combined with 4-OHT in MCF7 cells and their 4-OHT resistant derivatives (TRCs).

ACSS2 has been reported to promote autophagy that can increase cancer cell survival [9,35]. We and others have found that TAM and its metabolites can induce autophagy in breast cancer cells [17,18,36,37]. Thus, we speculated inhibition of ACSS2 could block or reduce pro-survival autophagy. To explore this, we monitored LC3B-II accumulation in cells treated with bafilomycin A1, which is an indicator of autophagic flux. We found that LC3B-II accumulated to a lesser extent in cells treated with bafilomycin A1 and 4-OHT when co-treated with ACSS2 inhibitor (Supplemental Fig. 2). This result supports the possibility that ACSS2 promotes survival in part by maintaining autophagy. In total, our results indicate ACSS1 and ACSS2, and presumably their corresponding effects on acetyl-CoA levels, metabolism, and/or autophagy, promote survival in estrogen deprived and TAM treated cells. Future studies should investigate the mechanisms by which ACSS1/2 may promote autophagy, metabolism, and survival in these cells.

Supplementary material

Supplemental Data Translational Oncology track changes.docx

CRediT authorship contribution statement

Sarah Calhoun: Data curation, Investigation, Writing – original draft. **Lei Duan:** Investigation, Data curation, Visualization, Writing – review & editing. **Carl G. Maki:** Visualization, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported in part by a grant from the National Cancer Institute (R01CA200232–05) and by a DoD breast cancer grant (Grant W81XWH-16–1–0025) to C.G.M.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101386.

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