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Gene Expression Profile and Signaling Pathways in MCF-7 Breast Cancer Cells Mediated by Acyl-CoA Synthetase 4 Overexpression

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Abstract

Aim: Breast cancer comprises a heterogeneous group of diseases that vary in morphology, biology, behavior and response to therapy. Previous studies have identified an acyl-CoA synthetase 4 (ACSL4) gene-expression pattern correlated with very aggressive tumors. In particular, we have used the tetracycline Tet-Off system to stably transfect non-aggressive breast cancer MCF-7 cells and developed a stable line overexpressing ACSL4 (MCF-7 Tet-Off/ACSL4).

As a result, we have proven that cell transfection solely with ACSL4 cDNA renders a highly aggressive phenotype in vitro and results in the development of growing tumors when injected into nude mice. Nevertheless, and in spite of widespread consensus on the role of ACSL4 in mediating an aggressive phenotype in breast cancer, the early steps through which ACSL4 increases tumor growth and progression have been scarcely described and need further elucidation. For this reason, the goal of this work was to study the gene expression profile and the signaling pathways triggered by ACSL4 overexpression in the mechanism that leads to an aggressive phenotype in breast cancer.

Methods: We have performed a massive in-depth mRNA sequencing approach and a reverse-phase protein array using MCF-7 Tet-Off/ACSL4 cells as a model to identify gene expression and functional proteomic signatures specific to ACSL4 overexpression.

Results and Conclusion: The sole expression of ACSL4 displays a distinctive transcriptome and functional proteomic profile. Furthermore, gene networks most significantly upregulated in breast cancer cells overexpressing ACSL4 are associated with the regulation of embryonic and tissue development, cellular movement and DNA replication and repair. In conclusion, ACSL4 is an upstream regulator of tumorigenic pathways. Because an aggressive tumor phenotype appears in the early stages of metastatic progression, the previously unknown mediators of ACSL4 might become valuable prognostic tools or therapeutic targets in breast cancer.

Keywords: Acyl-CoA synthetase 4; Gene signature; Transcriptome; Functional proteomics; Breast cancer

Introduction

Breast cancer remains the second most important cause of death (by cancer) among women [1]. Patients who cannot be cured are those in whom breast cancer has metastasized, that is, breast cancer cells have migrated and invaded other organs such as lung and bone. As no effective therapies are currently available, aggressive breast cancer constitutes a key field for both researchers and clinicians. It has been shown that both in breast cancer cell lines and in tumor samples the expression of acyl-CoA synthetase 4 (ACSL4) is directly correlated with aggressiveness in breast cancer and inversely correlated with estrogen receptor alpha (ERα) levels [2-4]. ACSL4 belongs to a five-member family of enzymes that esterifies mainly arachidonic acid (AA) into acyl-CoA [2-5]. Unlike the other ACSL isoforms, ACSL4 is encoded on the X chromosome and its expression is highest in adrenal cortex, ovary and testis [6-10], as well as in mouse and human cerebellum and hippocampus [11]. Studies on the physiological functions of ACSL4 have revealed possible roles in polyunsaturated fatty acid metabolism in brain, in steroidogenesis, in eicosanoid metabolism related to apoptosis and embryogenesis [8-14]. ACSL4 expression has also been associated with non-physiological functions such as mental retardation disorder [15,16] and cancer [2,3,17,18]. ACSL4 was first associated with cancer due to its abnormal expression in colon and hepatocellular carcinoma. Increased ACSL4 expression, both at mRNA and protein levels [18], in colon adenocarcinoma cells has been associated with the inhibition of apoptosis and an increase in cell proliferation when compared to adjacent normal tissue. ACSL4 has also been suggested as a predictive factor for drug resistance in breast cancer patients receiving Adriamycin-containing chemotherapy [19].

We have demonstrated a positive correlation of ACSL4 expression and aggressiveness in breast cancer cell lines, with the highest expression found in metastatic lines derived from triple-negative tumor breast cancer (MDA-MB-231 and Hs578T) [3]. Functionally, we have found that ACSL4 is part of the mechanism responsible for increased breast cancer cell proliferation, invasion and migration, both in vitro and in vivo [3,4]. We have further demonstrated that ACSL4 can be silenced to reduce cell line aggressiveness. The role of ACSL4 in the development of growing tumors found further support when tumor growth was inhibited through the inhibition of ACSL4 expression [5]. However, even if the role of ACSL4 in mediating an aggressive phenotype in breast cancer is well accepted, its underlying mechanisms have been scarcely explored. For this reason, the goal of this work was to study the gene expression profile and the signaling pathways triggered by ACSL4 overexpression in the mechanism that leads to an aggressive phenotype in breast cancer. Thus, we have performed a massive in-depth mRNA sequencing approach and a reverse-phase protein array using ACSL4-overexpressing MCF-7 cells, as a model to identify gene expression and functional proteomic signatures specific to ACSL4 overexpression.

Materials and Methods

Cell culture

The human breast cancer cell line was generously provided by Dr.

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Vasilios Papadopoulos (Research Institute of the McGill University Health Centre, Montreal, Canada) and obtained from the Lombardi Comprehensive Cancer Center (Georgetown University Medical Center, Washington D.C., USA). The tetracycline-repressible MCF-7 cell lines, designated MCF-7 Tet-Off empty vector, and MCF-7 Tet-Off-induced repression of ACSL4, designated MCF-7 Tet-Off/ACSL4, were obtained previously in the laboratory [3]. The cell lines were maintained in Dulbecco’s modified Eagle (DMEM) medium (GIBCO, Invitrogen Corporation,Grand Island, NY, USA) supplemented with 10% Fetal calf serum (PAA laboratories GmbH,Pasching, Austria) plus 100 U/ml penicillin and 10 mg/ml streptomycin (GIBCO, Invitrogen Corporation,Grand Island, NY, USA). Doxycycline (Sigma Chemical Co.,St. Louis, MO, USA), a more stable tetracycline analogue, was used to regulate the expression of the Tet-Off system. Sterile and plastic material for tissue culture was from Orange Scientific (Braine-l’Alleud, Belgium). All other reagents were of the highest grade available.

**RNA-Seq sample preparation and sequencing**

For each cell line, total RNA was extracted by Direct-zol RNA kit (Zymo Research, Irvine, CA, USA). RNA quality was assessed by agarose gel electrophoresis (visual absence of significant 28S and 18S rRNA degradation) and by spectrophotometric. RNA-Seq was performed by Zymos RNA research facility performing PolYA enrichment of the RNA samples. HiSeq 2 x 50 bp paired-ends reads from RNA-Seq of a human normal-tumor pair samples were analyzed first using the TopHat and Cufflinks software. TopHat (v2.0.6) was utilized for alignment of short reads to GRCh37, Cufflinks (v2.0.2) for isoform assembly and quantification, and commeRbund (v2.0.0) for visualization of differential analysis. Default parameters were used. The RNA-Seq quality control was performed using Dispersion, volcano, MA, Density, PCA, Scatter and Box plots.

**Quantitative reverse transcription-PCR (qRT-PCR)**

MCF-7 Tet-Off empty vector and MCF-7 Tet-Off/ACSL4 total RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer’s instructions. Any residual genomic DNA was removed by treating RNA with DNase I (Invitrogen, Carlsbad, CA, USA) at room temperature for 15 min, which was subsequently inactivated by incubation with 2.5 mM EDTA for 10 min at 65°C. Two µg of total RNA were reverse transcribed using random hexamers and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. For real-time PCR, gene specific primers were obtained from RealTimePrimers.com (Elkins Park, PA, USA). Real-time PCR was performed using Applied Biosystems 7300 Real-Time PCR System and 20 µl of a solution containing 5 µl of cDNA, 10 µM forward and reverse primers, and 10 µl of SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA) for each reaction. All reactions were performed in triplicate. Amplification was initiated by a 2-min preincubation at 50°C, 2-min incubation at 95°C, followed by 40 cycles at 95°C for 15 sec, 55°C for 15 sec and 72°C for 1 min, terminating at 95°C for the last 15 sec. Gene mRNA expression levels were normalized to human 18S RNA expression, performed in parallel as endogenous control. Real-time PCR data were analyzed by calculating the 2−ΔΔCt value (comparative Ct method) for each experimental sample.

**David and Ingenuity Pathways Analysis**

To identify the statistically significant biological functions and signaling pathways affected by the genes differentially expressed in our comparisons, we used Database for Annotation, Visualization and Integrated Discovery (DAVID) [20] and Ingenuity Pathways Analysis (IPA: Ingenuity Systems, Inc., Cambridge, MA, USA) [21]. IPA is the largest curated database and analysis system for understanding the signaling and metabolic pathways, molecular networks and biological processes that are most significantly changed in a dataset of interest. Ranking and significance of the biofunctions and the canonical pathways were tested by the p-value. Additionally, canonical pathways were ordered by the ratio (number of genes from the input data set that map to the pathway divided by the total number of molecules that exist in the canonical pathway). IPA also generated cellular networks where the differentially regulated genes were related according to previously known associations between genes or proteins, but independently of established canonical pathways. Top networks represent associative network functions based on a score that considers the −log (p-value), which aggregates the likelihood that genes in the network are found together due to random chance.

**Reverse phase protein assay (RPPA)**

RPPA was performed in the RPPA Core Facility - Functional Proteomics from MD –Anderson Cancer Center, University of Texas, TX, USA. Cellular proteins were denatured by 1% SDS (with betamercaptoethanol) and diluted in five 2-fold serial dilutions in dilution buffer (lysis buffer containing 1% SDS). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab) by Aushon 2470 Arrayer (Aushon BioSystems) [22].

**Statistical Analysis**

Data analysis was performed using GraphPad InStat Software 3.01 (La Jolla, CA, USA). Statistical significance was determined by analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparison Test and Spearman’s rank correlation coefficient was calculated using Social Science Statistics free statistics software.

**Results and Discussion**

**Differential gene expression triggered by ACSL4**

As ACSL4 plays a crucial role in tumor growth, we have undertaken a systematic study to identify genes with tumorigenesis capacity and elucidate the underlying signaling mechanism. Toward understanding the capacity of ACSL4 in the regulation of tumorigenesis, we have previously reported the involvement of AA lipoxygenase and cyclooxygenase metabolites in the action of ACSL4 [3,4] and, in this report, we focus on the identification of ACSL4-responsive genes using the RNA-Seq in MCF-7 Tet-Off/ACSL4 compared with an MCF-7 Tet-Off empty vector. This system is particularly valuable in enabling both the overexpression and the specific inhibition of ACSL4 through doxycycline treatment and has been previously used to develop tumors in xenograft models [3,4]. Results show a total of 26703566 RNA-Seq reads acquired from MCF-7 Tet-Off/ACSL4 and 22258811 reads acquired from MCF-7 Tet-Off empty vector. We next aligned the sequence reads to a human genome reference (GRCh37) using TopHat version 2.0.6, with results rendering 93.30% of the MCF-7 Tet-Off/ACSL4 reads and 92.90% of the MCF-7 Tet-Off empty vector reads as successfully mapped. The resulting read alignments (file format: BAM) were then assembled through Cufflinks version 2.0.2 for isoform assembly and quantification, and commeRbund (v2.0.0) for visualization of differential analysis (default parameters were used). The RNA-Seq quality control was performed using Dispersion, volcano, MA, Density, PCA, Scatter and Box plots. Gene expression levels were determined by measuring the sum of fragments per kilobase of exon model per million of reads mapped (FPKM), analyzed in each exon. To acquire more accurate results, data were filtered out whenever
estimated FPKM values in both MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector samples were less than 1.0 (cf. an FPKM value of 0.05 is commonly set as the lowest boundary of expression level). Only loci having a log2 (fold change) > 2 between MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector were considered. Ultimately, we observed that, from 32247 successfully sequenced loci, 3944 were significantly and differentially expressed in MCF-7 Tet-Off/ACSL4 samples. Among them, 2501 were upregulated and 1443 were downregulated. ACSL4 gene was one of the genes taken as a control of its overexpression and, as expected, it was one of the genes showing major differences between MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector. Table 1 shows the top genes which exhibit differential changes when ACSL4 is overexpressed. To determine the characteristic chromosomal location of genes controlled by ACSL4 expression, we used the CROC program [23] to examine the expression landscape by plotting the number of differentially expressed genes along the whole chromosomes. Results revealed that chromosome distribution patterns varied greatly with respect to gene density and, in particular, chromosome 1 showed the highest gene density among genes mapped (Figure S1). A total of 52 clusters were found –chromosome 1 showed the highest number of clusters (8)–, while the number of genes in clusters was 181.

**Enriched functional categories of gene networks relating to the transcripts regulated by ACSL4 overexpression**

To gain insights into biological cell properties, IPA was used to rank enriched functional categories of gene networks relating to the transcripts regulated in ACSL4-responsive gene sets acquired from the RNA-Seq data. The highest-scoring associated diseases and disorders are shown in Table 2, while cancer was the disease showing the lowest p-value among diseases and disorders. As a result, we verified 390 top biofunctions concerned with the ACSL4-induced transcriptome alteration in MCF-7 Tet-Off/ACSL4 cells. The most significantly tumorigenesis-related biofunctions (only p-values under 0.01) are shown in Table 3. In agreement with our previous results regarding ACSL4 effect on cell proliferation, invasion and migration [3,4], the top three biofunctions which were IPA-predicted to be increased in RNA-Seq data were cell movement migration and proliferation. Table S1 shows a detailed list of molecules altered in these biofunctions. Table 4 shows the top ten most significantly upregulated functions related to gene networks in the RNA-Seq –along with a list of the corresponding genes in each function network–, while Figure S2 shows the network corresponding to the top ten upregulated functions. DNA replication,
ALTERATION IN MCF-7 TET-OFF/ACSL4 CELLS.

TOP ASSOCIATED DISEASES AND DISORDERS ANALYZED BY IPA. TOP SIGNIFICANTLY ENRICHED PATHWAYS ANALYZED BY IPA.

Table 1: Identification of significantly upregulated and downregulated genes by ACSL4 overexpression using RNA-Seq.

| Gene Expression | transactivation 3.17E-04 | Increased 3.318 | 59 |
| Cellular Assembly | and Organization | of cytoplasm 4.73E-04 | Increased 3.714 | 113 |
| Carbohydrate Metabolism | sultation of polysaccharide 4.39E-04 | Increased 2.132 | 5 |
| Gene Expression | transactivation of RNA 9.23E-04 | Increased 3.718 | 54 |
| Cancer | proliferation of cancer cells 1.05E-03 | Increased 2.009 | 37 |
| Cellular Assembly | and Organization | organization of plasma membrane projections 1.20E-03 | Increased 2.128 | 49 |
| Cellular Growth and Proliferation | proliferation of connective tissue cells 1.45E-03 | Increased 2.628 | 54 |
| Cellular Development | branching of cells 1.60E-03 | Increased 3.043 | 17 |
| Cellular Movement | cell movement of tumor cell lines 2.20E-03 | Increased 2.034 | 67 |
| Cellular Movement | migration of tumor cell lines 2.26E-03 | Increased 2.139 | 55 |
| Cellular Movement | cell movement of tumor cells 2.41E-03 | Increased 2.530 | 19 |
| Cell Morphology | shape change of tumor cell lines 5.81E-03 | Increased 2.180 | 16 |
| Cellular Development | differentiation of stem cells 1.78E-03 | Increased 2.540 | 21 |
| Cancer | prostate cancer and tumors 1.17E-02 | Increased 2.200 | 54 |
| Cellular Assembly and Organization | growth of plasma membrane projections 1.18E-02 | Increased 2.866 | 40 |
| Cellular Movement | migration of tumor cells 1.26E-02 | Increased 2.089 | 20 |
| Cell Morphology | shape change of neurons 1.26E-02 | Increased 2.205 | 6 |

Table 2: Top associated diseases and disorders analyzed by IPA. Top significantly diseases and disorders concerned with the ACSL4-induced transcriptome alteration in MCF-7 Tet-Off/ACSL4 cells.

| Category | Diseases or Functions Annotation | p-value | Predicted Activation State | Activation z-score | # Molecules |
|----------|----------------------------------|---------|---------------------------|-------------------|------------|
| Cellular Movement | cell movement | 3.97E-07 | Increased | 4.685 | 182 |
| Cellular Movement | migration of cells | 4.65E-07 | Increased | 4.677 | 166 |
| Cellular Growth and Proliferation | proliferation of cells | 2.04E-06 | Increased | 2.390 | 295 |
| Cellular Assembly and Organization | formation of cellular protrusions | 7.15E-05 | Increased | 3.830 | 73 |
| Cellular Assembly and Organization | microtubule dynamics | 1.48E-04 | Increased | 4.169 | 94 |
| Cellular Assembly and Organization | organization of cytoskeleton | 1.89E-04 | Increased | 3.662 | 107 |

Table 3: Top associated biofunctions by IPA. Top significantly tumorigenesis-related biofunctions concerned with the ACSL4-induced transcriptome alteration in MCF-7 Tet-Off/ACSL4 cells. Only p-values under 0.01 calculated by right-tailed Fisher’s Exact test were considered significant. IPA uses the activation z-score algorithm to make predictions. The z-score algorithm is designed to reduce the chance that random data will generate significant predictions.
recombination, repair, gene expression and cancer showed the highest score. In particular, the finding that cellular development and embryogenesis are within the top ten biofunctions is interestingly in agreement with recent work showing the crucial role of ACSL4 pathways in embryo development in zebrafish [12] and Drosophila [14,24].

RNA-Seq data confirmed by analysis of gene expression changes by real-time RT-PCR

We further validated the gene expression changes found in RNA-Seq data and IPA by real-time RT-PCR in independent biologic repeats of samples from MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector under the same conditions used for RNA-Seq analysis (relative mRNA expression levels are shown in Figure 1). A comparison of fold changes between RNA-Seq and real-time RT-PCR for each gene is shown in Figure 2, which verifies that the real-time RT-PCR expression profiles were mostly in agreement with RNA-Seq data. Although there were small differences in the fold change values between the two methods of measurement, results were generally highly related (Spearman’s rank correlation coefficient was 0.55525 and the two-tailed value of \( p \) was 0.00001) strongly supporting the reliability of our RNA-Seq analysis. We next focused our attention on categories that were relevant to this study, subgrouped these genes by function and measured an example of each of them (i.e. cytokines, transcription factors, growth factors, integrin family and cytokine, Wnt signaling family, oncogenes, growth factor receptors and energy metabolism).

Most of these genes have central roles in the biology of cancer cells regarding proliferation, migration and invasion. ACSL4 increased the proliferation, migration and invasion. ACSL4 increased the most of these genes have central roles in the biology of cancer cells regarding proliferation, migration and invasion. ACSL4 increased the
throughput antibody-based technique developed for functional protein signature of the ACSL4 pathway by using RPPA, a high-throughput technique. A microarray experiment was performed on lysates derived from MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector. Lysates from doxycycline treated MCF-7 Tet-Off/ACSL4 (Doxy, 1 ug/ml, 48 h) cells, and were subjected to RPPA analysis. Data are presented as fold changes (only results with p-values under 0.05 are shown).

### Table 5: Top small nuclear RNA and microRNA regulated by ACSL4.

| Small nuclear RNA (some examples) | Gene Symbol | Location | Change |
|-----------------------------------|-------------|----------|--------|
| RNA, U12 small nuclear            | RNU12       | Nucleus  | Upregulated |
| RNA, U4 small nuclear             | RNU4-1      | Nucleus  | Upregulated |
| RNA, U4atac small nuclear         | RNU4ATAC    | Nucleus  | Upregulated |
| RNA, USA small nuclear (U12-dependent splicing) | RNU5A-1 | Unknown | Upregulated |
| RNA, USD small nuclear            | RNU5D-1     | unknown  | Upregulated |
| RNA, USE small nuclear            | RNU5E-1     | unknown  | Downregulated |
| small nuclear RNA, H/ACA box 8    | SNORA4      | unknown  | Downregulated |
| Micro RNA (some examples)         | Gene Symbol | Location | Change |
| microRNA 29a                      | miR-29      | Cytoplasm| Upregulated |
| microRNA 1290                     | miR-1290    | Cytoplasm| Upregulated |
| microRNA 25                       | miR-25      | Cytoplasm| Downregulated |
| microRNA let-7a-1                 | let-7       | Cytoplasm| Downregulated |
| microRNA let-7d                    | let-7       | Cytoplasm| Downregulated |

### Signal transduction pathways triggered by ACSL4 overexpression

In order to study the signaling pathway triggered by ACSL4 on the basis of RNA-Seq bioinformatic studies, we next defined a functional protein signature of the ACSL4 pathway by using RPPA, a high-throughput antibody-based technique developed for functional proteomic studies to measure phosphorylation states, as well as total levels of key signaling pathway intermediaries. This RPPA used 217 different antibodies directed to signaling proteins or directed to specific phosphorylated sites known to regulate protein signaling activity [22]. The analysis was performed on lysates derived from MCF-7 Tet-Off/ACSL4, MCF-7 Tet-Off empty vector and doxycycline-treated MCF-7 Tet-Off/ACSL4 cells, the latter used to specifically override ACSL4 expression. The pattern of protein expression and/or phosphorylation was remarkably different between MCF-7 Tet-Off/ACSL4 and MCF-7 Tet-Off empty vector. Lysates from doxycycline-treated MCF-7 Tet-Off/ACSL4 cells showed a pattern similar to that of MCF-7 Tet-Off empty vector, further supporting the role of ACSL4 in the effects observed. Figures 3 and 4 show the proteins that exhibited a significant increase or decrease, respectively, in expression or phosphorylation status. ACSL4 overexpression in MCF-7 breast cancer cells changed the pattern of expression or the pattern of phospho-dephosphorylation of about fifty proteins. These effects were reversed by doxycycline treatment, which confirms the specificity of the functional proteomic signature of ACSL4. We next performed a functional annotation analysis using the bioinformatic program DAVID on the basis of RPPA data. Figure 5 shows the DAVID scheme of pathways in cancer. ACSL4 overexpression stimulated the dephosphorylation of two proteins and the phosphorylation of thirteen proteins, among which it markedly
stimulated the phosphorylation of the mTOR pathway as previously described [22]. In accordance with the results mentioned above, ACSL4 overexpression also stimulates the expression of caveolin. Caveolin-1 is a ubiquitously expressed scaffolding protein which is enriched in caveolae—i.e. subtypes of lipid rafts—and which is involved in several cellular functions such as endocytosis, vesicular transport and signal transduction. Studies also revealed that caveolin-1 is an essential regulator of the invadopodia-mediated degradation of extracellular matrix, which indicates that caveolin-1 plays an essential role in cancer cell invasion [27]. Indeed, at least in breast cancer cell lines, caveolin-1 expression is predominantly observed in invasive cell lines and well correlated with invadopodia activity. These results correlate with those obtained in RNA-Seq, where cell movement showed the highest score (Table 3). Glycogen synthase kinase-3 alpha and beta (GSK3α and GSK3β), critical negative regulators of diverse signaling pathways, are two additional phosphoproteins whose levels exhibit an important increase in response to ACSL4 overexpression, and whose phosphorylation on Ser21 and Ser9, respectively, inhibits GSK3 activity. GSK3 has also been implicated in the negative regulation of FAK (focal adhesion kinase) activity [28]. As GSK3α has been shown to inhibit the Wnt signaling pathway, the inhibition of GSK3α activity by phosphorylation might suggest that Wnt signaling is part of the mechanism of action of ACSL4 overexpression. The aberrant regulation of the Wnt signaling pathway is a prevalent theme in cancer biology. From early observations that Wnt overexpression could lead to malignant transformation of mouse mammary tissue to the most recent genetic discoveries gleaned from tumor genome sequencing, the Wnt pathway continues to evolve as a central mechanism in cancer biology. Results from RNA-Seq also show that ACSL4 overexpression causes a strong reduction in the expression of WIF1. The protein encoded by this gene inhibits Wnt proteins, which are extracellular signaling molecules playing a role in embryonic development. Mammary cancer is a prominent example involving the Wnt pathway, particularly cancers classified as basal-like or triple-negative [29,30] which characteristically involve the expression of Wnt receptor FZD7 [31]. Accordingly, Yang et al. recently reported experiments in which knockdown of FZD7, in cell line models of triple-negative breast cancer, reduced the expression of Wnt target genes, inhibited tumorigenesis in vitro and greatly retarded the capacity of the MDA-MD-231 cell line to form tumors in mice [32]. These results suggest that Wnt ligands might drive certain breast cancers and are consistent with previous work from the Hynes laboratory [33]. In agreement with these data, ACSL4 overexpression increased the expression of WNT6 and WNT10A (Figure 1). The WNT6 gene is overexpressed in cervical cancer cell lines and strongly coexpressed with another family member, WNT10A, in a colorectal cancer cell line. WNT6 overexpression may play a key role in carcinogenesis and is clustered with WNT10A in chromosome 2q35 region. The Wnt pathway is also implicated in the activation of mTORC1 through TSC1/2. In our studies, Wnt signaling inhibited GSK3β, which normally phosphorylates and promotes TSC2 activity, and ACSL4 overexpression produced the stimulation of GSK3 on Ser9, which suggests that this mechanism of mTORC1 activation is also used by ACSL4 [22]. As mentioned above, ACSL4 overexpression increases the phosphorylation of GSK3α and β, and a requirement for ACSL4 has recently been demonstrated in dorsalventral patterning of zebrafish embryo [12] and embryogenesis and neurogenesis in Drosophila [14,24]. These results show that ACSL4 works through the inhibition of AKT-dependent GSK3 activity by increasing its phosphorylation. And, given the interplay between morphogenic signals in developing
embryos, the interaction of these pathways might be expected in cancer. ACSL4 overexpression also stimulates the protein levels of growth factors and their receptors, such as the insulin-like growth factor binding protein (IGFBP2). Among genes involved in cell cycle control, ACSL4 decreases the level of the ataxia telangiectasia (ATM) gene, a kinase that regulates cell cycle checkpoints by phosphorylating multiple proteins including histone H2AX, CHK1 and CHK2 kinases and p53. ATM is activated through auto- or transphosphorylation on Ser1981 and/or Ser1893 in response to DNA damage, particularly the induction of DNA double-strand breaks. ACSL4 also produces a substantial increase in cyclin-B1, the oncogen E3 ubiquitin protein ligase (MDM2), the eukaryotic translation factor 4E binding protein 1 (eEF2) and the GRB2-associated binding protein 2 (GAB2). In the energy area, genes regulated are the acetyl-CoA-carboxylase alpha and beta (ACC) and the succinate dehydrogenase complex subunit a flavoprotein (SDHA), a part of the respiratory chain. ACC participate in fatty acid synthesis and oxidation and are phosphorylated by AMP-activated protein kinase (AMPK) on Ser79 to inhibit their activity. ACSL4 overexpression decreases the activity of AMPK and thus decreases the phosphorylation of ACC. Finally, another gene increased by ACSL4 is the disheveled segment polarity protein 3 (DVL3), a member of a multi-gene family which bears strong similarities with the Drosophila disheveled gene, which in turn encodes a cytoplasmatic phosphoprotein that regulates cell proliferation. The idea of personalized medicine and molecular profiling for prognostic tests has led to a plethora of studies in the past 10 years, in search for genetic determinants of metastatic breast cancer. Such studies have identified gene sets, or "signatures", whose expression in primary tumors is associated with higher risk of metastasis and poor disease outcome for the patients. Current views on cancer cell mutations hold that there are two different types: driver mutations, which are behind cancer growth because they give tumor cells a growth advantage, and passenger mutations, which are just along for the ride. It has been suggested that ACSL4 overexpression is led by a driver mutation [18,34]; however, it is as capable per se of changing cancer cell phenotypes, ACSL4 overexpression may be thought of as a backseat driver factor which generates changes in gene expression and signaling pathways toward a highly aggressive phenotype, and which acts in addition to passenger mutations when the driver gene is mutated. In summary, this study derives an ACSL4 overexpression gene and functional proteomic signature which might reveal important information about novel mediators of breast cancer cell aggressiveness. Here we report that ACSL4 overexpression can trigger several different mechanisms to regulate the aggressiveness of breast cancer cells, including the pathways stimulated by growth factors, nutrients, cytokines and changes in energy metabolism. The major findings of the present study are: (a) ACSL4 overexpression induces changes in genes associated with tumorigenesis-related biofunctions; (b) the four biofunctions with the highest activation z-scores are: cell movement, growth and proliferation, protein and cell assembly and organization; (c) the inhibition of ACSL4 expression completely abolishes the changes observed in protein expression and phosphorylation-dephosphorylation, which demonstrates the specificity of ACSL4 function; (d) since ACSL4 is per se capable of changing cancer cell phenotype, this protein overexpression may be thought of as a backseat driver factor which generates changes in gene expression and signaling pathways toward a highly aggressive phenotype; e) as ACSL4 has been related to colon and hepatocellular carcinoma, besides breast carcinoma, the present findings suggest novel mediators, specifically for combined pharmacological treatment toward tumor growth inhibition. Altogether, the present results open the possibility to use the inhibition of ACSL4 as a therapeutic tool in combination with agents targeting key molecular elements involved in breast cancer. In addition, ACSL4 could be used as a predictive marker that may provide the basis for patient therapy.

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