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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The goal of this proposal was to determine whether there were differences in fatty acid activation between estrogen receptor positive (ER+) and receptor negative (ER-) breast cancer. As a result of support provided by this award, we have been able to expand this investigation to include other hormone receptor biomarkers, and have determined that expression of long-chain fatty acyl-CoA synthetase 4 (ACSL4) is negatively correlated with expression of ER, androgen receptor (AR), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), and in fact functions as a biomarker of quadruple negative breast cancer (QNBC). In addition, we have demonstrated that expression of ACSL4 is able to mediate estrogen resistance. We are currently assessing the utility of using ACSL4 as a biomarker in the prediction of hormone resistance.

15. SUBJECT TERMS
No subject terms provided.

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PROGRESS REPORT

INTRODUCTION

The subject of this project is the role of the fatty acid activating enzyme, long-chain acyl-CoA synthetase 4 (ACSL4), in defining the malignant phenotype in human breast cancer. Fatty acid activation involves the condensation of a CoA molecule with a fatty acid, and is a requirement for subsequent incorporation of the fatty acid into triglycerides and phospholipids as well as for oxidation of fatty acids to provide energy. ACSL4 is one of 5 mammalian isoforms of the enzyme and is characterized by specificity for arachidonic acid. Data derived from mining public information indicates that ACSL4 is differentially expressed in estrogen receptor alpha-negative (ER-) breast cancer. Using this information as a starting point, we have investigated several different aspects of the relationship between ACSL4 expression and various characteristics of breast cancer.

BODY

The research supported by this award for the period August 15, 2010 through August 14, 2011 has resulted in the generation of the data described below. In addition, a preliminary patent application was submitted. The published data, from the previous year, can be found in reference (*1*). The "Statement of Work" outlined in the proposal described 5 tasks associated with 3 specific aims. Progress with respect to each of these tasks is detailed below.

Specific Aim #1: Determination of the specific biochemical function of increased ACSL4 activity in ER- breast cancer cells in tissue culture

Task 1: Develop an MCF-7 cell line with inducible ACSL4 expression
        Having determined that ACSL4 expression was associated with ER- breast tumors and cell lines, we developed an inducible model of ACSL4 expression and performed a microarray analysis comparing mRNA expression in control and induced MCF-7 cells. We noted changes in the expression of thousands of genes as a result of ACSL4 expression, and overall the gene signature of the induced cells was consistent with conversion from a luminal to a basal-like subtype. In addition, the induced cells were no longer estrogen dependent, as shown in figure 1. This was accompanied by a reduction in expression of estrogen, androgen and progesterone receptors. **Thus, ACSL4 expression is not only a marker for hormone resistance, but also a mediator of that resistance.** We are currently in the process of establishing stable MCF-7 sublines that express ACSL4 for use in further evaluating alterations that result from ACSL4 expression.

Task 2: Assessment of lipid metabolism in control- and ACSL4-induced MCF-7 cells

This task has not yet been addressed. Due to the unstable nature of the inducible model   (cloned cells rapidly loss inducibility), we will carry out these experiments using a more stable model that express ACSL4.

Task 3: Determine the effect of knocking down ACSL4 in MDA-MB-231 on lipid metabolism

Using siRNA specific for ACSL4, we were able to reduce expression of ACSL4 protein by >95% as shown in the attached reprint of our recent publication (*1*). Under these conditions, there were no changes noted in either triglyceride or phospholipid metabolism, and no changes in fatty acid oxidation, even using arachidonic acid as substrate (data not shown). We have not yet determined whether the fatty acid profile of the triglycerides and phospholipids is altered, just as we have not yet measured the identities of the specific acyl-CoA's in control and knocked-down cells.

Specific Aim #2: Determination of the potential of ACSL4 as a target for development of chemotherapeutic regimens.

Task 4: Assessment of the role of ACSL4 in mediating cell proliferation, migration and invasion.

These experiments essentially revolve around determining the effect of targeting ACSL4 in breast cancer cells. Two models are utilized: 1] the ACSL4+ cell line, MBA-MB-231, and 2] the ACSL4- cell line, MCF-7. We initially compared cell proliferation, migration and invasion in control and ACSL4 knock-down (KD) MDA-MB-231 cells. As previously demonstrated (*1*), treatment of MDA-MB-231 cells with ACSL4-specific siRNA results in a >95% decrease in expression of ACSL4 protein. This alteration has no effect on the growth rate of the cells, as shown in the same publication.   However, treatment of cells with a pharmacologic inhibitor of ACSL4, triacsin C, which also inhibits ACSL1 and ACSL3, blocked proliferation of the cells. Furthermore, ablation of ACSL4 rendered the cells more sensitive to triacsin C (*1*). In addition, as described in last year's report, both cell migration and invasion are negatively impacted by a reduction in ACSL4 protein.

Alternatively, we measured the effect of induction of ACSL4 expression in MCF-7 cells, and determined that in this situation, ACSL4 appeared to positively impact the growth rate, as documented in last year's report. This effect was especially apparent in estrogen-depleted medium. Taken together, these data suggest that cells normally expressing ACSL4 have additional alterations, such as increased activity in alternate growth pathways, that render the effect of ACSL4 on growth imperceptible. However, cells, such as MCF-7, which are induced to express ACSL4 in isolation, manifest an effect of this expression on growth. This interpretation is supported by our published observation that a boost in the activity of the MAPK pathway in MCF-7 cells caused by overexpression of cRAF results in induction of ACSL4 expression (*1*).

Specific Aim #3: Determination of the prognostic potential of ACSL4 expression

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<u>Task 5: Assessment of ACSL4 protein expression in tissue microarrays of breast tumor samples.</u>

It is in this realm that we have made the most significant advance in the past year. An analysis of expression data for both breast cancer cell lines and tumor samples supports the idea that ACSL4 is a biomarker for quadruple negative breast cancer (QNBC). QNBC is that which is triple negative (ER-, PR- and HER-2-negative) as well as AR-negative. Figure 2 illustrates the correlation between biomarker status and ACSL4 expression in both cell lines and tumor samples (data taken from (2-5). Details of one of the cell line studies is shown in Table 1. Note the data in the rectangles. Our current hypothesis would suggest that those cells which express both a hormone receptor biomarker as well as ACSL4 are hormone-resistant, while those cells that express neither hormone receptor biomarkers nor ACSL4 might have a different phenotype from QNBC that are ACSL4-positive, and may have different treatment sensitivities. Figure 3 illustrates the relationship between ACSL4 expression and molecular subtype in a series of breast cancer cell lines. Again, one can see that ACSL4 expression is negatively correlated with expression of hormone receptor biomarkers, and like CD44$^+$/CD24^{low}, expression of ACSL4 is positively correlated with an aggressive phenotype. Exploring these relationships will be a part of this project going forward.

We are also currently setting up a clinical assay to evaluate ACSL4 expression in breast tumor tissue samples utilizing an immunohistochemical approach. The goal would be to utilize ACSL4 status in predetermining sensitivity to hormonal and other therapies.

KEY RESEARCH ACCOMPLISHMENTS

- Carried out a microarray study documenting changes induced in MCF-7 cells by forced expression of ACSL4. Determined that ACSL4 expression results in loss of estrogen dependence, accompanied by downregulation of ER, PR and AR expression.
- Determined that ACSL4 expression was negatively correlated with QNBC, and hypothesized that ACSL4 might be useful as a biomarker of hormone resistance.
- Submitted a preliminary patent application to use ACSL4 as a breast cancer biomarker in determining treatment options and prognosis.

REPORTABLE OUTCOMES

- Microarray analysis comparing control and ACSL4-induced MCF-7 cells
- Analysis of public expression databases resulted in determination that ACSL4 expression is negatively correlated with QNBC and might be useful as a biomarker.

CONCLUSION

As a result of support provided by this award, we have been able to investigate the relationship between ACSL4 expression and breast cancer phenotype, as well as to begin to address the question of the potential utility of ACSL4 as a biomarker for predicting therapeutic response. We have determined that there is a negative relationship between ACSL4 expression and hormone receptor expression in breast cancer and furthermore that ACSL4 expression might be a useful predictor of response to endocrine therapies. Its presence in tumors expressing one or more receptor biomarker might indicate resistance to endocrine therapies, while its absence in QNBC tumors might be indicative of a phenotype different from ACSL4-positive QNBC with a concomitant altered therapeutic response and/or prognosis. We can envision the possibility of ACSL4 levels being routinely measured in breast cancer samples and the results factored into subsequent treatment decisions.

REFERENCES

1.   **Monaco ME, Creighton CJ, Lee P, Zou X, Topham MK, Stafforini DM** 2010 Expression of Long-chain Fatty Acyl-CoA Synthetase 4 in Breast and Prostate Cancers Is Associated with Sex Steroid Hormone Receptor Negativity. Transl Oncol 3:91-98

2.   **Hess KR, Anderson K, Symmans WF, Valero V, Ibrahim N, Mejia JA, Booser D, Theriault RL, Buzdar AU, Dempsey PJ, Rouzier R, Sneige N, Ross JS, Vidaurre T, Gomez HL, Hortobagyi GN, Pusztai L** 2006 Pharmacogenomic Predictor of Sensitivity to Preoperative Chemotherapy With Paclitaxel and Fluorouracil, Doxorubicin, and Cyclophosphamide in Breast Cancer. J Clin Oncol 24:4236-4244

3.   **Hoeflich KP, O'Brien C, Boyd Z, Cavet G, Guerrero S, Jung K, Januario T, Savage H, Punnoose E, Truong T, Zhou W, Berry L, Murray L, Amler L, Belvin M, Friedman LS, Lackner MR** 2009 In vivo Antitumor Activity of MEK and Phosphatidylinositol 3-Kinase Inhibitors in Basal-Like Breast Cancer Models. Clinical Cancer Research 15:4649-4664

4.   **Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe J-P, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo W-L, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW** 2006 A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10:515-527

5.   **Hu Z, Fan C, Oh D, Marron J, He X, Qaqish B, Livasy C, Carey L, Reynolds E, Dressler L, Nobel A, Parker J, Ewend M, Sawyer L, Wu J, Liu Y, Nanda R, Tretiakova M, Orrico A, Dreher D, Palazzo J, Perreard L, Nelson E, Mone M, Hansen H, Mullins M, Quackenbush J, Ellis M, Olopade O, Bernard P, Perou C** 2006 The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7:96

Supporting Data

Figure 1: Expression of ACSL4 in MCF-7 Cells Causes Hormone Resistance
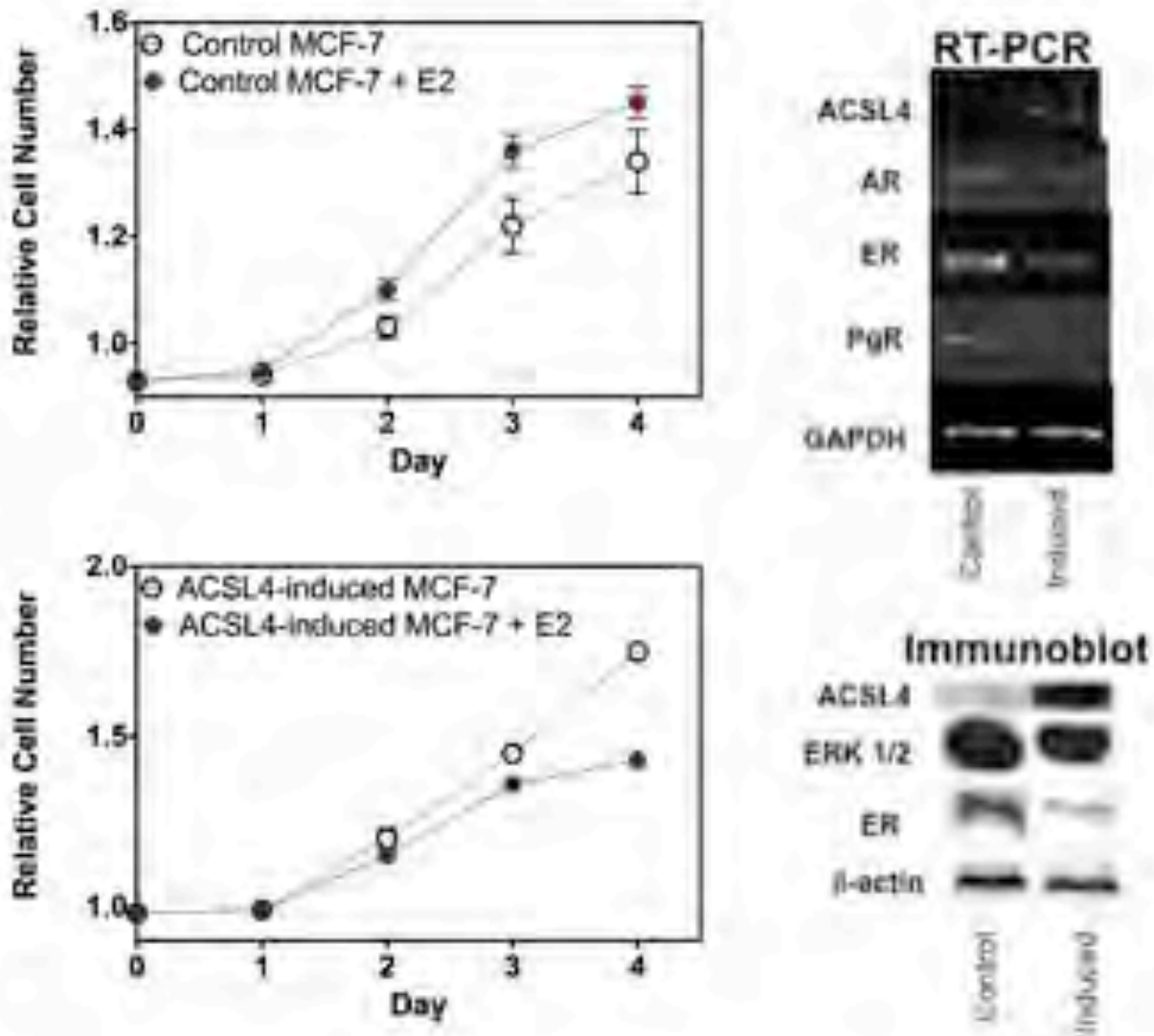
Cells were transfected with ACSL4 cDNA utilizing the TET-On system (Clontech). Stable clones were isolated and doxycycline (1ug/ml) added to induce expression of ACSL4. Proliferation data is comprised of triplicate determinations +/- 1 SD.

Comparison of ACSL4 expression levels in breast cancer cell lines and tumor samples. Panel A compares expression in a series of 50 cell lines as a function of hormone receptor biomarker expression. Data taken from **Neve, RM et al: Cancer Cell 10:515, 2006**. Panel B is comprised of data taken from another cell line study (**Hoeflich, KP et al: Clin Cancer Res 15: 4649, 2009**). Panel C details results from a study of 178 human breast tumor samples (**Hess, KR et al: J Clin Oncology 24: 4236, 2006**).
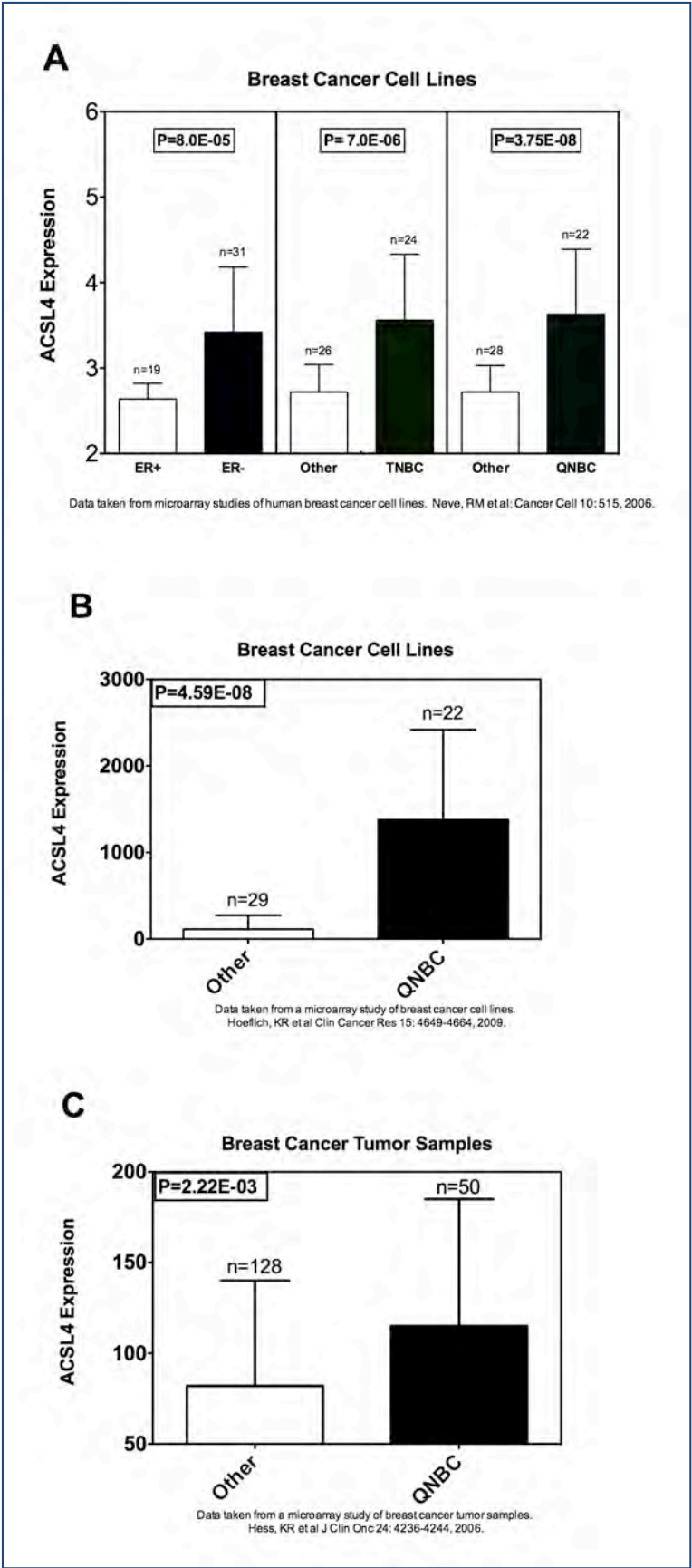

A

Breast Cancer Cell Lines

$P=8.0E-05$ $P= 7.0E-06$ $P=3.75E-08$

ACSL4 Expression

n=19   n=31   n=26   n=24   n=28   n=22

ER+   ER-   Other   TNBC   Other   QNBC

Data taken from microarray studies of human breast cancer cell lines. Neve, RM et al: Cancer Cell 10: 515, 2006.

B

Breast Cancer Cell Lines

$P=4.59E-08$

ACSL4 Expression

n=22

n=29

Other   QNBC

Data taken from a microarray study of breast cancer cell lines.
Hoeflich, KR et al Clin Cancer Res 15: 4649-4664, 2009.

C

Breast Cancer Tumor Samples

$P=2.22E-03$

ACSL4 Expression

n=50

n=128

Other   QNBC

Data taken from a microarray study of breast cancer tumor samples.
Hess, KR et al J Clin Onc 24: 4236-4244, 2006.

Table1: Biomarker Expression in Human Breast Cancer Cell Lines

Green = Positive for one or more receptor biomarkers.

Red = Quadruple negative breast cancer

In cell lines positive for a receptor biomarker, 84% are negative for ACSL4.

In cell lines negative for all four receptor Biomarkers, 86% are positive for ACSL4

Anomalies are in rectangles. Are the green anomalies hormone resistant? Are the red anomalies a distinct basal-like subclass?

Figure 3: Biomarker Expression as a Function of Molecular Subtype

	LUMINAL	CLAUDIN-LOW	BASAL-LIKE

ACSL4
ESR1
ERBB2
AR
PGR
CD44/24

1   2   3   4 5   6 7   8
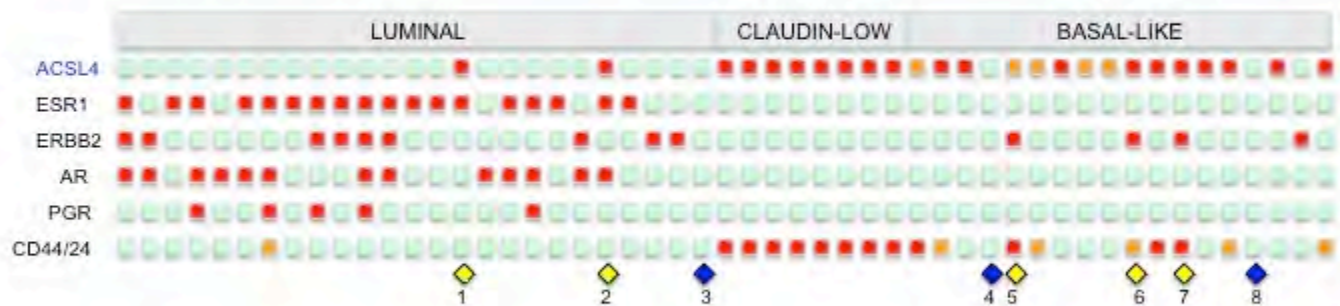
LEGEND:

☐ = negative for mRNA expression or overexpression
🟧 = borderline mRNA expression or overexpression
🟥 = positive for mRNA expression or overexpression
◇ = ACSL4 positive anomaly
◆ = ACSL4 negative anomaly

CELL LINES:
1. ZR75B
2. MDAMB415
3. HCC2185
4. HCC1187
5. HCC1569
6. SUM190PT
7. HCC1954
8. HCC1937

Data from: Neve, RM et al: Cancer Cell 10: 515, 2006.
Prat, A et al: Breast Cancer Research 12: R68, 2010

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