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Functional Characteristics of Tumor Associated Protein Spot14 and Interacting Proteins in Mouse Mammary Epithelial and Breast Cancer Cell Lines

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Thyroid Hormone Responsive Protein Spot14 (S14) is necessary for high rate de novo fatty acid synthesis. Elevated S14 is correlated with reduced disease free survival of women afflicted with breast cancer. The molecular mechanism of S14 remains illusive. Two models exist for S14 function: one suggesting transcriptional events and the other in metabolic processes. Our previous findings showed that S14 does not alter lipogenic gene expression in the cell lines investigated even if driven to the nucleus. S14 overexpression did not affect glycolytic or lipogenic enzyme levels in normal or ErbB2 tumor cells. However, S14 overexpression enhanced accumulation of total lipids evaluated by Bodipy staining and NMR analysis. A major finding in this report is that glycolytic and lipogenic enzyme abundance was altered in a serum dependent manner for normal but not ErbB2 cells. We found that mouse ErbB2 cells do not respond to progestin to induce S14 because they lack the progesterone receptor (PR). In contrast, S14 was sharply induced along with FASN by progestin in T47D human breast cancer cells known to express PR. Progestin stimulated total lipid accumulation and increased total fatty acids in T47D cells. Importantly, 13C(U) glucose tracer studies showed a progestin dependent increase of 13C incorporation into de novo palmitate. Together, these results show that S14 does not alter gene expression but functions to enhance the de novo fatty acid synthesis pathway in a progestin dependent way.

Subject Terms:
THRSP (Spot14), Cancer Metabolism, Fatty Acid Synthesis

Security Classification:
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Introduction

It is widely accepted that cancer cells avidly uptake and consume glucose (1). This increased carbon flux through glycolysis is a well-established characteristic of transformed cells, especially in tumor cells that exhibit high proliferation rates. Because of this phenotype, anabolic metabolism, such as de novo synthesis of fatty acids, is inherently active in most cancer cells (2, 3). Increased levels of de novo fatty acid synthesis have been recognized as a prominent tumor-associated characteristic since the 1950’s (4). More recently, the sole enzyme to synthesize de novo fatty acids, Fatty Acid Synthase (FASN), was observed to be elevated in a wide variety of human cancers by immunohistochemical staining relative to adjacent normal tissue (5). Recently, Thyroid hormone responsive protein spot 14 (THRSP, Spot14, S14) was also shown to be a prognostic indicator of poor clinical outcome (6). Increased S14 and FASN abundance in a various cancers was shown to be predictive of tumor aggressiveness; and, in women afflicted with clinically aggressive breast cancers, S14 and FASN overexpression is highly correlated with reduced disease free survival (7, 8). Much is understood regarding regulation of FASN, but little is known about the regulation of S14 or its molecular mechanism. When S14 is lost due to genomic knock out in mice (9) or siRNA knockdown in hepatocyte cell culture (10), a reduction of de novo fatty acid synthesis follows. Two lines of evidence exist for S14 function in modification of metabolism: one in the nucleus to regulate transcriptional/mRNA processes (11), the other in the cytosol at the protein level to alter lipogenic enzyme activity (12). This study attempts to determine what hormones regulate S14 gene expression, and what potential proteins interact with S14 to confer its function.

Project Review

The broad goals of this project were to examine the functional characteristics of tumor associated S14 and to identify potential interacting proteins to elucidate mechanism. The level of endogenous S14 gene expression and protein abundance is miniscule in all normal mammary and tumor tissue culture cells examined to date. This finding prompted generation of stable, doxycycline (dox) inducible S14 normal CiT3 and ErbB2 tumor cell lines. To test the effect of S14 overexpression on gene regulation, normal mammary epithelial cells under growth conditions stably overexpressing S14 showed only minor differences in expression of glycolytic and de novo fatty acid genes. Only the chief glucose transporter (Glut1), aldolase C, and pyruvate carboxylase had significantly different levels (annual report 2009). Although statistically significant for change, the differences were moderate (< 2-fold) and likely not due to S14 influence as a transcriptional activator. We determined that overexpressed S14 is not nuclear, unless driven to the nucleus by creating a S14-NLS fusion protein. S14-NLS was observed in the nucleus by immunofluorescence and sub-cellular fractionation techniques, but no effects were detected for genes reported as responsive to S14 (annual report 2010).

In addition to gene expression profiling, protein abundance was evaluated by immunoblot. Overexpression of S14 did not vary protein levels of glycolysis and lipogenic pathway enzymes noticeably. No differences were detected in de novo fatty acid gene expression or protein levels due to overexpression of S14 ErbB2 tumor cell lines (annual report 2010). We compared normal serum with delipidated serum (DLS), and DLS +/- Oleic Acid, for effects of S14 overexpression in normal CiT3 and ErbB2 tumor cell lines. We observed no S14 dependent differences at the gene and protein level for de novo fatty acid synthesis pathway components, suggesting that S14 abundance does not alter levels of these factors. However, in normal CiT3 cells, phosphorylated ACLY was decreased in DLS but increased with Oleic Acid when S14 is overexpressed.

Although no direct influence on gene expression or protein abundance due to S14 was observed, neutral lipid staining showed that CiT3 cells overexpressing S14 accumulated more lipid than controls (annual report 2009). Further analysis of the total lipid component using NMR Metabolomics showed significant increases in the quantity of intracellular (CH2) and (CH3) acyl chains (i.e. fatty acids). Together, these data demonstrated that S14 activity occurs not at the transcriptional level to directly influence gene expression, but at the level of enzyme activity to promote anabolic metabolism. We showed that S14 can homodimerize in mouse mammary cells, consistent with findings in the literature. Finally, we determined that S14 over expression conferred a slight growth advantage in CiT3-S14HA cells but not ErbB2 tumor cells with physiologic levels of glucose (annual report 2010). A large amount of evidence was gathered to demonstrate that mouse S14 does not act to regulate transcriptional activation in normal and tumor cell lines. Therefore, I hypothesize that S14 interacts with de novo fatty acid synthesis enzymes to carry out its function.
Body

We profiled the gene expression of S14 and FASN in a variety of different human breast cancer cell lines. Shown in Figure 1 is the mRNA copy number for S14 (left panel) and FASN (right panel) profiled from a broad diversity of human breast cancer cell lines growing under standard media conditions. Note the large variance in gene expression for FASN ranging from 20,000 to 550,000 copies per 25 ng total RNA among all cell lines. The amplitude of S14 gene expression is approximately 1,000 times smaller than FASN levels and is at the cusp of detection for these quantitative PCR conditions (e.g. 25 ng input total RNA). This result supports the previous findings in mouse cell lines wherein S14 gene expression is generally undetectable.

Progestin induces S14 gene expression and lipid accumulation- T47D cell line represents a HER2 negative luminal human breast cancer, and is classified as a lipogenic phenotype breast cancer cell line. We determined that both S14 and FASN are coordinately induced after 24-hour treatment of the synthetic progestin R5020 in T47D breast cancer cells. Figure 2 shows normalized gene expression data for S14 that increased 5.87 fold and FASN that increased 1.3 fold (p < 0.0001) relative to vehicle (ethanol) alone. This observation indicates that progestin alone can induce both S14 and FASN, which may in turn initiate de novo fatty acid synthesis. To examine if the R5020 mediated coordinate increase of S14 and FASN enhanced lipogenesis of T47D cells, we stained for neutral lipid droplets using bodipy. Figure 3 shows a qualitative enhancement of neutral lipid droplet staining (green) in T47D cells following 48 hours of 10 nM R5020 treatment; nuclei are stained with DAPI (blue) for reference. Although more lipid is accumulated in response to R5020 stimulation, bodipy immunofluorescence can not discriminate between cellular fatty acids synthesized via the de novo pathway and fatty acids taken up from the culture medium (serum).


gc-ms chromatograms and mass spectra of fatty acids- In order to discriminate between de novo fatty acids and serum-derived fatty acids (preformed), Gas Chromatography Mass Spectrometry (GC-MS) methods were developed. GC-MS differs from NMR analysis of lipid fractions in that GC-MS distinguishes between fatty acids based on acyl chain length. We tested if the progestin stimulated induction of S14 and FASN in T47D cells promotes de novo fatty acid synthesis using GC-MS to both discriminate and quantify fatty acids from the cell. Chromatography resolves individual fatty acid pentafluorobenzyl esters of discrete chain lengths based on the specific retention time for each.
Individual fatty acid standards were purchased from a commercial vendor, mixed together, and the mixture was subjected to GC-MS analysis. Figure 4 shows the total ion chromatogram (TIC, inset) and the extracted ion m/z spectra for individual fatty acids of various chain lengths (10, 12, 14, 16, and 18) confirming the retention times for fatty acids extracted from the cells. Further, the unique m/z values of internal fatty acid standards distinguish these molecules from unlabeled fatty acids extracted from the cell. These labeled standards consisted of tri-deuterated 10:0 174 m/z, 12:0 202 m/z, 14:0 230 m/z, 18:0 286 m/z, 18:1 283 m/z, di-deuterated 18:2 281 m/z, and 1,2,3,4\(^{13}\)C (4) 16:0 (259 m/z). The mass spectrometer readily differentiated the unique m/z for each heavy atom standard of a given chain length. Adding the heavy atom standard to each sample allows more precise quantitation of fatty acids of a given chain length since the standard and unknown analyte are measured simultaneously.

Briefly, T47D cells were treated for 24 hours +/- R5020 and analyzed for total fatty acid content by extracting the total lipids from the cells. Cells were grown until about 70% confluence in the presence of synthetic progestin R5020 or vehicle. Cells were trypsinized, spun down and washed twice with ice cold PBS before lysis with ice cold 2:1 Methanol-Water. Lysed cells were cleared by centrifugation at 13,000 x G for 10 minutes at 4 C, supernatants were collected in new glass tubes, and total lipids were extracted using 1 mL of 2:1 isoctane-ethyl acetate. Total lipids were taken to dryness using a Savant Speed Vacuum, and lipid pellets were resuspended in 500 uL of methanol. 600 uL of 1 M NaOH was added to saponify the lipids into free fatty acids for 1 hour, afterwards samples were acidified with 600 uL of 1 M HCL and extracted with 1 mL of isoctane. Fatty acids were taken to dryness by vacuum centrifugation and derivatized with 1% pentafluorobenzyl bromine for 1 hour, resuspended in 500 uL of methanol. 600 uL of 1 M NaOH was added to saponify the lipids into free fatty acids.

Fatty acids were taken to dryness by vacuum centrifugation and derivatized with 1% pentafluorobenzyl bromine and 1% N,N-Diisopropylethylamine for 30 minutes at room temperature. Samples were dried down and pellets were resuspended in 100 uL of isoctane for GC-MS analysis.

Fatty acids of the de novo fatty acids synthesis pathway are known to be \( \leq 16 \) carbons in chain length, while fatty acids imported from the serum are known to be \( \geq 18 \) carbons in chain length (called preformed). Total fatty acid analysis refers to quantification of all fatty acids in the cell, including those in triglycerides, membrane phospholipids, lipid rafts, cholesteryl-esters, and free fatty acids. Figure 5 shows the total fatty acid analysis of lipid extracted from T47D cells following 24 hours of vehicle or R5020 for palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), and linoleic acid (18:2). The results show increased amounts of total fatty acids analyzed, with significance for both de novo fatty acids (16:0 and 16:1) and for preformed fatty acids (18:0, 18:1, and 18:2). This result indicates that addition of progestin R5020 is capable of increasing both de novo fatty acid synthesis and accumulation of preformed fatty acids from the serum in T47D cells.

\(^{13}\)C(U) glucose tracer incorporation- We tested whether the progestin mediated increase in palmitic acid (16:0) was specifically due to activity of the de novo fatty acid synthesis pathway. In order to test differences in FASN activity in vivo, a \(^{13}\)C(U) glucose tracer assay was developed that directly measures \(^{13}\)C incorporation into de novo synthesized \(^{13}\)C-palmitate in the context of living cells. This method relies on the unique capability of GC-MS to quantitate \(^{13}\)C incorporation into de novo synthesized palmitate based on discrimination using the
specific m/z of labeled palmitate from unlabeled palmitate (e.g. > 256 m/z for incorporated from the non-incorporated 255 m/z, respectively). Any 13C incorporation will be detected in the mass spectrometer as an increase in the m/z above unlabeled palmitate (less the contribution of naturally occurring 13C). For example, unlabeled palmitate has m/z of 255, and if 10 13C carbon atoms are incorporated the m/z ion is detected at 265 m/z. We chose ubiquitously labeled 13C glucose as a heavy atom tracer to evaluate 13C incorporation into palmitate, because it is well established that cancer cells avidly take up glucose as a source of energy and de novo fatty acid synthesis.

Carbon originating in glucose is modified by the enzymes of glycolysis and ultimately is converted into the common entry molecule to the de novo fatty acid synthesis pathway, citrate. The cartoon in Figure 6 shows many of the enzymes along the glycolysis pathway, including the chief glucose transporter Glut-1, and how the carbon from glucose is converted into citrate. Metabolic intermediate molecules are shown in italics and enzymes are shown in blue. Figure 6 also depicts the uptake of preformed fatty acids from the serum, via the primary fatty acid transporter FatP, and how preformed fatty acids are combined with the de novo fatty acids into a triglyceride droplet for lipid accumulation. The three enzymes of the de novo pathway are ATP Citrate Lyase (Acly), Acetyl-CoA Carboxylase 1 alpha (Acc1α), and Fatty Acid Synthase (FASN). Of the three, FASN is absolutely required for the synthesis of palmitate in mammalian cells.

FASN activity in vivo was evaluated in T47D cells using 5.5 mM 13C(U) glucose for 24-hour treatment time-point (Figure 7). Total fatty acids from 13C(U) glucose treated cells to screen for 13C incorporation into de novo synthesized fatty acids. We found that 13C(U) glucose readily incorporated into palmitic acid extracted from the total lipid fraction after 24-hours of labeled glucose administration. The incorporation of 13C from ubiquitously labeled glucose into palmitic acid is expressed in terms of the Atomic Percent Excess (APE). The changes in APE in figure 7 are significant for + 1 (7%), + 2 (3.5%), and + 3 (3.7%) incorporation of 13C into palmitate (+1, +2, and +3 refer to the number of 13C atoms that have incorporated into the palmitate molecule). Thus, ions in addition to unlabeled palmitate detected (e.g. above natural abundance) can only originate from direct incorporation via cellular de novo palmitate synthesis originally derived via the glycolysis pathway. This result shows that de novo fatty acid synthesis pathway activity is elevated by treatment with R5020 relative to vehicle only.
Key Research Accomplishments

1. S14-HA overexpression did not induce gene expression in CiT3-S14HA cells.
2. NLS-S14 localized to the nucleus, verified using two independent methods.
3. NLS-S14 driven to the nucleus did not induce expression of S14 target genes in CiT3 cells.
4. FLAG-S14 did not induce expression of S14 target genes in CiT3 cells.
5. S14 self associates, as determined by two independent methods; the first report that mouse S14 homodimerizes.
6. The de novo fatty acid synthesis pathway is stabilized when serum lipids are limited in normal CiT3 cells, but that ErbB2 tumor cells did not respond to lipid depleted serum.
7. S14 overexpression conferred a growth advantage when serum lipids were limited only in CiT3-S14HA cells, but not in either 78617-S14HA or 85815-S14HA ErbB2 tumor cells.
8. ErbB2-S14HA tumor cells do not respond to progestin R5020 because they do not express the Progesterone Receptor (PR).
9. Determined that S14 is regulated in human T47D breast cancer cells by the progestin R5020.
10. Showed qualitatively that neutral lipid droplet accumulation is enhanced by R5020 treatment in T47D cells.
11. Learned to independently perform/analyze quantitative GC-MS detection of fatty acids of various chain lengths.
12. Generated palmitate standard regression curve for FASN activity assay and determined optimal internal standard concentration for the FASN assay.
13. Quantified the amounts of total fatty acids from cell extracts using GC-MS technology to show a R5020 dependent increase in the de novo fatty acid palmitate.
14. Developed a method to evaluate FASN enzyme activity by using $^{13}$C(U) glucose tracer to directly quantify $^{13}$C palmitic acid synthesis to show that 13C incorporation into palmitate is enhanced by R5020 in T47D cells.

Reportable Outcomes

1. Data were presented at the Molecular Biology Program seminar (October 2009)
2. Data were presented at the Molecular Biology Program retreat (November 2009)
3. Data were presented at the Gordon research Conference for Mammary Gland Biology (June 2009)
4. Data were presented at the Mammary Gland Program Project Retreat (January 2010)
5. Data were presented in the Pathology Research in Progress Seminar (March 2010)
6. Data were presented at the Breast Cancer Group Seminar (April 2010)
7. Data were presented at the Mammary Gland Program Project Retreat (January 2011)
8. Data were presented in the Pathology Research in Progress Seminar (March 2011)
9. Data were presented at the Breast Cancer Group Seminar (May 2011)
10. Data were presented at the Gordon research Conference for Mammary Gland Biology (June 2011)
11. Data were presented at the Department of Defense Era of Hope (August 2011)
12. Data were presented at the Milk and Lactation Conference (January 2012)

Key Training Accomplishments

1. Learned to perform co-immunoprecipitation techniques
2. Use of contemporary linear ion trap liquid chromatography (LTQ-LC) mass spectrometer (Agilent) for identification of co-IP proteins
3. Learned proteomics software, MASCOT and SCAFFOLD to identify peptide fragments and compare replicate data
4. Learned to perform cell proliferation assays
5. Learned to perform Luciferase reporter assays
6. Capability to measure gene expression at the copy number level
7. Use of state of the art Applied Biosystems 7500 Fast thermocycler for quantitative real-time PCR data acquisition
8. Learned to perform immunofluorescence on paraffin embedded tissue and fixed cell culture samples
9. Use of state of the art Olympus IX81 inverted motorized microscope with spinning disk attachment for deconvolution fluorescent images
10. Learned to extract aqueous and lipid metabolites from cells for NMR metabolomic analysis
11. One- and two-dimensional 1H-MR spectra were obtained using a Bruker 500 MHz DRX spectrometer (Bruker Biospin, Fremont, CA) using an inverse TXI probe. For metabolite identification in water soluble and lipid mammary gland extracts, a two-dimensional (2D)-H, C-HSQC (heteronuclear single quantum correlation) technique was used
12. Learned total lipid extractions in preparations for GC-MS, including saponification of fatty acids from the total lipid fraction.
13. GC-MS sample analysis was performed by negative ion chemical ionization (NICI) GC/MS using the Finnigan DSQ GC-MS system with a ZB-1 column (15 m - 0.25 mm inner diameter 0.10 mm film thickness) from Phenomenex.

Conclusions
The function of tumor-associated protein S14 has long been associated with the synthesis of fatty acids de novo, but little is understood about S14 mechanism. Conflicting paradigms exist regarding the molecular function of S14 and enhanced de novo fatty acid synthesis; one that suggests modification to transcription events in the nucleus (6) and the other suggests S14 works with metabolic proteins (12). In our hands, S14 was never observed to alter the expression of lipid metabolism genes in normal mouse mammary epithelial cells or in mouse ErbB2 mammary cancer cells. Even when S14 was driven to the nucleus, we still did not observe any change in reported S14 responsive genes in these cells. All results to date support that S14 molecular mechanism in mammary cells does not function at the transcriptional level.

Another goal of this project was to investigate what signaling factors regulate the expression to S14. No clear evidence of S14 regulation was observed in mouse cell lines by prolactin, hydrocortisone, R5020, or thyroxine (T3) at the gene expression level and using the human S14 promoter luciferase reporter. However, we determined that S14 is regulated by synthetic progestin R5020 in the T47D human breast cancer cell line. Using this cell line, we showed that S14 was induced nearly 5.8 fold, and that this induction was coincident with a 1.3 fold increase of FASN. This result demonstrates that the progesterone receptor in human T47D cells regulates S14 gene expression.

Previously we showed that S14 overexpression in normal mouse CiT3 cells promoted neutral lipid accumulation based on Nile Red/Bodipy cytoplasmic lipid droplet staining and NMR metabolomics (annual report 2009). Currently, we showed that the progestin R5020 promotes lipid droplet accumulation in T47D cells in a similar way to the S14 overexpressing mouse cells. However, due to difficulty of sample preparation and the duration of running NMR experiments using our core facility, we developed new techniques to continue this study. Hence, GC-MS methods were developed to quantify the total fatty acids extracted from cells as a major tool in evaluating the S14 influence on de novo fatty acid synthesis. In retrospect, we learned that GC-MS is far more informative than NMR for studies of fatty acids. Using GC-MS, we can now quantify simultaneously both the de novo fatty acids and fatty acids from the serum that are preformed. It is not possible using NMR metabolomics to make this distinction between de novo and preformed fatty acids.

Using T47D cells, we determined that progestin treatment stimulated the de novo fatty acid synthesis pathway in two ways: first, we quantified the fatty acids with 16 carbons, which are known to be the primary product of de novo fatty acid synthesis. Secondly, we traced $^{13}$C-glucose incorporation into 16 carbon fatty acids, which must occur via de novo fatty acid synthesis. Both increases in de novo fatty acids were determined to be progestin dependent in T47D cells. We are currently planning experiments to test the role of S14 using knock down constructs to prevent R5020 mediated S14 induction using GC-MS to quantify fatty acids. We will also perform a time-series to determine the optimal $^{13}$C(U) glucose incubation time needed to quantify the rate of $^{13}$C enrichment into de novo palmitate synthesis. We hope to prepare these data for future publication.

It is interesting to note that the level of endogenous S14 is very low in gene expression and not detectable at the protein level in all cultured cells examined. Considering that S14 is dispensable in the majority of tissue...
culture cells we have evaluated to date, further studies of S14 will require experiments using mouse models or human tissue explants, which are outside the scope of this project. For example, binding partners for S14 may not be expressed in tissue culture cells, but instead S14 and interacting proteins could require both the physiology and architecture within the animal. It seems that cell culture systems cannot effectively model the biology of the S14 affected cancers. We have determined that S14 does not influence gene expression, and have acquired some additional correlative data regarding the function of S14 in tissue culture.

References

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Appendix

Conference Abstracts in Chronological Order:

**Rudolph MC**, Serkova NJ, Anderson SM. Spot14, prognostic marker of aggressive breast cancers, has functional interactions with glycolysis enzymes to increase de novo fatty acid synthesis in mammary epithelial cells. *Gordon Research Conference for Mammary Gland Biology, June 2009*


**Michael Rudolph**, Elizabeth Wellberg, Andrew Lewis, Chris Johnson, Robert Murphy, Steven Anderson. THRSP is Necessary for Complete Activation of de novo Fatty Acid Synthesis Enzyme Acetyl-CoA Carboxylase 1 in Lactating Mammary Epithelium. *Mammary Gland Program Project Retreat, January 2011*


**Michael Rudolph**, Elizabeth Wellberg, Andrew Lewis, Andrea Merz, Chris Johnson, Natalie Serkova, Robert Murphy, and Steven Anderson. THRSP is Necessary for Complete Activation of de novo Fatty Acid Synthesis Enzyme Acetyl-CoA Carboxylase 1 in Lactating Mammary Epithelium. *Gordon Research Conference for Mammary Gland Biology, June 2011*


**Michael Rudolph**, Elizabeth Wellberg, Andrew Lewis, Andrea Merz, Chris Johnson, Natalie Serkova, Robert Murphy, and Steven Anderson. THRSP is Necessary for Complete de Novo Fatty Acid Synthesis in Lactating Mammary Epithelium. *Milk and Lactation Conference, January 2012*

Publications in Funding Cycle (Chronological Order):


Positions and Honors:

Teaching Experience: Mentor for pre-graduate students of the Graduate Experiences for Minority Students (GEMS) program and the Minority Access Program (2007-2011).

Honors: Gary J. Miller Award (2010), Outstanding Research by a graduate student. Given by the Faculty of the Pathology Department at the University of Colorado Anschutz Medical Campus.