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Award Number: W81XWH-08-1-0596

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

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REPORT DATE: September, 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2009	Annual Summary	1-Sep-2008 to 31-Aug-2009

4. TITLE AND SUBTITLE
Functional Characteristics of Tumor-Associated Protein Spot14 and Interacting Proteins in Mouse Mammary Epithelial and Breast Cancer Cell Lines

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0596

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Michael C. Rudolph

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AND ADDRESS(ES)
University of Colorado Health Science Center

Aurora CO. 80045

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Material Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Thyroid Hormone Responsive Protein Spot14 (S14) is known to be necessary for high rate de novo fatty acid synthesis in tissues that synthesize lipid; however, S14 is also correlated with poor prognosis of breast cancer. The molecular mechanism of S14 remains illusive, but two paradigms exist: one that implicates S14 in transcriptional events and the other in metabolic processes. In vivo, S14 null mammary epithelial cells (MECs) at lactation day 4 showed changes in gene expression for various glycolysis, pentose phosphate shunt, and de novo fatty acid synthesis mRNAs; but unexpectedly, loss of Spot14 *increased* these mRNAs thereby contradicting previous reports from the literature. Despite literature evidence for direct involvement in regulating gene expression, S14 over-expression studies showed only minor mRNA changes in normal mouse CiT3 cells. S14 may not directly influence mammary epithelial gene expression. In contrast, Nile Red and Bodipy staining of cytoplasmic lipid droplets showed a S14 dependent increase, and metabonomic profiling in short term S14 overexpression revealed significantly increased CH2 acyl lipids. Despite this shift toward lipogenic metabolism, no changes in the abundance of glycolytic or lipogenic enzymes were observed, suggesting that S14 may operate with enzymes. Mass Spectrometry identified a handful of glycolytic enzymes and motor proteins that putatively associate with S14. In CiT3 mammary epithelial cells, S14 seems to function at the protein level along the glycolysis pathway to somehow tip metabolism towards fatty acid synthesis.

15. SUBJECT TERMS
THRSP (Spot14), Cancer Metabolism, Fatty Acid Synthesis,

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*
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Introduction

Cancer cell metabolism differs substantially from quiescent, normal cell metabolism[1]. While regulation of anabolic metabolism (i.e. lipid biosynthesis) is important to normal mammary epithelial function during lactation, breast cancers that are characterized by elevated lipogenesis correlate with reduced disease free survival of afflicted women[2]. Two prognostic indicators are fatty acid synthase (FASN) and thyroid hormone responsive protein Spot14 (S14)[3]. 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 [4] or siRNA knockdown in cell culture[5], 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[6], the other in the cytosol at the protein level to alter lipogenic enzyme activity[7]. The proposed study attempts to understand what factors contribute to the gene expression of S14 and to identify potential S14 interacting proteins that confer its function.

Body

The overarching goals of this proposal are to examine the functional characteristics of tumor associated Spot14 and to identify its interacting proteins. Initially, endogenous mRNA levels of S14 were evaluated. Normal mouse mammary epithelial cells (CiT3), two mouse ErbB2 mammary tumor cell lines (78617 and 85815), and stable rtTA-S14HA CiT3 cells were evaluated for endogenous S14 gene expression (Figure 1). In the normal CiT3 and ErbB2 tumor lines, S14 mRNA was very low; while in the stable cells S14 mRNA was 100 times greater. This expression difference in the stable cell lines is likely slight ‘leakiness’ of the rtTA promoter. Note the two fold increase (p = 0.041) in S14 mRNA in normal CiT3 cells between growth (GR, Insulin and EGF) and “differentiation media” (DIP, Dexamethasone, Insulin and Prolactin). Interestingly, the increase in rtTA-S14HA transgene mRNA between growth and differentiation was nearly two fold and also significant (p = 0.026). Increased in S14 mRNA is consistent with previous gene expression measurements of S14 in the mouse mammary epithelium between pregnancy and lactation[8].

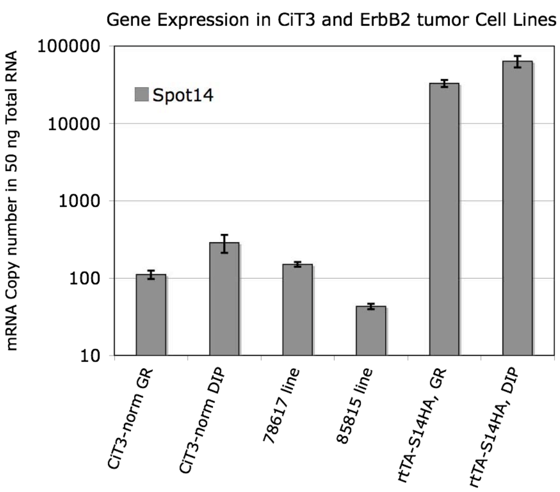


Figure 1. Quantitative PCR gene expression of mouse Spot14 in normal CiT3, breast cancer cell lines (78617, 85815), and stable doxycycline inducible CiT3 cell lines (rtTA-S14HA). Normal and rtTA CiT3 cells were examined under growth or differentiation conditions. Data are presented on a log scale due to dynamic range. Error bars are SEM, n = 3.

Measurements of endogenous S14 in normal CiT3 cells and ErbB2 tumor cell lines completed part of task 1 of objective 1 in the original proposal. S14 protein was not detected by immunoblotting in any of these cell lines (data not shown). These data suggest that DIP can induce endogenous S14 mRNA, however, no luciferace reporter assays have been completed. The generation of stable rtTA-S14HA CiT3 cells satisfies part of task 1 & 2 of objective 2, and I have made stable 78617 and 85815 rtTA cell lines, in which I will introduce the pTetSplice-S14HA vector to make stable rtTA-S14HA 78617 and 85815 ErbB2 tumor cell lines.

Conflicting reports in the literature exist regarding the reported nuclear influence of S14 on regulation of metabolic gene expression[3] and the cytosolic effects on synthesis of fatty acids[7]. An early report by Brown et al[5] showed evidence in primary hepatocytes that S14 could function at the pre-translational level to mediate a high glucose/thyroid hormone response. Therefore, the mRNAs measured by Brown et al., plus additional glycolysis and de novo fatty acid biosynthesis genes were selected and evaluated in S14 over-expressing CiT3 cells to determine if S14 affects gene expression. Figure 2 shows qPCR gene expression in either normal or rtTA-S14HA CiT3 cells. Panel A shows that addition of 1.0 ug/mL doxycycline has no effect on the metabolic genes that were measured. Malic Enzyme (ME1) was previously shown to be a target of S14 activity in rat primary hepatocytes[5], and recently Chou et al. showed a S14 dependent increase of ME1 promoter activity using a luciferase reporter in HeLa cells. In CiT3 cells overexpressing S14HA, ME1 mRNA was not affected regardless of growth or DIP condition. The only differential genes in response to S14 over-expression were

Glut1 (Glucose transporter 1), AldoC (aldolase C) and PCx (pyruvate carboxylase). Interestingly, expression differences happened only under growth medium not following DIP medium. This observation likely shows the difference in metabolic demands for cell proliferation (insulin, EGF signaling) as opposed to TAG production following differentiation (insulin, prolactin signaling). Further, no clear trend was established to support S14 as an activator of transcription, suggesting the changes in expression of these genes could be a byproduct of S14 influence in metabolism rather than having a direct influence on transcription.

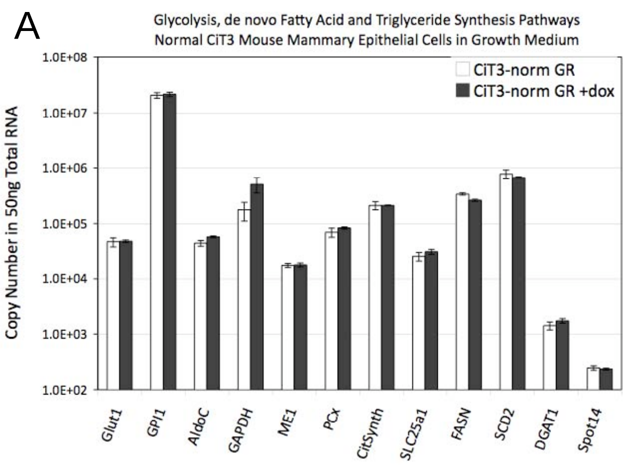
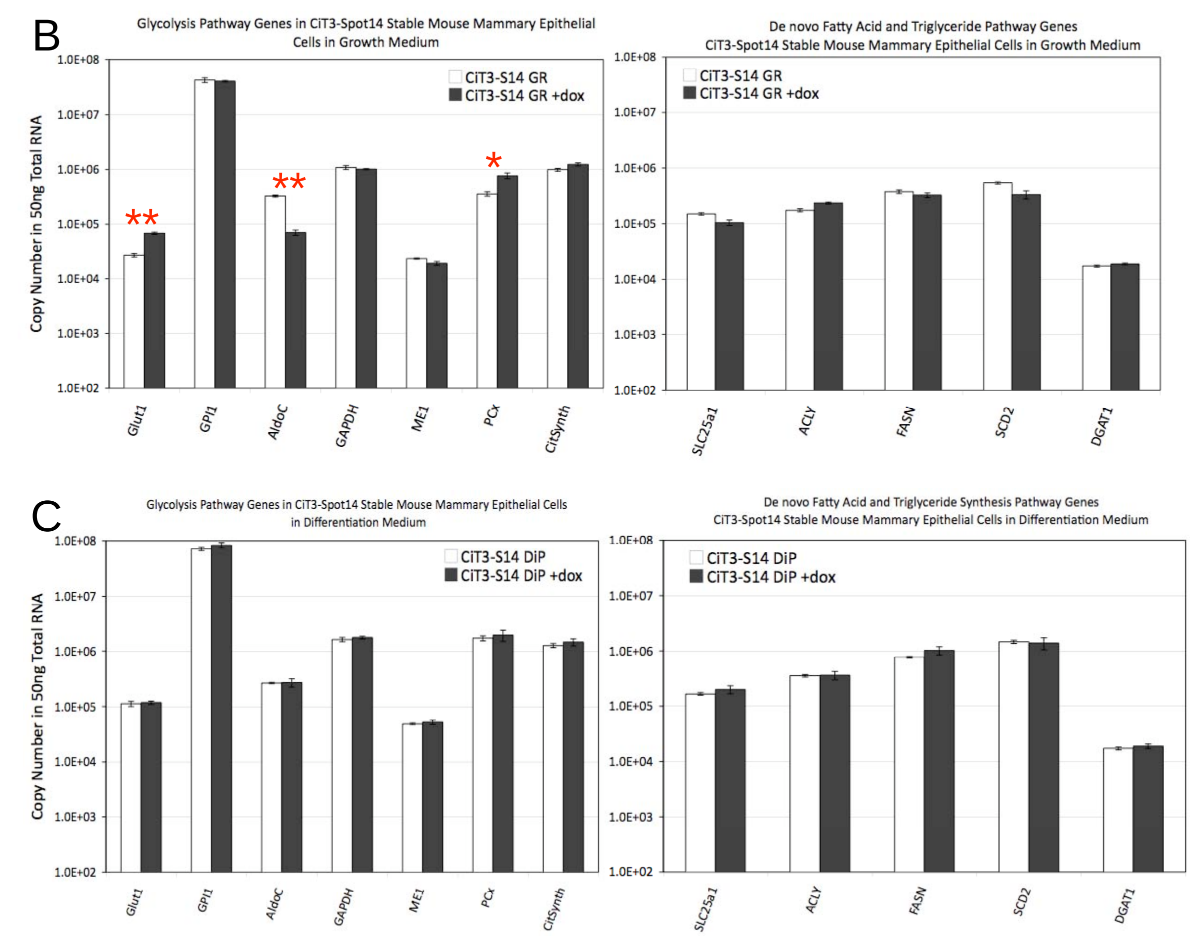


Figure 2. qPCR glycolysis and de novo fatty acid synthesis pathway gene expression in normal and rtTA-S14HA CiT3 cells. Panel A shows that gene expression in normal CiT3 cells is not affected by addition of 1 ug/mL doxycycline to growth medium. Panel B shows the effect of S14HA over-expression on metabolic gene expression in rtTA-S14HA CiT3 cells under growth conditions, while panel C shows differentiation conditions. Only Glut1 (glucose transporter), AldoC (aldolase C) and PCx (pyruvate carboxylase) changed with S14 over-expression, and only under growth conditions. Error bars are SEM, and * = $p \leq 0.05$, ** = $p \leq 0.01$.



In order to understand if S14 over-expression may affect these metabolic components at the protein level, immunoblots for key enzymes along the metabolic pathways were performed. Figure 3 shows the protein levels for selected metabolic enzymes immunoblotted for in rtTA-S14HA CiT3 cells +/- dox induction of S14. No clear differences in protein level were detected as a function of S14HA overexpression (+dox), with the exception of perhaps PFK-L (phosphofructokinase) in DIP. Glut1 and PCx were not examined at the protein level due lack of antibody availability at the time. Although S14HA overexpression appeared to have no direct influence on glycolysis and lipogenic gene expression or protein levels in CiT3 cells, a lipogenic phenotype is still observed.

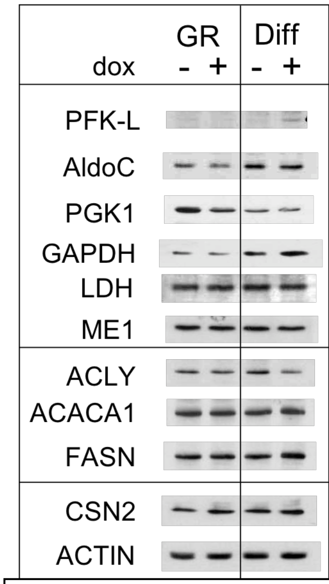


Figure 3. Immunoblots for proteins along the glycolysis and fatty acid synthesis pathways. rtTA-S14HA CiT3 cells were cultured under growth or differentiation conditions + or – dox induction of S14HA. Actin is shown as a loading control.

A neutral lipid stain was used to identify cytoplasmic lipid droplets (CLD), which harbor intracellular triglycerides in these cells and readily stain with Nile Red to identify these vacuoles. Figure 4 shows Nile Red fluorescence in rtTA-S14HA CiT3 cells under DIP conditions -/+ S14 overexpression. CLDs are clearly present with and without S14, however, when S14 is overexpressed, CLDs appear more numerous per cell as well as larger. The Nile Red staining was repeated using a different neutral lipid binding dye (Bodipy) under growth conditions that gave the same results (not shown). These data are merely qualitative, so in order to quantify the functional effect of S14 overexpression, NMR based metabolomics was used. The literature reports that antisense knock down of S14 blocks 14C-acetate incorporation into fatty acids in primary hepatocytes and in T47D breast cancer cells following tracer incubation [9, 10]. Importantly, the metabolomics data showed significant increases in the quantity of intracellular $(CH2)_n$ and (CH3) acyl chains that was enriched in the lipid fraction, while lactate and total -CH2/CH3 (acetyl molecules) were increased in the water soluble molecules (Figure 5).

rtTA-S14HA CiT3 cells were differentiated for 24 hours, afterwards media was switched to 5mM glucose/7mM 13C-acetate, 2% charcoal stripped FCS, and DIP, + and – dox, n = 3 per condition. Essentially, S14HA was induced for the 24-hour incubation period of 13C-acetate to investigate 13C incorporation into fatty acids. Data were collected and analyzed as previously [8, 11]. A significant increase in lipid acyl chains was measured, however, the 13C-acetate tracer was not enriched in these samples, which contradicts the 14C-acetate incorporation into data that Martel et al showed in T47D breast cancer cells. This result suggests that acetate incorporation into fatty acid chains is not affected by 24 hours S14 overexpression in DIP medium, even though fatty acids were significantly increased. Due to limitations of NMR based metabolomics, the chain length of the fatty acid cannot be determined; meaning that this technique did not discriminate between fatty acids synthesized de novo and preformed fats taken up from the medium. To address this issue a substrate other than 13C-acetate is required. 13C-glucose will be used as the tracer to determine if S14 affects glucose carbon conversion directly into fatty acids. Interestingly, glucose consumption and lactate excretion was identical in either growth or DIP conditions, even though the intracellular concentration lactate was significantly elevated in the presence of S14HA. In combination with the

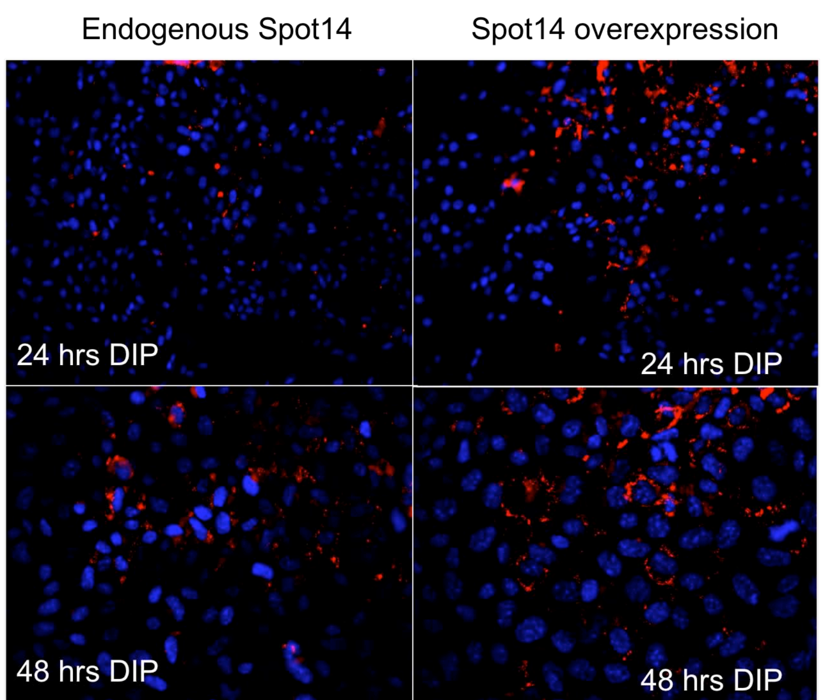


Figure 4. Nile Red cytoplasmic lipid droplet staining in rtTA-S14HA CiT3 cells following 24 and 48 hours of differentiation medium, +/- dox induction of S14HA.

Nile Red/Bodipy CLD staining, these data begin to suggest that S14 could function to shift metabolism to lipogenesis, but do so without changing mRNA and protein levels or using acetate as the de novo fatty acid substrate.

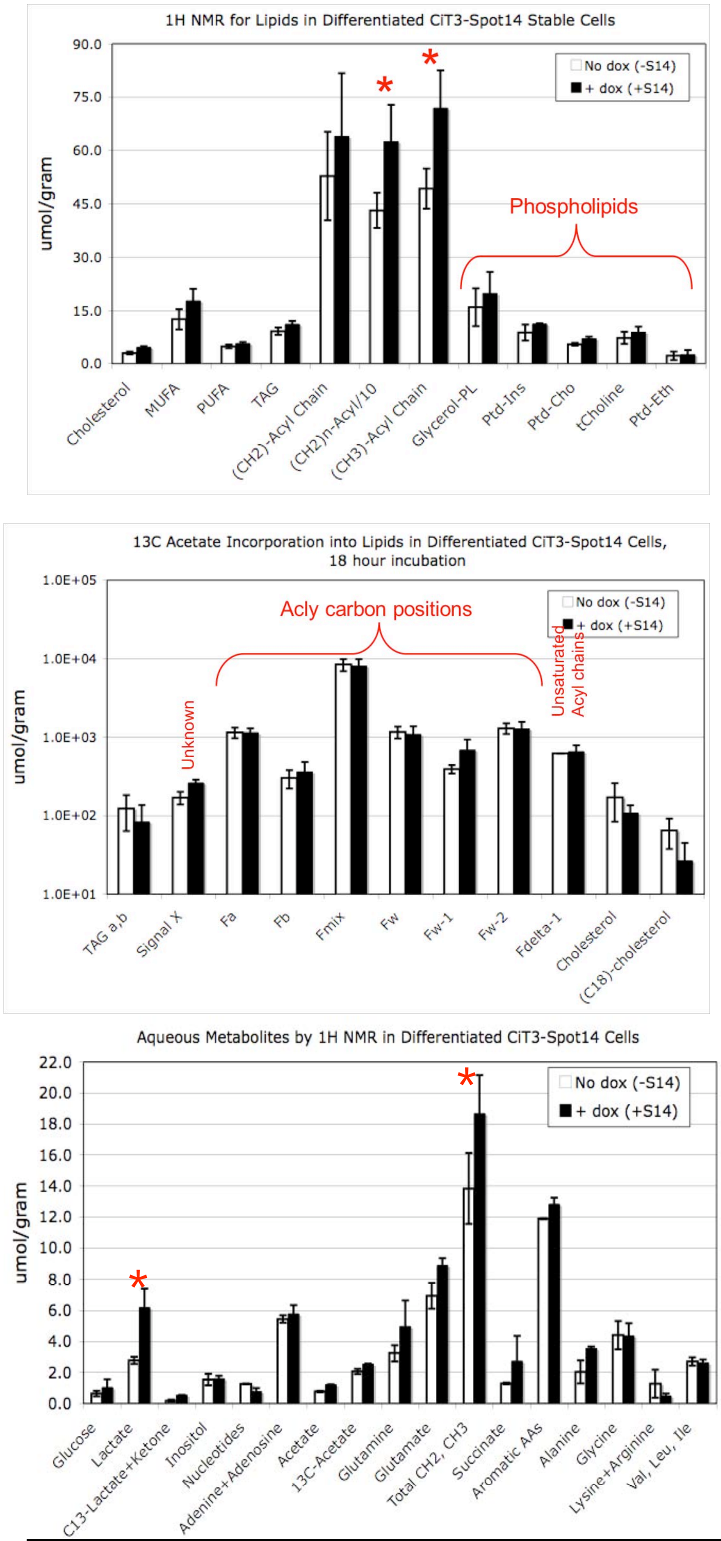


Figure 5. NMR metabolomic data in DIP rtTA-S14HA CiT3 cells showing concentrations of perchloric acid extracted intracellular metabolites. Top panel shows 1H-proton data from the lipid fraction, middle panel show 13C incorporation data in the lipid fraction, and bottom panel shows the 1H-proton data from the water soluble fraction. Error bars are SEM, n = 3.

Objective 2 of the original proposal seeks to identify proteins that may interact with S14 to confer its function. To address this issue, an unbiased strategy was needed: S14HA immunoprecipitation and mass spectrometry was used to identify proteins that are enriched in the pull down. Essentially, rtTA-S14HA cells were grown under DIP conditions for 48-hours +/- dox induction of S14HA. The S14HA containing protein complexes were immunoprecipitated (IP) using anti-HA-agarose beads, and the complexes were eluted via low pH. Table 1 shows the proteins identified by mass spectrometry using this strategy colored according to functional category. Functional categories included cytoskeletal/motor, glycolysis, nucleic acid binding, and other. S14 was the top hit identified indicating that S14HA bound to the anti-HA-agarose beads as intended, which was also verified using standard immunoblotting techniques (data not shown). It is interesting to note that this screen identified some proteins that exist in the nucleus that could modify pre-translational levels of mRNA, but that no differences in gene expression were detected for the DIP culture conditions (Figure 2c). Importantly, a handful of glycolytic enzymes were identified in S14HA cytosolic complexes, including phosphofructokinase 1, aldolase A, lactate dehydrogenase C, and pyruvate kinase M2. These factors often have increased activity and are known to be associated with cancer cell metabolism [1, 12]. Additional verification of proteins that complex with S14 will be verified from further IPs using standard immunoblotting technique. This suggests that S14 could function to modulate glucose metabolism at the enzyme level in CiT3 mammary epithelial cells, but that the growth condition should also be investigated due to differences in signaling/metabolism. Lastly, this IP will be performed in the ErbB2 tumor cell lines that overexpress S14HA to identify S14 associated proteins in these cells.

Objective 3 in the original proposal was to use chromatin immunoprecipitation to determine whether S14 and/or its interacting proteins are loaded at promoters to upregulate mRNA of glycolysis and lipid synthetic enzyme genes. No work is completed on objective 3 at this point. Although these studies are being extended into the tumor cell lines, currently there is no evidence that S14HA overexpression directly influences the panel of metabolic genes interrogated in normal mammary cells in growth or DIP. Should the tumor cell line data demonstrate S14 mediated metabolic gene expression differences this objective remains feasible. However, if no differences in metabolic gene expression are determined in tumor cell lines, then objective 3 may require significant restructuring.

In the case that modification of objective 3 happens, permission will be gained from the Army Contracting Officer Representative prior to any changes to the statement of workflow. Potential future directions could include mutations to the coil domain of S14 to abrogate dimer/oligomerization in vitro to assess if dimerization is required for metabolic function. Moreover, an n-terminal tag of S14 protein will be created to verify that the c-terminal HA tag is not affecting migration to the nucleus, which would abolish S14 transcriptional influences. To that end, immunohistochemistry (IHC) will be performed to demonstrate the cellular localization of S14 in all cell lines. In combination with coverslip grown cells for CLD staining, this procedure is easily adapted to include IHC, which can also be extended to investigate co-localization of S14 with the enzymes identified by mass spectrometry.

Table 1. Proteins identified by mass spectrometry that co-immunoprecipitated specifically with the S14HA construct overexpressed in DIP medium. Functional categories are colored.

Name	Symbol	prot_hits	prot_score	prot_mass
Thyroid hormone-inducible hepatic protein (Spot 14 protein) (SPOT14) (S14 protein) - Mus musculus (Mouse)	THRSP	2	386	17253
Keratin, type I cytoskeletal 14 (Cytokeratin-14) (CK-14) (Keratin-14) (K14) - Mus musculus (Mouse)	K1C14	10	242	53176
Keratin, type II cytoskeletal 6A (Cytokeratin-6A) (CK 6A) (K6a keratin) (Keratin-6 alpha) (mK6-alpha) - Mus mu	K2C6A	15	217	59641
Keratin, type I cytoskeletal 17 (Cytokeratin-17) (CK-17) (Keratin-17) (K17) - Mus musculus (Mouse)	K1C17	18	198	48417
Myosin-9 (Myosin heavy chain 9) (Myosin heavy chain, nonmuscle IIa) (Nonmuscle myosin heavy chain IIa) (N	MYH9	20	182	227414
6-phosphofructokinase type C (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofructo-1-	K6PP	21	182	86427
Vimentin - Mus musculus (Mouse)	VIME	24	141	53712
6-phosphofructokinase, muscle type (EC 2.7.1.11) (Phosphofructokinase 1) (Phosphohexokinase) (Phosphofru	K6PF	26	128	86070
Galectin-9 - Mus musculus (Mouse)	LEG9	30	117	40409
Actin, alpha cardiac muscle 1 (Alpha-cardiac actin) - Mus musculus (Mouse)	ACTC	31	113	42334
Histone H1.4 (H1 VAR.2) (H1e) - Mus musculus (Mouse)	H14	33	108	21964
Nuclease sensitive element-binding protein 1 (Y-box-binding protein 1) (Y-box transcription factor) (YB-1) (CCA	YBOX1	34	103	35709
Keratin, type I cytoskeletal 18 (Cytokeratin-18) (CK-18) (Keratin-18) (K18) (Cytokeratin endo B) (Keratin D) - M	K1C18	37	101	47475
Alpha-actinin-4 (Non-muscle alpha-actinin 4) (F-actin cross-linking protein) - Mus musculus (Mouse)	ACTN4	36	101	105368
Keratin, type II cytoskeletal 5 (Cytokeratin-5) (CK-5) (Keratin-5) (K5) - Mus musculus (Mouse)	K2C5	42	87	61957
Alpha-actinin-2 (Alpha actinin skeletal muscle isoform 2) (F-actin cross-linking protein) - Mus musculus (Mouse	ACTN2	44	85	104215
Alpha-actinin-3 (Alpha actinin skeletal muscle isoform 3) (F-actin cross-linking protein) - Mus musculus (Mouse	ACTN3	43	85	103605
Heat shock-related 70 kDa protein 2 (Heat shock protein 70.2) - Mus musculus (Mouse)	HSP72	46	83	69983
Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Aldolase 1) - Mus musculus (Mouse)	ALDOA	50	76	39787
L-lactate dehydrogenase C chain (EC 1.1.1.27) (LDH-C) (LDH testis subunit) (LDH-X) - Mus musculus (Mouse)	LDHC	52	76	36231
Keratin, type II cytoskeletal 6G (Cytokeratin-6G) (CK 6G) (K6g keratin) (Keratin-K6irs) (mK6irs1/Krt2-6g) (Kerat	K2C6G	55	75	57860
RNA and export factor-binding protein 2 - Mus musculus (Mouse)	REFP2	57	74	23773
40S ribosomal protein S28 - Mus musculus (Mouse)	RS28	67	59	7893
Lactadherin precursor (Milk fat globule-EGF factor 8) (MFG-E8) (MFGM) (Sperm surface protein SP47) (MP47)	MFGM	66	59	52376
Mitochondrial 28S ribosomal protein S29 (S29mt) (MRP-S29) (Death-associated protein 3) (DAP-3) - Mus mus	RT29	65	59	45070
60S ribosomal protein L31 - Mus musculus (Mouse)	RL31	70	58	14454
Putative RNA-binding protein Luc7-like 1 - Mus musculus (Mouse)	LUC7L	68	58	44307
Desmin - Mus musculus (Mouse)	DESM	74	54	53522
Pyruvate kinase isozyme M2 (EC 2.7.1.40) - Mus musculus (Mouse)	KPYM	75	54	58420
Keratin, type II cytoskeletal 8 (Cytokeratin-8) (CK-8) (Keratin-8) (K8) (Cytokeratin endo A) - Mus musculus (Mo	K2C8	76	53	54531
Eukaryotic translation initiation factor 1A, X-chromosomal (eIF-1A X isoform) (eIF-4C) - Mus musculus (Mouse)	IF1AX	80	44	16564
Histone H1.5 (H1 VAR.5) (H1b) - Mus musculus (Mouse)	H15	81	44	22562
Kinesin-like protein KIF3B (Microtubule plus end-directed kinesin motor 3B) - Mus musculus (Mouse)	KIF3B	83	44	85350
Kinesin-like protein KIF3C - Mus musculus (Mouse)	KIF3C	82	44	90131
Peripherin - Mus musculus (Mouse)	PERI	84	43	54349

Cytoskeleton/Structural
Motor Proteins
Metabolism
Nucleic Acid Binding
Other

Key Research Accomplishments

1. Generated rtTA-S14HA stable CiT3 normal mouse mammary epithelial cells
2. Generated stable rtTA 78617 and 85815 mouse ErbB2 tumor cell lines
3. Determined S14HA construct is functional for lipogenic phenotype (Nile Red/Bodipy)
4. Determined S14HA overexpression does not directly influence gene expression in DIP conditions
5. Performed NMR metabolomic analysis with 13C-acetate uptake/incorporation assay
6. Immunoprecipitated S14HA construct to identify interacting proteins

Reportable Outcomes

1. Attended the AACR annual meeting (April 2009)
2. Data were presented at the Mammary Gland Gordon Research Conference (May 2009)
3. Data were presented in the Pathology Research in Progress Seminar (March 2009)
4. Attended the AACR Cancer Metabolism meeting (September 2009)
5. Data were presented at the Breast Cancer Group Seminar (September 2009)
6. Data will be presented at the Molecular Biology Progress Seminar (October 2009)

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 regarding the mechanism of S14 exist to induce lipogenic metabolism; one that suggests modification to transcription events in the nucleus[3] and the other suggests S14 works with cytoskeletal/enzyme proteins[7]. The progress outlined in this report demonstrates that overexpression of S14HA did not affect gene expression of the metabolic mRNAs interrogated, but rather S14HA appears to function at the cytoskeletal/enzyme level in normal mouse mammary cells. This effect will be confirmed in the ErbB2 tumor cell lines next. Three lines of evidence show S14HA overexpression shifts cells towards anabolic metabolism and the latter paradigm: first, Nile Red/Bodipy cytoplasmic lipid droplet staining showed larger and more numerous droplets compared to controls, but this was qualitative. Second, NMR metabolomics quantified a S14HA dependent increase in CH2 acyl and CH3 acyl lipids that did not incorporate 13C-acetate, but because 13C was not differential there was no way to distinguish between de novo synthesized fatty acids and preformed fats taken up from the medium. Third, the co-IP mass spec data revealed cytoskeletal proteins, motor proteins and glycolytic enzymes enriched specifically with S14HA overexpression, but that nucleic acid binding proteins were also identified. Further, these studies will focus on S14HA overexpression in the ErbB2 tumor cell lines to determine if these trends are unique to the normal mammary epithelial cell line.

Tumor cells shift their energy utilization in characteristic ways that distinguish cancer metabolism from normal cell metabolism. Generally these changes affect how fast a tumor cell grows or whether a tumor is sensitive to signals that tell it to die. That tumors dramatically alter their metabolic pathways to affect energy utilization suggests these pathways may become attractive cancer therapeutics in the future. To that end, tumor cells avidly uptake glucose, placing S14 function directly into this pathway according to this preliminary data presented above. The shift in glucose utilization by S14 seems to support increased lipid synthesis. The high rate of lipid biosynthesis may contribute to additional changes in the biology of the tumor cell such as the ability of tumor cells to metastasize. Human breast tumors that have elevated S14 and that make excess lipid have a poorer clinical response in terms of patient survival. This correlation, in combination with presented data makes S14 a reasonable therapeutic target.

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