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TITLE: Defining the Role of Post-Translational Modifications in SRC-3-Mediated Repression of mRNA Translation

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Breast cancer is the most common, malignant cancer of women in North America; however, there are a number of criteria that determine the prognosis of each cancer. Breast cancer development and progression is influenced by gene expression patterns of both the tumor cells, as well as cells within the tumor microenvironment, such as macrophages. Steroid receptor coactivator 3 (SRC-3) is a molecule that has been shown to influence gene expression both within breast cancer cells and within macrophages. SRC-3 is a potent oncogene in breast cancer cells, functioning as a coactivator of hormone-driven transcription, and a translational repressor of proinflammatory cytokines, such as TNF-α, in response to lipopolysaccharide (LPS) stimulation of macrophages. I hypothesized that SRC-3 may inhibit tumor-promoting TNF-α in macrophages activated by paracrine signaling from breast cancer cells, as well; however, our in vitro data do not show any dependence on SRC-3 or its two related proteins, SRC-1 or SRC-2, for macrophage-induced breast cancer cell invasion. Thus, we decided to further characterize the members of the SRC family within breast cancer cells and not macrophages. Our laboratory discovered that an isoform of SRC-3, Delta 4, is a strong inducer of breast cancer cell metastasis, and our preliminary data indicate that it may be a predictor of basal breast carcinomas, the most aggressive type of breast cancer. Additionally, I developed a diagnostic tool for measuring minute amounts of oncogenic SRC-3 within tumor cells. By understanding how different members of the SRC family are expressed during breast cancer, we aim to create tools for personalized medicine, providing tumor-specific targets to be tested for future cancer intervention.
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Introduction

The development and progression of breast cancer is influenced by a multitude of factors, including the particular gene expression patterns of each tumor, as well as cells within the tumor microenvironment [1-5]. Breast tumors, in general, can be classified as luminal or basal dependent on whether they share gene expression profiles with the luminal epithelial layer or underlying basal layer of normal breast ducts [6]. Patients with luminal breast tumors have a significantly higher survival rate than those with basal tumors [5]; thus, classification of tumors is a useful prognostic tool. Gene expression changes within the tumor, itself, are not the only predictors of tumor progression. Cells within the tumor microenvironment are also influential. For example, tumor-associated macrophages infiltrate premalignant mammary tissue as part of an inflammatory response and promote tumorogenesis by expressing proinflammatory cytokines, such as TNFα [2, 4, 7]. Chronic expression of TNFα correlates with increased tumor grade, and co-culture of macrophages with breast cancer cells promotes breast cancer cell migration and invasion in a TNFα-dependent manner [8-10]. My studies focus on a molecule that controls gene expression in both tumor cells and macrophages. Steroid receptor coactivator 3 (SRC-3) is a transcriptional coactivator and an oncogene within breast cancer cells [11] but a translational repressor of proinflammatory mRNAs TNF alpha, IL-6, and IL-1beta in macrophages activated by lipopolysaccharide (LPS) [12]. I proposed that SRC-3 also inhibits TNFα translation in macrophages activated by signaling molecules secreted from breast cancer cells, thus, serving as a tumor suppressor. One way that SRC-3 could be directed towards transcriptional activation or translational repression is through the addition of specific, post-translational modifications (PTMs). Our preliminary data suggest that there is a PTM-switch on the SRC-3 protein that parses its function between transcriptional activation and translational repression. Additionally, our laboratory published a paper describing the function of an alternatively spliced form of SRC-3, termed Delta 4 [13] with an alternative, oncogenic function. SRC-3Δ4 is localized to the cell membrane and serves as a bridging factor between epidermal growth factor receptor (EGFR) and focal adhesion kinase (FAK). EGF is secreted from macrophages, and SRC-3Δ4 is critical for transducing the signal from EGF-bound EGFR to focal adhesion kinase (FAK) within breast cancer cells. Activation of FAK promotes cell migration and tumor metastasis [13]. We aimed to identify if particular PTM-modified forms or the alternatively spliced form of SRC-3 are associated with oncogenic or tumor suppressive activities of SRC-3 and assess their relative expression level in subclasses of breast tumors and macrophages. Additionally, we have included analyses on the other two members of the SRC family: SRC-1 and SRC-2. SRC-1 is also a known oncogene that promotes cancer cell migration and invasion [14], while not much is known about SRC-2 in breast cancer. These data will provide useful prognostic tools and pave the way for more personalized medicine.

Body
Specific Aim 1: Define if specific PTMs affect SRC-3’s function as a translational repressor.

By mass-spectrometry, we identified 26 uncharacterized post-translationally modified amino acids from SRC-3 proteins immunoprecipitated from HeLa cells. Through site-specific mutagenesis, I created PTM-mutant versions of SRC-3 for each of these sites, as shown in Figure 1. I planned to test if any of these PTM mutants alters SRC-3’s ability to repress translation. Our laboratory previously created a RAW 264.7 macrophage cell line stably transfected with a plasmid encoding a CMV-driven luciferase gene fused with the 3’UTR of TNFα (pCMV-Luc-TNFα 3’ UTR). I originally proposed to transiently transfect plasmids that express the wildtype or PTM-mutant versions of SRC-3 into these cells, plus and minus treatment with LPS. I planned to measure the activity of the reporter gene by luciferase assay, as a read-out of translation, and normalize these values to total protein content. We have already identified two PTM mutant forms of SRC-3 that alter SRC-3’s function as a translational repressor: SRC-31-6A and SRC-3R1171/1177A (Figure 2). We previously published that phosphorylation of six amino acids on the SRC-3 protein increases its transcriptional activity by enhancing the interaction between SRC-3 and nuclear receptors or other cofactors [15], while methylation of SRC-3 at R1171/1177 disrupts the interaction between SRC-3 and transcriptional coactivators [16]. Our preliminary data indicate that phosphorylation of these six key ‘transcriptional

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Figure 1. Table depicting unpublished SRC-3 PTMs identified by mass spectrometry from HeLa cells. The mutations that I have made for each amino acid are indicated.

Figure 2. AA Residue/Mutation Modification

Specific Aim 1: Define if specific PTMs affect SRC-3’s function as a translational repressor.
activation' sites is inhibitory for SRC-3-mediated translational repression, as
mutation of these phosphorylation sites (SRC-31-6A) enhances SRC-3-mediated
translational repression (Fig. 2A). Additionally, mutation of SRC-3 methylation sites,
R1171/1177, also enhances SRC-3 mediated repression (Fig. 2B). However, this
reporter is no longer responding to transiently transfected SRC-3, and we have not
been able to test the PTM-mutants created.

As an alternative approach, we initiated a
collaboration with Dr. Rick Lloyd’s laboratory to employ a different translational
reporter screening method. Their laboratory created a reporter construct with a
Tet-inducible promoter (TRE) driving expression of a luciferase gene with two sets
of RNA motifs in its 3’UTR: four MS2 and four BoxB RNA hairpin repeats. These
repeats serve as binding sequences for specific RNA binding proteins: the lambda N
protein binds specifically to the BoxB RNA hairpin repeats and the MS2 coat protein
binds the MS2 repeats. The Lloyd laboratory took advantage of these specific RNA-
protein interactions in order to tether proteins to the 3’UTR of the luciferase gene
for the purpose of testing the activity of proteins in translation of luciferase. For
example, they created a TIA-1-lambda N cDNA expression vector, which also contains
GFP for easy visualization. When coexpressing both constructs, TIA-1 efficiently
represses translation of the luciferase gene (communication with Lloyd laboratory).
We transiently co-transfectd SRC-3, or its vector control, alongside TIA-1-lambda N
and the reporter construct into HeLa cells to determine if SRC-3 enhances TIA-1-
mediated repression. However, we could not see any overexpression of SRC-3 with
this system; therefore, results could not be concluded. To remedy this problem, we
have generated a Lentivirus that expresses SRC-3. Viral transduction of SRC-3 is
substantially more efficient that traditional plasmid-based transfection techniques.
We will retry these assays with this expression system. However, this is not an ideal
screening strategy, as a different virus for each PTM-mutant would need to be
created. Recreation of the original screening reporter would yield a better tool.

Figure 2. Specific PTM mutations enhance SRC-3 mediated translational repression. RAW cells stably
expressing a pCMV-driven luciferase gene fused with the
3’UTR of TNFα were transiently transected with (A) either
wildtype SRC-3 (pCMV-SRC) or
phosphorylation-defective SRC-3
(pCMV-SRC3 1-6A); or (B) either
wildtype SRC3 (pSG5-SRC3) or
methylation-defective SRC-3
(pSG5-SRC3 1171/1177A) and
treated with or without LPS.

A. B.
Since I am interested in PTMs that may serve as a switch between SRC-3’s function as a transcriptional coactivator and a translational repressor, I also screened the PTM mutant constructs in transcriptional coactivation assays. Wildtype or PTM-mutant versions of SRC-3 were cotransfected with ERα expression vector and the ERE (estrogen response element)-E1b-Luc reporter construct into HeLa cells treated with vehicle or estradiol (E2). As seen in Figure 3 and previously published data [17, 18], widtype SRC-3 readily coactivates ERα-driven transcription in response to E2. However, none of the previously uncharacterized PTM mutants significantly inhibited or enhanced this function in HeLa, MCF7, Ishikawa, LNCaP, MG-G3, or T47-D cells (Figure 4 and data not shown). Additionally, this panel of PTM mutants was tested for it’s ability to coactivate GR, PR, and NFkB in HeLa cells, and no significant difference was observed between the wildtype or PTM mutant forms of SRC-3 (data not shown.) Thus, these data likely eliminate these newly
characterized PTMs from a role in transcriptional coactivation, yet retain the possibility for a role in translational repression.

**Specific Aim 2: Determine the correlation of SRC family members and specific PTM forms on their activity in human breast cancers.**

*i) Profile expression levels of SRC proteins and functionally-relevant PTM-modified forms of SRC-3 in breast cancer cells and macrophages.*

Numerous publications have concluded that SRC-3 and SRC-1 are overexpressed in human breast tumors [19]; however, the same characterization of SRC-2 or SRC-3Δ4 has not been conducted. Moreover, the expression of each of these members has not been fit with the established method of breast cancer subclassification. Subclassification of breast cancer provides crucial information that influences given prognosis and treatment. Two broad categories of breast cancer are often described: luminal and basal. We utilized 33 breast cancer cell lines that have previously been classified in this manner to determine if there is any correlation between expression of SRCs and the type of breast cancer. **Figure 4** shows that there is wide variation in mRNA expression of SRC-3, SRC-3Δ4, and SRC-1; however, there is much more limited variation in the expression of SRC-2.

![Figure 4](image)

**Figure 4.** Quantitative RT-PCR was performed to measure expression of SRC-3 (A), SRC-3Δ4 (B), SRC-1 (C), and SRC-2 (D) mRNA. Expression of each transcript was normalized to GAPDH mRNA.
We analyzed our data to determine if these proteins are associated with either luminal or two subcategories of basal types of breast cancer [6]. We did not find any significant correlations **Figure 5**. Interestingly, though, we observed a very tight correlation between full-length and SRC-3Δ4 mRNAs: $R^2 = .99$.

![Figure 5](image.png)

**Figure 5.** Categorization of SRC-3 (A), SRC-3Δ4 (B), SRC-1 (C), SRC-2 (D) mRNA in reference to luminal, basal A, and basal B breast cancer cell lines. mRNA levels were normalized to GAPDH mRNA.

Next, we determined if there is any correlation between mRNA expression of SRCs and the expression of receptors commonly assayed for prognostic purposes: ERα, progesterone receptor (PR), human epidermal growth factor receptor 2 (Her-2), EGFR, and those triple negative for ERα, PR, and Her-2. ERα and PR are associated with luminal breast cancers and a better prognosis, Her-2 expression has its own subclassification which has a worse prognosis than luminal cancers, and EGFR is often overexpressed in the most aggressive of breast cancers, basals [1, 5]. Triple negative breast cancers also have a very poor prognosis, as these cancers lack the expression of receptors that can be targeted for therapy [5]. The status of ERα, PR, and Her-2 was obtained from published literature and the ATCC website [6, 20, 21]. EGFR levels were determined by a taqman protein PCR assay described further in this section. We did not see any specific correlation between SRC mRNA expression and receptor status (**Figure 6**).
Although there were no luminal/basal or receptor correlations with SRC mRNA levels, protein levels may be more indicative of a functional relationship. Thus, we are performing western blots to analyze the expression of SRC proteins in several cell lines and quantifying our results. Preliminary results are shown in Figure 7.

Figure 6. Correlation between SRC-3 (A), SRC-3Δ4 (B), and SRC-1 mRNA expression and the presence or absence of ERα, PR, Her2, EGFR, or in those triple negative for ERα, PR, and Her-2. P-values were determined by a student’s T-test. No correlation was seen with SRC-2 mRNA either, data not shown.
From these analyses we were able to determine that there is no correlation between full-length SRC-3 and luminal/basal classification; however, there is a significant correlation between SRC-3Δ4 protein levels and basal classification. We also correlated protein expression of SRC-3 and SRC-3Δ4 with receptor status and observed that SRC-3Δ4 protein expression correlates with cell lines overexpressing EGFR (Figure 8). Thus, SRC-3Δ4 protein levels may be an important prognostic factor for this aggressive sub-type of breast cancer.

Figure 7. Delta 4 protein levels correlate with basal classification. Full-length SRC-3 (A) and SRC-3 Delta 4 (B) protein levels were assessed in luminal and basal human breast cancer cell lines by western blot analysis with the same antibody. We used the molecular weight to differentiate between the two forms. Protein levels were quantified and normalized to GAPDH. The normalized data is presented in box and whiskers plots. P-values were determined by a student’s T-test.
Expression of SRC-1 and SRC-2 protein is still being determined in these cell lines and expression levels have not been profiled in macrophages. We are also looking to classify specific PTM forms of SRC-3 in human breast cancer cells. By classifying SRC-3 in tumors this way, we will have an idea if particular PTMs forms of SRC-3 are associated with certain subcategories of cancers. If so, detection of these forms of SRC-3 could prove to be a powerful diagnostic tool, and specific forms of SRC-3 could provide druggable targets, specific to the various types of breast cancer. We have generated antibodies to specific phosphorylation sites of SRC-3 known to be important for SRC-3’s ability to coactivate transcription and function as an oncogene [15]. PTMs that regulate SRC-3’s function as a translational repressor have yet to be identified. Once PTMs of interest are identified they will be screened by western blot in both human breast cancer cell lines and macrophages. We hypothesize that different PTM forms of SRC-3 will be present in breast cancer cell lines than macrophages, corresponding with its diverse functions in these two cell types. It will also be interesting to determine if there is any SRC-3Δ4 expression in macrophages or if it is restricted to breast cancer cells where it functions as an oncogene.

Figure 8. Quantification of SRC-3 (A) and SRC-3Δ4 (B) protein levels in correlation with ERα, PR, and EGFR protein levels. SRC-3 and SRC-3Δ4 were quantified by western blot and normalized to GAPDH. P values determined by a student T-test.
ii) Create diagnostic tools for quantitatively assessing SRC protein levels in breast cancer.

It is often difficult to obtain enough tumor tissue to adequately score breast cancer markers for prognostic and diagnostic tumors. Additionally, recent developments in the literature suggest that quantification of breast cancer markers in small populations of cancer cells, such as circulating tumor initiating cells, would be beneficial [22]. Therefore, we sought to develop an assay for SRC proteins that would be useful for sensitive quantification of protein levels. ABI/Invitrogen has developed an assay for quantifying protein levels by PCR. Briefly, two antibodies that recognize different epitopes of the same protein are biotinylated. One antibody is conjugated to a 5’ oligo and the other to a 3’-oligo. When both antibodies bind the target protein in a cell lysate, the oligos are ligated by forced proximity. One can then PCR across these oligos. First we developed this assay for EGFR, then we developed a probe for SRC-3 (Figure 9). Both probes could be useful tools in the clinic.

A.

![Graphs showing relative expression of EGFR](image)

B.

![Bar graph showing relative SRC-3 protein levels](image)
iii) Determine the contribution of the PTM-modified and alternatively spliced forms to the activity of SRC-3.

We hypothesize that the level of SRC protein in each cell line tested will be indicative of that cell's dependence on that protein. In breast cancer cells, SRC-3Δ4 is an oncogene promoting migration and invasion. We sought to determine if the level of SRC-3Δ4 correlates with that cell's dependence on it for these activities. We are determining the % migration of each cell line in response to serum. Thus far, we see a trend indicative that the more SRC-3Δ4 there is, the more invasive the cell line.

We have also optimized SRC-3 and SRC-3Δ4 siRNA conditions in each of these cell lines (Figure 11). Next, we will knock-down these proteins and test the cells’ migration rates. We will cross-reference this data with their expression levels in these lines. These data will tell us if screening patients for the amount of Delta 4 will truly be predictive of the patient’s response to an inhibitor of each of these molecules, the ultimate long-term goal of these studies. Similar assays knocking down the other members of the SRC protein family and testing PTM-mutant forms of SRC-3 followed by proliferation and migration assays will be informative to predict how effective it will be to target cancers with other SRC-specific inhibitors.
Specific Aim 3: Determine if SRC-3 protein expressed in macrophages influences metastatic properties of breast cancer cells and if this effect is PTM-specific.

i) Determine if loss of macrophage-expressed SRC-3 affects breast cancer cell invasion or migration.

Previously, researchers found that co-culturing macrophages with breast cancer cell lines MCF-7 and SK-BR3 promotes the invasive properties of these breast cancer cell lines in a TNFα-dependent manner [8]. I established my own macrophage-brest cancer cell coculture system, in vitro, in order to test if macrophage-expressed SRC-3 inhibits breast cancer cell invasion or migration. In this in vitro system, loss of neither SRC-3 (Figure 12), SRC-2 (Figure 13), nor SRC-1 (Figure 14) in macrophages significantly affected their ability to enhance breast cancer cell migration or invasion. Thus, these data do not support our original hypothesis.
Figure 12. SRC-3 does not inhibit nor enhance breast cancer cell invasion. Either 4T1 cells (A) or MtLn3 cells (B) were cocultured with either no cells (0 in Fig. 7B), the respective control cell line, 4T1 (A) or MtLn3 cells (B), macrophages from wildtype mice, or from SRC-3 -/- mice. Invasion of the breast cancer cells was assayed by measuring the absorbance of crystal violet dye eluted from the invaded cells.

Figure 13. Loss of macrophage-expressed SRC-1 does not reproducibly alter macrophage-induced breast cancer cell invasion. 4T1 cells (A-C) or MtLn3 cells (D) were cocultured with either no cells (0), wildtype macrophages, or SRC-1 -/- macrophages. In C and D 3000pg/mL versican, a macrophage activator, was added to the media. Invasion of the breast cancer cells was assayed by measuring the absorbance of crystal violet dye eluted from the invaded cells at A560nm.
However, TNFα, the hypothesized target of SRC-3, is not induced in this coculture system. To address this problem, I collaborated to create 3D organoids from mammary epithelial cells (MECs) derived from either wildtype or MMTV-polyoma middle T (PyMT) mice. PyMT mice spontaneously develop breast tumors and are a common mouse model used to study breast cancer [23]. DeNardo et al showed that coculture of PyMT organoids with tumor-associated macrophages enhances the invasive properties of the organoids [24]. Through collaboration with Dr. Jeff Rosen’s laboratory, we initially established an ex vivo model system that demonstrates macrophage-induced invasiveness of 3D organoids created from pre-malignant p53-/- mouse mammary cells of high (PN1a) and low (PN1b) tumor forming potential (Figure 15). MECs from PyMT mice are yet to be tried; however, the p53-/- MECs in this experiment nicely demonstrate how macrophages can be

**Figure 14.** Loss of macrophage-expressed SRC-2 does not significantly alter macrophage-induced breast cancer cell invasion or migration. MtLn3 cells were seeded in SFM inside cell culture inserts either precoated with Matrigel (A and B) to measure invasion or without (C and D) to measure migration. These cells were then cocultured with either no cells (0), MtLn3 cells, wildtype macrophages, or SRC-3-/- macrophages grown in complete media. In B and D, 3000 pg/mL versican was added to the complete media in all of the wells. Invasion or migration of the breast cancer cells was assayed by measuring the absorbance of crystal violet dye eluted from the invaded cells at A560nm.
induce an invasive tumor morphology. Unfortunately, our collaborators have not been able to consistently repeat this data; thus, we have not been able to test how loss of SRC-3 affects the invasiveness of tumor cells.

I believe the best approach would be to make a macrophage-specific SRC-3 knockout mouse in a PyMT mouse model in order to compare the migration and invasion rates of breast cancer cells with and without SRC-3 present in the tumor-associated macrophages.

**Key Research Accomplishments**

1. Analysis of PTM mutant forms of SRC-3 revealed no effect on SRC-3’s function as a transcriptional coactivator. Technical difficulties have
obstructed us from determining their effects on SRC-3’s function as a translational repressor.

2. SRC-1, SRC-2, SRC-3, and SRC-3Δ4 mRNA levels were quantified in a large panel of breast cancer cell lines and revealed no correlation with either breast cancer subclassification or receptor status.

3. Data generated thus far reveal an association between SRC-3Δ4 protein levels, the basal type of breast cancer, overexpression of EGFR, and more migratory cell lines.

4. SRC-1, SRC-2, and SRC-3 are not essential in macrophages for macrophage-induced breast cancer cell invasion, in vitro.

5. I created a diagnostic tool for quantitatively measuring small quantities of EGFR and SRC-3 in tumor cells.

**Recordable Outcomes**

1) Poster presentation at the Department of Defense Breast Cancer Research Program Era of Hope Conference.

2) Review article published:

3) A manuscript with the majority of these findings in currently in preparation for submission.

**Conclusions**

We found that the identified PTM sites on the SRC-3 protein are not essential for its function as a transcriptional coactivator in breast cancer cells. We have not been able to test how these same sites affect SRC-3’s function as a translational repressor in macrophages due to technical difficulties. Our in vitro data indicate that SRC-3 may not be functioning in macrophages as originally hypothesized. If macrophage-expressed SRC-3 was repressing tumor-promoting TNFα levels raised by paracrine signaling from breast cancer cells, we would expect to see an increase in migration of breast cancer cells cocultured with macrophages lacking SRC-3. However, we do not see any difference in the migration rates of breast cancer cells cocultured with wildtype or SRC-3 knockout macrophages. A caveat to this conclusion is that we do
not believe TNFα levels secreted by the macrophages are being adequately induced by the breast cancer cells in our in vitro system to appropriately test our hypothesis. I believe that the best in vivo system would be to compare the migration and invasion rates of breast cancer cells in mice that contain wildtype and SRC-3 knockout macrophages. We also tested how macrophage-expressed SRC-1 and SRC-2 may effect breast cancer cell migration and saw no difference in breast cancer cell migration in cells cocultured with wildtype or siRNA-treated macrophages. Thus, we thought it wise to focus our attention back on how SRC proteins influence breast cancer progression inside the breast cancer cell and not within the macrophage. We further characterized the mRNA and protein expression of the SRC family members, including a splice form of SRC-3, Delta 4. Thus far, our data indicate that SRC-3Δ4 may be a good prognostic factor for basal breast tumors. Based on the protein analysis completed in this report, we see a correlation between SRC-3Δ4 protein levels, EGFR overexpression, and migration.

So what: By decoding the various forms of SRC proteins that exist in different types of breast cancer and the tumor environment, we believe that more personalized drugs could be developed to inhibit the active, tumorigenic forms of SRCs.

References