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14. ABSTRACT The signal transducer and activator of transcription (STAT) 5b is involved in tumorigenic signaling in a number of cancers including breast cancer. However, its importance in breast cancer migration has not been investigated. This research aims to determine the role of STAT5b in breast cancer migration. Knockdown of STAT5b inhibited migration of human breast cancer cells to serum by 60-80%. This inhibition could be rescued by re-introduction of wild-type STAT5b as well as a transcriptionally inactive Y699F-STAT5b mutant. This suggests that the transcriptional activity of STAT5b is not required for its pro-migratory function. Knockdown of STAT1 also inhibited migration of MDA-MB-231 cells but knockdown of STAT3 or STAT5a had no effect. This data demonstrates that STAT5b is integral to breast cancer cell migration and that this effect may occur through a novel, non-transcriptional function. Further elucidating the mechanism by which STAT5b promotes migration of breast cancer cells may lead to more effective therapies for preventing and treating metastasis.

15. SUBJECT TERMS
breast cancer, STAT5b, EGFR, migration, invasion, metastasis

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Introduction

Breast cancer is the second most common cancer in American women. Despite improvements in detection and the development of new treatment strategies, the American Cancer Society estimates that over 180,000 new cases of breast cancer will be diagnosed, and 40,000 women will die from breast cancer this year alone. Because many cancers arise from dysregulation of signaling pathways found in normal cells, one of the difficulties in treating cancers is identifying cancer-specific therapeutic targets. Many tumorigenic signaling pathways converge on common nuclear transcription factors and therefore, targeting these downstream proteins may be efficacious [1]. One such group of transcription factors is the signal transducer and activator of transcription (STAT) family. STATs are a family of transcription factors activated by cytokines and/or growth factors. Activation of STATs 1, 3, and STAT5a/b occurs in a variety of cancers including breast cancer [2, 3]. In general, STAT1 appears to function as a tumor-suppressor while activation of STAT3 and STAT5a/b promote tumorigenesis [4-8]. Both STAT3 and STAT5b promote motility of prostate cancer cells, but their role in breast cancer cell migration has not been examined [9, 10]. STAT5b is a downstream effector of epidermal growth factor receptor (EGFR) signaling [11-13]. Therefore, we sought to investigate the potential role of STAT5b in the migration of two highly aggressive, EGFR-overexpressing breast cancer cell lines, BT-549 and MDA-MB-231, using siRNA knockdown. We found that STAT5b was required for the migration of both of these cell lines. These results establish an important pro-migratory function of STAT5b in EGFR-overexpressing breast cancer cells, further defining its role in tumorigenesis and supporting is potential as a therapeutic target for the treatment of breast cancer.

Body

Task 1: Determine the role of EGF-induced STAT5b activity in breast cancer metastasis

A. Investigate the effects of EGF-induced STAT5b activation on migration and invasion of breast cancer cells

As reported in the year one annual summary report, the design of our migration studies was modified from that originally proposed. First, our studies utilized fetal bovine serum as a chemoattractant, rather than epidermal growth factor (EGF), to stimulate migration of both BT-549 and MDA-MB-231 breast cancer cells, as this was a more potent migratory stimulus for these cells. Secondly, our studies were designed to answer whether STAT5b is necessary for breast cancer cell migration, rather than if it is sufficient to stimulate migration. Therefore, we used siRNA knockdown of STAT5b in highly migratory breast cancer cells as opposed to transient overexpression of STAT5b in non-migratory normal breast epithelial cells.

STAT5b knockdown inhibits breast cancer cell migration - Knockdown of STAT5b to undetectable levels in BT-549 human breast cancer cells inhibited their migration to serum by approximately 70% (Figure 1A). To ensure that this effect was not unique to one cell line, we tested the effect of STAT5b siRNA on migration of MDA-MB-231 cells, another highly aggressive, migratory breast cancer cell line. In these cells, specific knockdown of STAT5b also inhibited migration to serum, by approximately 50% (Figure 1B).

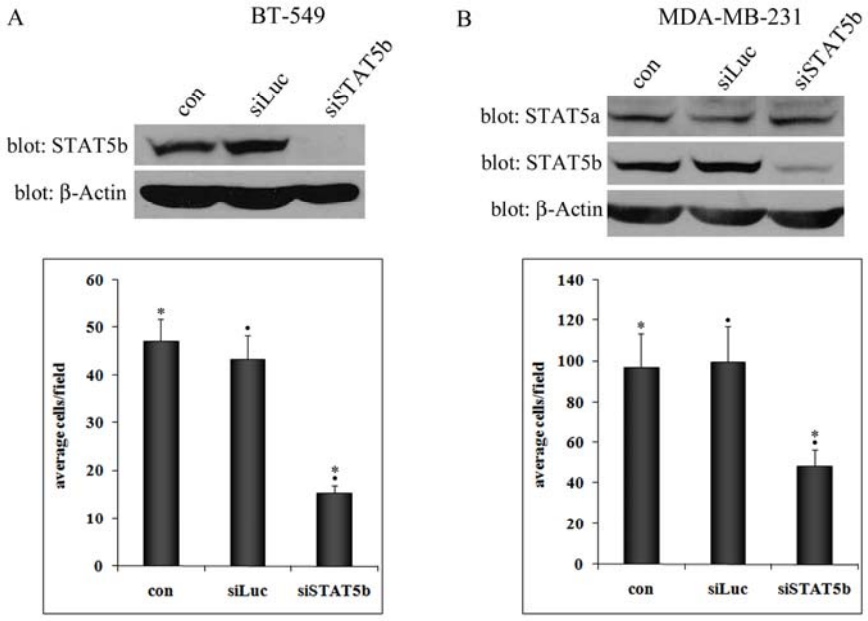
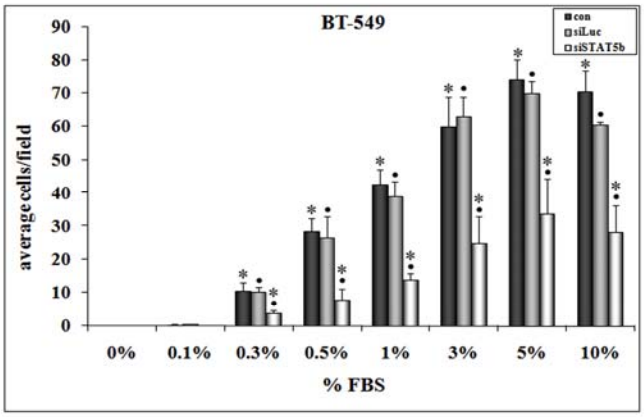


Figure 1: Knockdown of STAT5b inhibits breast cancer cell migration
(A) BT-549 breast cancer cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc) or siSTAT5b SMARTpool (siSTAT5b) using Oligofectamine. Seventy-two hours following transfection, cells were plated in serum-free media in Boyden Chambers. Media containing 1% FBS was placed in the lower chambers to serve as a chemoattractant. After three hours the cells were fixed, stained with crystal violet, and the number of migratory cells was counted. Four fields were counted on each of two filters. Results are graphed as the average number of migratory cells per field $\pm$ SE. The average cells per field $\pm$ SE for five independent experiments are as follows: con (47.15 $\pm$ 4.62), siLuc (43.50 $\pm$ 4.80), siSTAT5b (15.48 $\pm$ 1.51). Student's *t* test was used to determine statistical significance between the following: con and siSTAT5b *, $p < 0.0001$; siLuc and siSTAT5b •, $p < 0.0001$. (B) MDA-MB-231 cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), or siRNA specific to STAT5b (siSTAT5b siGENOME SMARTpool oligonucleotide #3) and migration was measured in the same manner as BT-549 cells except that Boyden Chamber assays were performed using 10% FBS in the lower chambers for six hours. The average cells per field $\pm$ SE for four independent experiments are as follows: con (97.09 $\pm$ 16.21), siLuc (99.81 $\pm$ 17.02), siSTAT5b (48.47 $\pm$ 7.84). Student's *t* test was used to determine statistical significance between the following: con and siSTAT5b *, $p = 0.0017$; siLuc and siSTAT5b •, $p = 0.0015$. (A, B) *Upper panels* - Whole cell lysates from siRNA-transfected BT-549 and MDA-MB-231 breast cancer cells were collected seventy-two hours post-transfection and immunoblotted with antibodies specific for STAT5a or STAT5b and β–actin as a loading control.

In the BT-549 cells, we determined the optimal migration conditions for Boyden Chamber transwell assays to be migration to 1% serum over 3 hours. Since these cells require a low dose of serum to migrate, we utilized them to determine whether increasing concentrations of serum could overcome the effect of STAT5b knockdown. As seen in Figure 2A, knockdown of STAT5b significantly inhibited migration of BT-549 cells by 60-80% at all serum concentrations ranging from

0.1% to 10%. Thus, increasing the concentration of serum components does not overcome the effect of STAT5b knockdown on inhibiting migration. However, migration is not completely abrogated upon knockdown of STAT5b in either cell line. The cells retain their capacity to migrate at a similar "basal" level across all serum concentrations tested (Figure 2A). To determine whether this residual migration is chemokinesis, or random movement, we performed a checkerboard assay. In this assay, BT-549 cells were plated in upper wells of Boyden Chambers in the presence of media containing varying concentrations of serum. Serum concentrations were also varied in the lower chambers in order to create positive serum gradients, in which serum concentrations are higher in the lower chambers, or negative serum gradients in which serum concentrations are higher in the upper chambers. BT-549 cells migrate chemotactically to serum, seen by an increase in the average number of migratory cells in the presence of a positive serum gradient (Figure 2B). Chemokinetic movement is migration that occurs in the absence of a serum gradient, quantitated in Figure 2B as the values in bold, located between the two lines. In our experiments, an average of approximately 15-26 cells per field randomly migrate. This number is similar to the migration we observed in BT-549 cells in the presence of STAT5b knockdown (Figures 1A, 2A). Therefore, STAT5b knockdown inhibits chemotactic migration of BT-549 breast cancer cells, while the remaining migration can be attributed to chemokinesis.

A



B

% Serum in Top Chamber

% Serum in Bottom Chamber	0	0.5	1.0	3.0
0	**0 ± 0**	1.25 ± 0.95	5.75 ± 0.85	0.5 ± 0.5
0.5	*32 ± 7.56*	**21.25 ± 3.97**	13.25 ± 0.48	15.25 ± 2.62
1.0	*77 ± 9.26*	*31.75 ± 2.69*	**26.5 ± 7.24**	22.75 ± 5.79
3.0	*114.25 ± 7.03*	*81.75 ± 1.80*	*56.25 ± 9.59*	**15 ± 2.12**

Figure 2: Knockdown of STAT5b inhibits chemotactic breast cancer cell migration independent of serum concentration

(A) BT-549 breast cancer cells were transfected with siRNA as described in Figure 1. Seventy-two hours following transfection, cells were plated in serum-free media in Boyden Chambers. Media containing varying concentrations of FBS (0.1%-10%) was placed in the lower chambers to serve as a chemoattractant. After three hours, cells were fixed, stained, and the number of migratory cells per field was counted. Four fields were counted on each of two filters. Results are graphed as the average number of migratory cells per field ± SE. The average cells per field ± SE for three independent experiments are as follows: 0.1% FBS – con (0.38 ± 0.07), siLuc (0.46 ± 0.15), siSTAT5b (0.08 ± 0.04); 0.3% FBS – con (10.71 ± 2.49), siLuc (10.38 ± 1.45), siSTAT5b (3.88 ± 0.98); 0.5% FBS – con (28.29 ± 4.01), siLuc (26.58 ± 6.30), siSTAT5b (7.88 ± 3.31); 1% FBS – con (42.71 ± 4.31), siLuc (39.00 ± 4.54), siSTAT5b (13.92 ± 1.86); 3% FBS – con (59.96 ± 8.94), siLuc (62.88 ± 5.85), siSTAT5b (24.67 ± 8.15); 5% FBS – con (74.21 ± 6.12), siLuc (69.92 ± 3.59), siSTAT5b (33.83 ± 10.57); 10% FBS – con (70.54 ± 6.46), siLuc (60.54 ± 0.82), siSTAT5b (28.21 ± 7.99). Student's *t* test was used to determine statistical significance between the following: con and siSTAT5b *, $p \leq 0.0114$; siLuc and siSTAT5b •, $p \leq 0.0104$. (B) BT-549 breast cancer cells were plated in Boyden Chambers with media containing varying concentrations of serum in both the upper and lower chambers, as indicated. After three hours, cells were fixed, stained, and counted as described above. Results are listed as average cells per field ± SE for three independent experiments. Values in bold, located between the two lines, indicate chemokinetic conditions.

The effect of STAT5b knockdown on migration is not due to indirect effects on proliferation or survival - It is well established that STAT5b promotes cell cycle progression and survival of breast cancer cells [14-19]. To determine whether knockdown of STAT5b affected cell cycle, survival, or proliferation under the conditions of our migration experiments, we performed trypan blue assays and flow cytometry on knockdown cells seventy-two hours post-transfection. In both BT-549 and MDA-MB-231 cells, knockdown of STAT5b did not significantly alter the total number of adherent cells or the viability of adherent cells over a six hour time period, which is the maximum length of our Boyden Chamber migration assays (Table 1). We also examined the distribution of cells in each phase of the cell cycle following STAT5b knockdown. In BT-549 breast cancer cells, knockdown of STAT5b

increased the percentage of cells in G1 and decreased the percentage of cells in S phase compared to control siLuc knockdown (Figure 3A). A different effect was seen in MDA-MB-231 breast cancer cells, where knockdown of STAT5b increased the percentage of cells in S phase and decreased the percentage of cells in G2 (Figure 3B). These experiments were done simultaneously with Boyden Chamber migration assays in which knockdown of STAT5b inhibited migration in both cell lines. Therefore, while STAT5b knockdown does influence cell cycle distribution, these effects are minimal and do not correlate with the decrease in migration observed. Thus, we conclude that the inhibition of migration upon knockdown of STAT5b is not a result of secondary effects on adherence, viability, or the cell cycle.

Cell Line	siRNA	Adherent Cells (% ± SE)	Viable Adherent Cells (% ± SE)	Non-viable Adherent Cells (% ± SE)
BT-549	con	68.23 ± 5.73	75.03 ± 10.98	24.97 ± 10.98
	siLuc	59.03 ± 4.64	75.37 ± 5.43	24.63 ± 5.43
	siSTAT5b	58.39 ± 4.61	72.93 ± 8.01	27.07 ± 8.01
MDA-MB-231	con	79.22 ± 2.66	87.13 ± 1.70	12.87 ± 1.70
	siLuc	82.12 ± 1.79	91.72 ± 0.91	8.28 ± 0.91
	siSTAT5b	74.14 ± 5.34	84.33 ± 4.30	15.67 ± 4.30

Table 1: The effect of STAT5b knockdown on migration is not due to loss of viability or adhesion
Results listed are for three independent experiments. Student's *t* test was used to determine statistical significance between con and siSTAT5b and siLuc and siSTAT5b. None of these values were statistically significant.

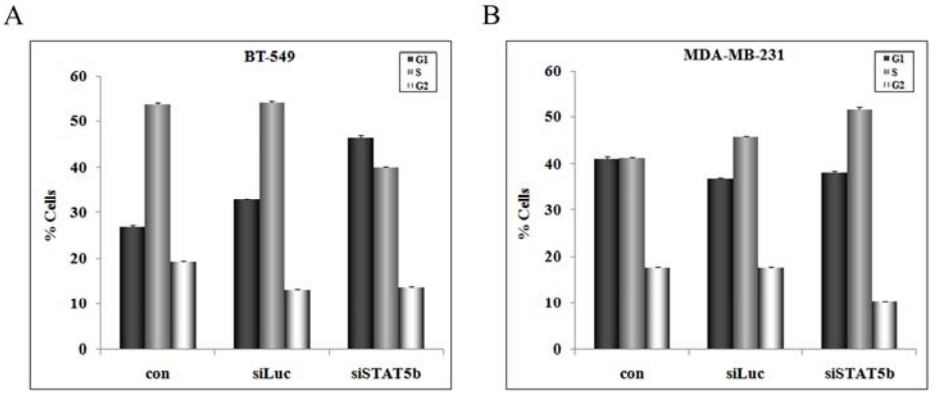


Figure 3: Cell cycle distribution following STAT5b knockdown
(A) BT-549 and (B) MDA-MB-231 breast cancer cells were transfected with siRNA as described in Figure 1. Seventy-two hours following transfection, cells were collected and fixed in 70% ethanol for 2 hours at 4°C. After fixation, cells were stained with a solution of 40µg/ml (BT-549) or 20µg/ml (MDA-MB-231) propidium iodide in 0.1% Triton X-100 in phosphate-buffered saline for one hour at room temperature, and flow cytometry was performed. Results are graphed as the percentage of cells in each phase of the cell cycle and represent an average of three triplicates from one experiment.

Inhibition of migration upon STAT5b knockdown can be rescued by re-introduction of STAT5b - To eliminate the possibility of off-target effects of STAT5b knockdown, we performed knockdown-rescue experiments. MDA-MB-231 cells were simultaneously transfected with STAT5b-specific siRNA and hemagglutinin (HA)-tagged wild-type or transcriptionally inactive Y699F forms of STAT5b. The rescue constructs were rendered immune to knockdown by introducing four silent point mutations in the siRNA targeted sequence. Consistent with previous experiments, knockdown of STAT5b inhibited migration of MDA-MB-231 cells by approximately 58%. Re-introduction of wild-type STAT5b restored migration to approximately 76% of control levels. The difference between rescue and control levels of migration was not statistically significant, confirming that the inhibition of migration upon STAT5b knockdown is due to a direct effect of STAT5b on migratory pathways.

Knockdown-rescue experiments can also be used to establish which regions or functions of a protein are necessary for mediating an observed effect. Therefore, we introduced a Y699F-STAT5b mutant to determine whether transcriptional activity of STAT5b is required for promoting migration. The Y699F-STAT5b mutant cannot be phosphorylated on the conserved tyrosine residue necessary for formation of an active dimer and subsequent transcriptional activity [20]. We have previously shown that knockdown of STAT5b inhibits transcription from the STAT5a/b-specific Spi2.1 luciferase reporter and that STAT5b transcriptional activity can be restored by co-transfection of siRNA-immune wild-type STAT5b, but not the Y699F-STAT5b mutant [21]. In migration assays, re-introduction of Y699F-

STAT5b into MDA-MB-231 cells rescued migration to the same level as rescue with wild-type STAT5b (Figure 4A). Together, these results suggest that the canonical transcriptional activity of STAT5b is not necessary for its pro-migratory function.

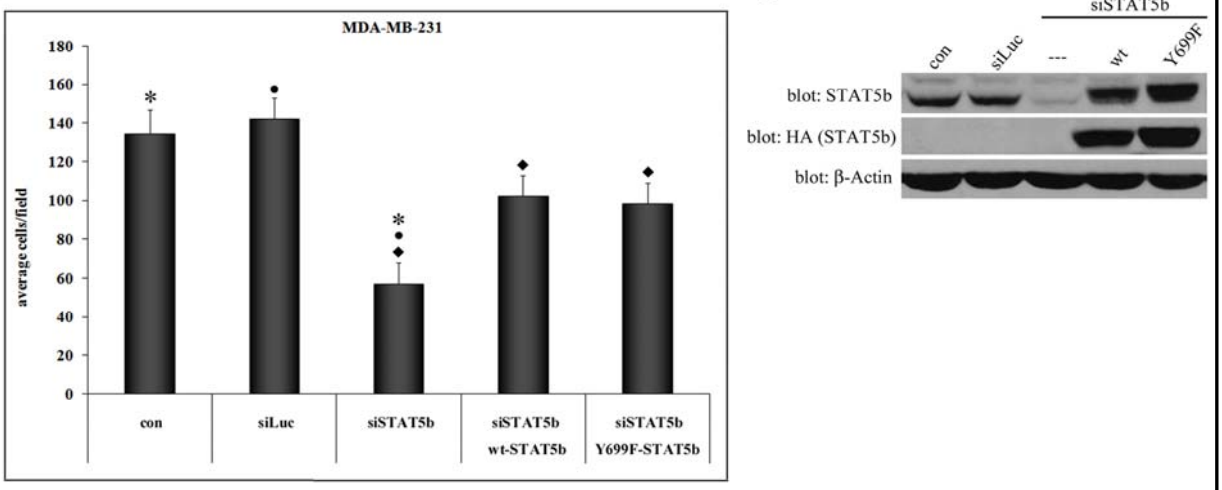


Figure 4: Re-introduction of both wild-type and transcriptionally inactive STAT5b rescues the migration defect caused by STAT5b knockdown

(A) MDA-MB-231 breast cancer cells were transfected with no siRNA (con), control siRNA to luciferase (siLuc) or siRNA specific to STAT5b (siSTAT5b) alone or in the presence of HA-tagged wild-type or Y699F-STAT5b constructs engineered to be immune to siRNA knockdown. Seventy-two hours following transfection, Boyden Chamber assays were performed for six hours as described in Figure 1. Results are graphed as average migratory cells per field ± SE for four independent experiments, and are as follows: con (134.47 ± 12.41), siLuc (142.31 ± 10.76), siSTAT5b (57.16 ± 10.88), siSTAT5b + wt-STAT5b (102.58 ± 10.09), siSTAT5b + Y699F-STAT5b (98.44 ± 10.49). Student's *t* test was used to determine statistical significance between the following: con and siSTAT5b *, p = 0.0034; siLuc and siSTAT5b •, p = 0.0014; siSTAT5b and siSTAT5b + wt-STAT5b ♦, p = 0.0222; siSTAT5b and siSTAT5b + Y699F-STAT5b ♦, p = 0.0341. Differences between siLuc and either siSTAT5b + wt-STAT5b or siSTAT5b + Y699F-STAT5b were not statistically significant. (B) Whole cell lysates from transfected MDA-MB-231 cells were collected seventy-two hours post-transfection and immunoblotted with antibodies specific for STAT5a, STAT5b, HA, or β–actin as a loading control.

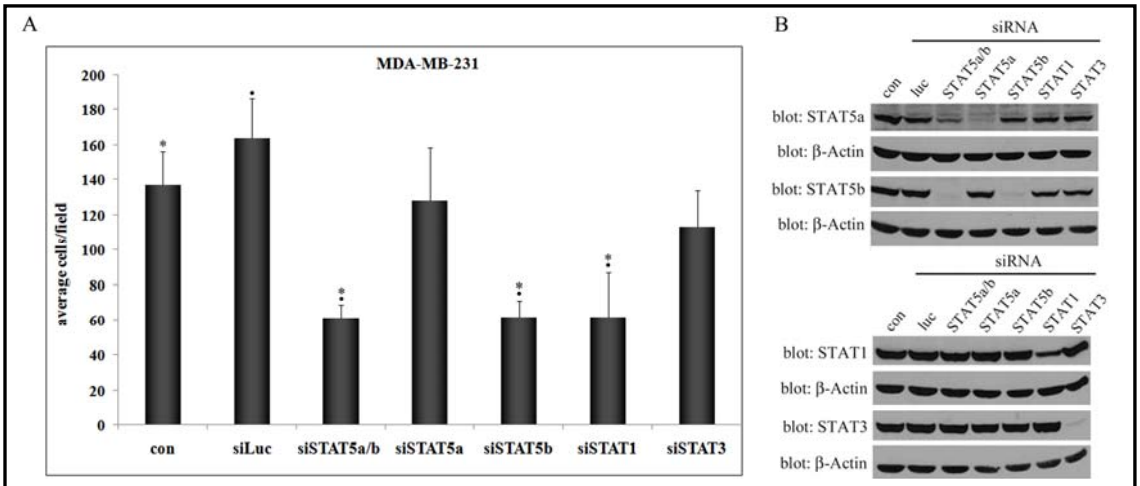


Figure 5: Knockdown of STAT5b or STAT1, but not STAT5a or STAT3, inhibits breast cancer cell migration

(A) MDA-MB-231 cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), or siRNA specific to STAT5a/b (siSTAT5a/b), STAT5a (siSTAT5a), STAT5b (siSTAT5b), STAT1 (siSTAT1), or STAT3 (siSTAT3). Seventy-two hours following transfection, Boyden Chamber migration assays were performed for six hours as described in Figure 1. Results are graphed as average cells per field ± SE for six independent experiments (with the exception of siSTAT5a which is the average of five independent experiments, and siSTAT1 which is the average of four independent experiments). Results are as follows: con (136.58 ± 19.78), siLuc (163.96 ± 22.02), siSTAT5a/b (61.02 ± 7.48), siSTAT5a (127.93 ± 30.69), siSTAT5b (61.79 ± 8.98), siSTAT1 (61.69 ± 25.09), siSTAT3 (113.25 ± 20.49). Student's *t* test was used to determine statistical significance between the following: con and siSTAT5a/b *, p = 0.0051; con and siSTAT5b *, p = 0.0063; con and siSTAT1 *, p = 0.0457; siLuc and siSTAT5a/b •, p = 0.0013; siLuc and siSTAT5b •, p = 0.0016; siLuc and siSTAT1 •, p = 0.0167. Comparisons of con and siSTAT5a, con and siSTAT3, siLuc and siSTAT5a, and siLuc and siSTAT3 were not statistically significant. (B) Whole cell lysates from siRNA-transfected MDA-MB-231 cells were collected seventy-two hours post-transfection and immunoblotted with antibodies specific for STAT5a, STAT5b, STAT1, or STAT3, and β–actin as a loading control.

Knockdown of STAT1, but not STAT5a or STAT3, inhibits breast cancer cell migration - STAT5a and STAT5b are highly homologous and are both activated in breast cancer, and previous work has shown that Prl-induced STAT5a activation suppresses migration of BT-20 and T-47D breast cancer cells [22] . Thus, we investigated the role of STAT5a, and other related STATs, STAT1 and STAT3, on migration in our model system. We chose to use the MDA-MB-231 breast cancer cell line because they are highly migratory and express all four STAT proteins. The siSTAT5b SMARTpool siRNA (Dharmacon) used in the BT-549 cell line, while efficient at knocking down STAT5b, also knocks down STAT5a by approximately 60% in MDA-MB-231 cells (Figure 5B, *lane 3*). To specifically target STAT5b in the MDA-MB-231 cells, we used one of the individual oligonucleotides from the SMARTpool, which had no effect on STAT5a levels (Figure 5B, *lane 5*). This single oligonucleotide had been used in previous knockdown experiments in the MDA-MB-231 cell line (Figure 1B). The total SMARTpool was utilized to determine the effect of dual knockdown of STAT5a and STAT5b in our MDA-MB-231 model system. It is of note that the BT-549 cell line does not express STAT5a, and therefore, the siSTAT5b SMARTpool was used in these cells without issue (Figure 1A).

As seen previously, knockdown of STAT5b inhibited migration of MDA-MB-231 cells by greater than 50%. In contrast, knockdown of STAT5a had no effect on migration of these cells (Figure 5A). Furthermore, knockdown of STAT5a in combination with STAT5b did not enhance the inhibition of migration due to knockdown of STAT5b alone. Similar to STAT5a, knockdown of STAT3 did not affect migration of MDA-MB-231 cells. Knockdown of STAT1 significantly inhibits their migration, to levels similar to that seen with STAT5b knockdown (55%). In summary, STAT5b and STAT1 are necessary for migration of EGFR overexpressing breast cancer cells, whereas STAT3 and STAT5a are not.

B. Determine if STAT5b is important for metastasis of breast cancer cells *in vivo*

No *in vivo* animal studies have been performed to date.

Task 2: Elucidate the mechanism by which STAT5b promotes breast cancer progression

A. Determine if STAT5b regulates transcription of tumor-secreted proteins Cyr61 and AM

B. Establish whether STAT5b is necessary for Cyr61- and AM- mediated tumor cell migration and invasion

No additional studies have been performed for Task 2 to date.

<u>Future Studies</u>

Future studies will be aimed at elucidating the mechanism by which STAT5b promotes migration of BT-549 and MDA-MB-231 breast cancer cells. First, wound healing assays will be performed in STAT5b knockdown cells with real-time live cell imaging. Wound closure rates and cell morphology will be compared between control and knockdown cells. In addition, immunofluorescence will be utilized to determine the subcellular localization of STAT5b in cells spread on fibronectin. Co-localization of STAT5b with focal adhesions, actin, and tubulin will be examined. These experiments will provide insight into a possible mechanism(s) by which STAT5b promotes migration of these cells.

In addition, the presence of STAT5b-STAT1 heterodimers in BT-549 and MDA-MB-231 cells will be evaluated. Co-immunoprecipitations of STAT5b and STAT1 will be performed in both cell lines to isolate potential heterodimers. Also, GST pulldown assays using GST-tagged wild-type and Y699F-STAT5b will be performed to compare proteins associated with wild-type and transcriptionally inactive STAT5b. Alternatively, nickel isolations of cells transiently transfected with His-tagged wild-type or Y699F-STAT5b may be employed.

Materials and Methods

Cell culture - BT-549 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were passaged twice per week and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All tissue culture reagents were purchased from Invitrogen (Gaithersburg, MD).

siRNA Transfection - BT-549 and MDA-MB-231 cells were transfected with siGENOME SMARTpool siRNA targeting human STAT5b, STAT1, or STAT3, individual custom oligonucleotides specific for STAT5a or STAT5b (siGENOME STAT5b SMARTpool duplex #3), or luciferase duplex control, all purchased from Dharmacon (Lafayette, CO). Transfections were performed using Oligofectamine (Invitrogen) as per manufacturers' instructions. For knockdown-rescue experiments, cells were transfected simultaneously with siSTAT5b SMARTpool duplex #3 and HA-tagged wild-type or Y699F STAT5b engineered to be immune to knockdown by introduction of four silent point mutations in the siRNA target sequence. These point mutations were introduced using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) and constructs were sequenced to verify mutations.

Immunoblotting - Cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris, pH 7.4, 1% deoxycholate, 1% Triton X-100, 5mM EDTA) containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and sodium orthovanadate (Sigma, St. Louis, MO). Protein lysates were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose (Pall Corporation, Pensacola, FL). Membranes were blocked in TBST (150mM NaCl, 0.1% Tween 20, 50mM Tris, pH 8.0) containing 5% nonfat dry milk and incubated with primary antibodies in TBST/5% milk. STAT1, STAT5a and STAT5b specific polyclonal antibodies were developed in our lab as previously described [11, 23]. STAT3 polyclonal antibody and β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA monoclonal antibody was obtained from the University of Virginia hybridoma facility. Secondary antibodies were applied in TBST and were HRP-conjugated sheep anti-mouse or donkey anti-rabbit (GE Healthcare, Piscataway, NJ). The enhanced chemiluminescence detection kit (GE Healthcare) was used to detect antibody binding. Acrylamide was from Bio-Rad (Hercules, CA), pre-stained molecular weight standards were from Sigma, and all other reagents were of reagent or molecular biological grade from Sigma.

Trypan Blue Assays - Seventy-two hours following siRNA transfection, $4x10^5$ cells were transferred to new plates containing DMEM/ 0.1% bovine-serum albumin (BSA) (Sigma) for 6 hours at 37^oC. Media was collected, cells were washed in phosphate-buffered saline (PBS) (Invitrogen), and the media and washes were combined to isolate non-adherent cells. Adherent cells were trypsinized and collected separately. Both fractions were stained with trypan blue (Fisher, Herndon, VA) and the total number of cells and number of trypan blue positive cells were counted via hemacytometer.

Propidium Iodide Staining and Flow Cytometry - Approximately $1x10^6$ cells were collected and fixed in 70% ethanol for two hours on ice. Fixed cells were washed in PBS and stained in a solution of 20µg/ml (MDA-MB-231 cells) or 40µg/ml (BT-549 cells) propidium iodide (Sigma) in 0.1% Triton X-100 containing 200µg/ml DNase-free RNase A (Qiagen, Germantown, MD) for one hour at room temperature. Cells were analyzed on a FACSCalibur bench cytometer (Becton Dickinson, San Jose, CA) using ModFit software.

Boyden Chamber Migration Assays - BT-549 and MDA-MB-231 cells were transfected with siRNA as described above. Seventy-two hours post-transfection, $5x10^4$ BT-549 cells or $1x10^5$ MDA-MB-231 cells were plated in serum-free (DMEM/0.1% BSA) media into the upper chambers of BD BioCoat Matrigel Control Chambers (BD Biosciences, San Jose, CA), and various concentration of FBS were placed in the lower chamber. Plates were incubated at 37^oC and migration was allowed to proceed for 3-6 hours. After this time, non-migratory cells in the upper chambers were removed with cotton swabs, and the remaining cells were stained with 0.1% crystal violet (Sigma) in 20% ethanol. Cells were counted using a Zeiss Invertoskop light microscope and graphed as average cells per field.

Key Research Accomplishments

- Construction of wild-type and Y699F-STAT5b plasmids immune to siRNA knockdown and optimization of knockdown-rescue experiments
- STAT5b, STAT5a, STAT1, and STAT3 can be specifically and efficiently knocked down in breast cancer cells which express all four STAT family members
- STAT5b plays an integral role in the migration of breast cancer cells to serum
- Knockdown of STAT5b inhibits chemotactic migration of breast cancer cells
- Knockdown of STAT5b does not alter viability, adherence, or cell cycle distribution of breast cancer cells over a 6 hour time period
- Loss of migration upon STAT5b knockdown is rescued with re-introduction of either wild-type or a transcriptionally inactive Y699F-STAT5b mutant
- Knockdown of STAT1 inhibits migration of breast cancer cells to serum
- Knockdown of STAT5a and STAT3 have no effect on breast cancer cell migration to serum

Reportable Outcomes

- Oral presentation
  25th Annual American Cancer Society Seminar of Cancer Researchers in Virginia (November 2007; Richmond, VA):
  Role of STAT5b in Breast Cancer Cell Migration
  University of Virginia
  Departments of Microbiology and Medicine and the Cancer Center

- Poster presentation
  2008 DOD Era of Hope Meeting (June 2008; Baltimore, MD)
  P36-12: Role of Signal Transducer and Activator of Transcription 5B in Breast Cancer Cell Migration
  Teresa M. Bernaciak and Corinne M. Silva
  University of Virginia
  Departments of Microbiology and Medicine and the Cancer Center

- Journal article submission
  Bernaciak TM and Silva CM: **Signal transducer and activator of transcription 5b (STAT5b) is an important signaling component of human breast cancer cell migration.**
  (submitted to Breast Cancer Research)

Conclusion

Our studies demonstrate an integral role for STAT5b in breast cancer cell migration. While a role for STAT5b in breast cancer cell proliferation and survival has already been established, this work is the first to show a positive regulatory role for STAT5b in breast cancer migration. Therefore, STAT5b may not only function in the initiation of tumorigenesis, through its pro-proliferative and pro-survival signaling, but may also promote tumor progression by stimulating migration. Future studies will elucidate the mechanism by which STAT5b exerts its effect on migration, thereby broadening our understanding of how STAT5b promotes tumorigenesis and possibly metastasis. This will facilitate the long-term goal of defining conditions whereby STAT5b would be an effective therapeutic target for the treatment of breast cancer.

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