Optical Imaging of Metabolic Adaptability as a Biomarker for Metastatic Potential in Breast Cancer Cells

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Abstract

Breast cancer metastasis is the main cause for mortality in breast cancer patients. However, knowledge of metastatic recurrence is limited, and there is a need to understand metastatic recurrence in order to treat breast cancer patients more effectively. Highly invasive metastatic breast cancer has shown to exhibit metabolic adaptability, transitioning from glycolysis to oxidative phosphorylation in the presence of microenvironmental stress. NADH and FAD are naturally occurring cofactor products during glycolysis and oxidative phosphorylation, respectively, and they are of particular importance during these metabolic processes due to their endogenous fluorescence. Measuring the ratio of fluorescence intensities of these cofactors through a redox ratio allows for the quantification of the comparative levels of glycolysis and oxidative phosphorylation occurring in breast cancer cells. Two-photon imaging was used to assess the redox ratio of 4T1KOT, a variant of highly metastatic 4T1 cells with deletion of the TWIST gene, a gene known to promote metastasis. This thesis reports that 4T1KOT cells, compared to metastatic 4T1 cells, have a decreased redox ratio at normoxia conditions, and they also lose their metabolic adaptability under hypoxia-reoxygenation stress. This study demonstrates that metabolic adaptability to hypoxia-reoxygenation stress could be used as a biomarker for breast cancer metastatic potential.
Background

Cancer metastasis to other organ systems is the leading cause of mortality in breast cancer patients [1]. Currently, there is very little knowledge of metastatic recurrence in breast cancer; it is necessary to understand markers of metastatic recurrence in breast cancer so that patients receive appropriate treatment based on metastatic potential of the primary tumor [2]. A current method for assessing the benefits of chemotherapy, and thus the risk of metastatic recurrence, is the Oncotype DX. The Oncotype DX uses an analysis of 16 breast cancer genes identified in literature as important indicators of metastatic recurrence and compares these genes with 5 reference genes not linked to metastatic recurrence, producing a score to assess the benefit of chemotherapy treatment [3]. However, the Oncotype DX is expensive (~$4000 per test), not available at all medical centers, and not able to be used for all tumor receptor statuses and sizes [4]. Therefore, there is still a need to determine markers for metastatic potential in breast cancer cells.

A common metabolic characteristic of cancer cells is the Warburg Effect, a discovery that tumor cells tend to rely more on glycolysis over oxidative phosphorylation in order to meet their energy needs [5]. However, recent studies have concluded that highly invasive cancer cells favor oxidative phosphorylation over glycolysis, using the extra energy to migrate to other organs in the body [6-7]. One of these studies explored metabolic features of metastatic cancer cells, particularly regarding the changes in metabolism that highly invasive breast cancer cells exhibit in response to microenvironmental stress [7]. Compared to cancer cells proliferating in either the breast tissue or another target organ, cancer cells migrating through the bloodstream
exhibited higher levels of oxidative phosphorylation, while the proliferating cancer cells were more glycolytic (Figure 1) [7]. A clinical application would need to be able to detect invasive cancers based on metabolic activity of the primary tumor and not circulating cancer cells. The study thus investigated primary cancer cells from three invasive breast cancer cell lines and found that hypoxia-reoxygenation, a microenvironmental stressor, upregulated PGC-1α expression in these proliferating cancer cells (Figure 1); PGC-1α is a gene that promotes oxidative phosphorylation [7]. Furthermore, acute hypoxia has been noted in another study to produce an environment more conducive to migration for cancer cells [8]. The study by LeBleu et. al [7] made us ask the question of whether this increase in oxidative phosphorylation triggered by microenvironmental stress was inherent of all breast cancer cells or just metastatic breast cancer cells.

**Figure 1**

**A:** Primary cancer cells (PCC’s), circulating cancer cells (CCC’s), and lung metastatic cancer cells (MCC’s) are illustrated [7].

**B:** Heat map illustrating expression of common genes between PCC’s, CCC’s, and MCC’s that are related to oxidative phosphorylation. A Z-score approaching 1 (red) corresponded with greater oxidative phosphorylation levels. Migratory CCC’s exhibited much greater levels of oxidative phosphorylation compared to proliferative PCC’s and MCC’s [7].
PGC-1α expression levels of three highly invasive breast cancer cell lines under two conditions: Normoxia (21% O₂) and Hypoxia-Reoxygenation (48 hours 1-2% O₂, 24 hours 21% O₂). The metastatic cell lines exhibited metabolic adaptability, increasing their oxidative phosphorylation in the presence of microenvironmental stress (hypoxia-reoxygenation) [7].

Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are two major cofactor products that occur in glycolysis and oxidative phosphorylation, respectively (Figure 2). These two cofactors are of importance due to their endogenous fluorescence [9]. During glycolysis, NAD is reduced to NADH in the cytoplasm; during oxidative phosphorylation, which occurs in the mitochondria, NADH is oxidized to NAD⁺ and FADH₂ is oxidized to FAD [4]. Because NADH and FAD are the only forms of these cofactors that have endogenous fluorescence, a redox ratio of fluorescence intensities of NADH and FAD (Figure 2), calculated through optical imaging methods like two-photon microscopy, allows for a quantification of the relative levels of glycolysis and oxidative phosphorylation occurring in the cell [10]. A past study in our lab found that the redox ratio changed in response to common mitochondrial drugs that affect the electron transport chain [4].

**Figure 2**

**A:** The schematic on the left illustrates the roles of fluorescent NADH and FAD during metabolism. The equation on the right illustrates how we define our redox ratio.
ratio, with a higher redox ratio indicating greater levels of oxidative phosphorylation occurring in the cell.

B: This experiment tracked the changes in redox ratio in responses to common mitochondrial drugs. Oligomycin inhibits Complex II, which drives down [FAD], decreasing redox ratio. FCCP drives the consumption of NADH by Complex I, increasing the redox ratio. Antimycin A/Rotenone inhibits Complex I, decreasing the redox ratio [4].

A recent study in our lab sought to assess the change in redox ratio in a panel of isogenic triple negative breast cancer cells with varying metastatic potential. The results of the study found that not only does the normoxic redox ratio decrease as metastatic potential decreases, but also the metabolic adaptability decreases as metastatic potential decreases [4]. This study caused us to further investigate the possible link between metabolic adaptability to microenvironmental stress and metastasis by posing the question of how deletion of a metastasis-promoting gene in highly invasive 4T1 cells would affect their metabolic adaptability.

TWIST is a gene that has been linked to metastatic potential through epithelial to mesenchymal transition [11]. A past study demonstrated that inhibition of TWIST in 4T1 cells resulted in significantly less metastatic nodes in the lungs of the Balb/c mice. Furthermore, this study noted that 70% of human invasive lobular carcinomas have been found to express TWIST, signifying the clinical relevance of this metastasis-promoting gene [11].

CRISPR/Cas9 gene editing allows for simple, permanent removal of target gene sequences. Derived from bacterial immune defense, the CRISPR/Cas9 system, combined with a 20-nucleotide single guide RNA (sgRNA) for base pair recognition, utilizes the Cas9 enzyme to cleave the desired target 20-base pair DNA sequence [12].
This method of DNA modification allows for the development of a permanent 4T1 cell line with the TWIST gene deleted.

**Methods**

**Breast Cancer Cell Culture**

Breast cancer cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) while also adding 10% fetal bovine serum (FBS), 2 mM of L-Glutamine, 1% penicillin-streptomycin (Pen-strep), and 1% nonessential amino acids (NEAA) [4]. Cells were placed in an incubator with conditions of 37° C, 21% O₂, and 5% CO₂. Upon reaching 70-80% confluency, cells were passaged into a new T75 flask.

**CRISPR/Cas9 Deletion of TWIST and Clonal Selection**

Using the sgRNA guide tool provided by the Zhang laboratory (MIT, Cambridge, MA), the 20-base pair sgRNA was identified to target the TWIST gene, also known as the Twist1 gene, in 4T1 mouse cells. The sgRNA was cloned into pCas-Guide-EF1a-GFP plasmids through the services of OriGene (Rockville, MD). These plasmids were expanded using E. Coli bacteria, and plasmid isolation from E. Coli was achieved using the QIAGEN Plasmid Maxi Kit. For plasmid transfection, 4T1 cells were seeded in a 6-well plate at a concentration of one million cells/well and incubated for 24 hours. 10 μg of plasmid were added to Lipofectamine 3000 and added to the 4T1 cells. After 24 to 48 hours, green fluorescent protein (GFP) signal was detected using a Nikon TiE fluorescence microscope workstation with a CoolSnap HQ2 camera; signals exhibiting GFP expression indicated the transfected cells, as the plasmid contained
GFP. Transfected 4T1 cells were suspended in a PBS solution at a concentration of two million cells per mL. The transfected cells were filtered through a 50 μm filter into a FACS tube. Cell sorting was achieved through the FACS Aria III System (BD Biosciences, San Jose, CA). The 5% of transfected cells that exhibited the greatest GFP expression were sorted to have cells with the highest CRIPSR/Cas9 plasmid concentration. The sorted cells were incubated and cultured for 7 to 14 days in the same incubation settings described above. The cell colonies were then cloned into 13 clones to produce a population that had the least TWIST expression.

**Flow Cytometry Preparation Protocol to Detect TWIST Expression**

The protocol is summarized below (Figure 3). A primary TWIST antibody (TWIST2C1a) was purchased from Santa Cruz Biotechnologies (Dallas, TX). In addition, an appropriate fluorescent secondary antibody (sc-2078) was also purchased through Santa Cruz. After obtaining 3 million cells in media, cells were centrifuged 400xg for 5 minutes to remove residual media; all subsequent centrifuges were at the same settings. The cells were resuspended in 1 mL PBS and centrifuged. The cells were then resuspended in 1 mL of 5x TF Perm/Wash Buffer (diluted in DI water) and centrifuged. Resuspension then occurred in 250 μL of 4x TF Fix/Perm (diluted in diluent buffer) and incubated in the dark at 4⁰ C for one hour. The cells were washed and centrifuged twice with 1 mL TF Perm/Wash. After resuspension in 500 μL of Perm/Wash, the volume was split into 3 polystyrene tubes, each labelled either experimental, isotype control, or negative control. 30 μL of primary antibody was added to the experimental tube, and all tubes were incubated in the dark at 4⁰ C for 1 hour.
After 2 more washes after incubation, 4 μL of secondary antibody was added to the experimental and isotype control tubes and incubated with the same conditions used for the primary antibody. The cells were then washed twice again and resuspended in 500 μL of Perm/Wash. Flow cytometry was performed using a flow cytometer from BD Biosciences.

**Figure 3**: Flow chart for preparation of cells for flow cytometry analysis for TWIST expression.
Lysate Preparation for Western Blot Analysis for TWIST Expression

Breast cancer cells were cultured and placed in a 6-well plate with a seeding density of 250,000 cells/well and incubated for 24 hours. After incubation, 75 μL of protein buffer was added to each well and accumulated using a cell scraper. The solution was filtered in a microcentrifuge tube and centrifuged at 3500 RPM for 3 minutes. The lysate solution was stored at -80º C until Western Blot Analysis was performed.

Normoxia and Hypoxia-Reoxygenation Protocols for Two-Photon Microscopy

Normoxia conditions were defined as the oxygen level during standard incubation (21% O₂), while hypoxiareoxygenation conditions were defined as one hour of chronic hypoxia (0.5% O₂) followed by one hour of reoxygenation in the incubator (21% O₂). Hypoxia-reoxygenation was achieved through the Oxycycler C42, a system that controls various levels of oxygen, nitrogen, and carbon dioxide through software; both the system and software were created by BioSpherix (Parish, NY).

Two-Photon Microscopy of 4T1-TWIST Knockout (4T1KOT) Cells

4T1KOT cells were cultured and plated on glass slides in 6 individual 35 mm petri dishes at a seeding density of 150,000 cells/mL. The cells incubated for approximately 72 hours until they reached 70-80% confluency. 3 petri dishes were designated for the normoxia condition, while 3 petri dishes were designated for the hypoxia-reoxygenation condition. 3 fields of view were taken for each dish, resulting in 9 total images for each condition. Images were taken on a custom-built multiphoton
microscope using a 20X water immersion objective lens with a 4X optical zoom. Images were 512 x 512 pixels with a 16-bit depth and a field of view of 130 μm x 130 μm. NADH was excited at 755 nm with resulting emission spectra of 460/40 nm; FAD was excited at 860 nm with resulting emission spectra of 525/40 nm. NADH and FAD were imaged at power settings of 30 mW and 40 mW, respectively. Images were analyzed in MATLAB by taking the average NADH and FAD intensity of 6 randomly circled cells in the image and calculating the redox ratio using these intensities values. The two-photon microscope protocol is summarized below (Figure 4).

Figure 4: Two-photon imaging protocol for 4T1KOT Cells. Power settings for NADH and FAD are listed under their respective names in parentheses.
Statistical Analysis

One-way ANOVA was used for analysis for the Western Blots, and a nested, two-way ANOVA was used for the redox ratio data.

Results

TWIST Expression Decreases as Metastatic Potential Decreases

Figure 5: Bar plot and Western Blot illustrating TWIST expression in 4T1, 4T07, and 168FARN. The figure also notes the metastatic potential of the cell lines, and the data suggests that TWIST expression could be linked to metastatic potential. Ponceau S is the reference protein used in the Western Blot. Error bars indicate the standard error of the mean TWIST expression level. The representative blots are derived from the same gel and image. * indicates p<0.05.
CRISPR/Cas9 editing produces a 4T1 cell line with decreased TWIST expression

Figure 6

A: Bar plot and Western Blot images of 4T1 and several of the possible 4T1KOT clones created through clonal selection. Clones were named after the respective well in which they were originally seeded in a 96-well plate. Ponceau S is the reference protein for the Western Blot data. Error bars indicate standard error of the mean TWIST expression level for each cell line.

B: Bar plot and Western Blot images results comparing TWIST expression of 4T1 to Clone A2, the clone we chose to be our 4T1KOT cell line. The results are normalized, with the 4T1 TWIST expression denoted as 1. 4T1KOT was achieved through CRISPR/Cas9 deletion and clonal selection. Error bar indicates the standard error of the mean TWIST expression level. Western blot images are derived from the same gel and image; the original image is shown in Figure 6A. * indicates significance at the level p < 0.05.
Deletion of TWIST from 4T1 Cells Decreases Redox Ratio and Eliminates Metabolic Adaptability

**Figure 7**

**A:** Redox Images of 4T1, 168FARN, and the 4T1 TWIST Knockout (4T1KOT) cell lines during normoxia and hypoxia-reoxygenation. The images show an increase in redox ratio for 4T1 after hypoxic stress, while no change in redox ratio for the less metastatic 168FARN. In addition, normoxia redox ratio is decreased for the non-metastatic breast cancer cell line 168FARN. 4T1KOT produces redox images similar to 168FARN.

**B:** Bar plot of the redox ratio of the three cell lines during normoxia and hypoxia-reoxygenation conditions. The data suggests both a decrease in optical redox ratio and a loss of metabolic adaptability upon loss of metastatic ability due to
TWIST deletion. Error bars indicate the standard deviation of the mean redox ratio. *** Denotes statistical significance at the level p<0.001.

C: Bar plot of the post-hypoxia change in redox ratio of the three cell lines, our measure of metabolic adaptability. Error bars indicate the standard deviation, calculated as $\sqrt{(s_1)^2 + (s_2)^2}$, where $s_1$ and $s_2$ are standard deviations of the redox ratio of the cell line at normoxia and hypoxia-reoxygenation, respectively.

4T1 and 168FARN images were gathered during a previous study in our lab [4].

Discussion / Conclusion

While the flow cytometry protocol could detect positive TWIST expression in 4T1 cells (Appendix), we opted to do Western Blots due to the efficiency that the procedure provides that flow cytometry didn’t. Lysates could be frozen down and used at the same time conveniently; however, all 13 clones, 4T1 cells, and a negative control cell line would have to be 70-80% confluent in culture at the same time for flow cytometry, which is very difficult to obtain in terms of cell culture.

Lessons learned from producing the flow cytometry protocol were very important for possible future uses. The protocol provided by the Zaharoff lab at the University of Arkansas originally called for a 0.2% Triton solution to act as the detergent; however, the detergent was killing our 4T1 cells prior to flow cytometry. After consultation with graduate students in the Zaharoff lab, we changed the detergent from a 0.2% Triton solution to the 4X TF Perm/Fix in order to simultaneously permeabilize and fix the cells to avoid cell death. Another issue we ran into was the identification of appropriate antibodies. We ultimately found a very commonly used TWIST primary antibody in literature; while many companies offered TWIST antibodies, Santa Cruz had by far the most publications with their antibody.
The Western Blot data regarding three breast cancer cell lines of varying metastatic potential is in consistency with the description of TWIST as a promoter of metastasis [11]. The Western Blot data of the 4T1KOT clones illustrates that we have developed a population of 4T1 cells that have varying levels of TWIST expression, and this provides us with the ability to add more data points to assess metabolic adaptability of 4T1 cells as a function of TWIST expression levels in the future. It is important to note that 4T1KOT (Clone A2), the clone we chose to model as our TWIST knockout cell line, still has some expression of TWIST. We believe this TWIST expression is due to the heterogeneity of the cell line, as some 4T1 cells with TWIST not deleted are likely to exist past the CRISPR/Cas9 process.

Based on the redox data, we have shown that metabolic adaptability in response to our hypoxia-reoxygenation stress test is a marker for metastatic potential. To support past research that compared metastatic and non-metastatic breast cancer cell lines, this study deleted TWIST, a metastasis-promoting gene, from the 4T1 genome [4,11]. Deletion of this metastasis-promoting gene not only reduced the normoxia redox ratio of 4T1 cells, but it also eliminated 4T1’s metabolic adaptability to the hypoxia-reoxygenation stress test. Comparatively, the 4T1KOT’s metabolic profile during normoxia and hypoxia-reoxygenation now resembles non-metastatic 168FARN [4].

Future studies will seek to examine the 4T1KOT cell line in vivo. We will be injecting mice with 4T1 and 4T1KOT cells to assess metastatic nodes in the lung, an experiment similar to the study done by Yang et. al [11]. In addition, future studies will seek to assess the redox ratio of human breast cancer cell lines of varying metastatic ability and determine their metabolic adaptability to the hypoxia-reoxygenation stress
test. Future work will also focus on redox imaging of primary tumors derived from mice under the same conditions as *in vitro* studies.

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- Website: http://crispr.mit.edu:8079/?

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Appendix

Figure 8:
Flow cytometry graphs that demonstrate 4T1 TWIST expression. “4T1-Negative” is a flow cytometry plot of 4T1 cells that received neither the primary nor the secondary antibody. “4T1-Iso” is a flow cytometry plot of 4T1 cells that received only the secondary antibody; this plot accounts for any non-specific binding of the secondary antibody that would contribute to a false positive TWIST expression. “4T1-Sample SC” is the flow cytometry plot of 4T1 cells incubated with both primary and secondary antibodies; SC stands for Santa Cruz, the company that provided the antibodies. Secondary antibody fluorescence was detected in the FITC Channel (Max excitation = 494 nm, Max emission = 520 nm), and positive TWIST expression was defined as the portion of the plot located in Quadrant P4. The plots demonstrate that the flow cytometry protocol described in the Methods section was successful in identifying TWIST expression in 4T1 cells.
References