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Characterizing the role of Nucleosome Remodeling Factor (NURF) in tumorigenesis and metastatic progression using mouse models of breast cancer.

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A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

4T1	Metastatic Mouse Breast Cancer Cell line
66cl4	Metastatic Mouse Breast Cancer Cell line
67NR	Non-metastatic Mouse Breast Cancer Cell line
6-TG	6-Thioguanine
ATP	Adenosine tri-phosphate
Bptf	Bromodomain PHD-finger-containing Transcription Factor
BRCA	Breast Cancer Susceptibility Gene
CpG	Cytosine-Guanine Dinucleotides
CTL	Cytotoxic T-Lymphocyte
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DP	Double Positive
ECM	Extracellular Matrix
EMT	Epithelial Mesenchyma Transition
ESC	Embryonic Stem Cell
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
IACUC	Institutional Animal Care and Use Committee
ISWI	Imitation Switch/SNF

KD	Knockdown
MDSC	Myeloid-Derived Suppressor Cell
MEF	Mouse Embryonic Fibroblast
MHC	Major Histocompatibility Complex
Mnase	Micrococcal Nuclease
NK	Natural Killer
NSG	NOD SCID Gamma, immune compromised mouse
NURF	Nucleosome Remodeling Factor
PBS	Phosphate Buffered Saline
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium
RT-qPCR	Reverse Trascriptase quantitative Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
shRNA	Short-hairpin RNA
SNF2L	Sucrose nonfermentable-2-like
SWI/SNF	Switch/Sucrose nonfermentable
TRI	Trizol® Reagent

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Abstract

CHARACTERIZING THE ROLE OF NUCLEOSOME REMODELING FACTOR (NURF) IN TUMORIGENESIS AND METASTATIC PROGRESSION USING MOUSE MODELS OF BREAST CANCER

By Suehyb G. Alkhatib, B.S.

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

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Increasingly the role of epigenetic machinery as a bridge between underlying DNA sequence and cellular phenotype is being discovered. The establishment of a myriad of unique cellular types sharing identical gene sequences in a multicellular organism gives a broad sense for the inherent role of epigenetic influence on cell differentiation. Importantly, the epigenetic mechanisms involved in establishing cell identity unsurprisingly contribute to diseased states, including cancer. Recent research continues to elucidate contributory roles of epigenetic mechanisms, such as DNA methylation, histone modification, and microRNA regulation, in human cancers. Additionally, chromatin remodelers, such as the Nucleosome Remodeling Factor (NURF), have been identified as important regulators for normal cell biology. While much has been done to identify and characterize the role of NURF chromatin remodeling

complex as a key regulator of development in a number of model organisms, little has been published on the implications of NURF in diseases such as cancer. Our preliminary data shows dysregulation of E-cadherins, N-cadherins, and MHC-I genes in Bptf (an essential subunit of NURF) knocked down murine breast cancer cell lines. These proteins have well documented roles in the development and metastatic progression of cancers. To study the effect of Bptf knockdown on the development and progression of cancer we injected Bptf knocked down mouse breast cancer cell lines, 4T1, 66cl4, and 67NR, into syngenic BALB/c mice. Our findings reveal decreased tumor growth in 66cl4 and 67NR as measured by tumor weight at 3-4 weeks post injection. Tumor growth did not appear to be significantly affected in 4T1 challenged mice. However, mice inoculated with Bptf knockdown 4T1 cell lines have decreased metastasis to lungs as compared to control while metastasis of 66cl4 tumors to the lungs appear unaffected. To assess the role of the immune system in decreasing tumor growth in BALB/c mice, we injected 66cl4 tumors into NOD-SCID-Gamma (NSG) immune deficient mice. The tumors from these mice show no difference in tumor growth between Bptf knockdown and control tumors, implicating a role for the immune system regulating the decreased tumor weight in BALB/c mice. To delineate which immune cell effector may impede breast cancer carcinogenesis, we performed an *in vitro* natural killer (NK) cell cytotoxicity assay against 66cl4 tumors and found greater susceptibility to NK killing in Bptf knockdown tumors.

Chapter 1: Introduction

Cancer Statistics

According to the National Cancer Institute Surveillance Epidemiology and End Result report in 2008, the prevalence of cancer in the United States of America was nearly 12 million people. An estimated 1.6 million Americans will be diagnosed with cancer in 2011, of these more than a third are expected to die of their cancer (1). The International Agency for Research on Cancer, an arm of the World Health Organization, paints an equally sobering picture of cancer statistics worldwide. A 2008 IARC report estimated the number of newly diagnosed cancer cases to be 12.7 million worldwide, with the second highest incidence occurring in North America. That same year 7.6 million people died of cancer (2). Clearly, the economic burden of cancer treatment is extensive.

The Challenges of Cancer

Cancer describes a large number of diseases exhibiting complex and variable phenotypes that generally involve an abnormal growth of cells, termed neoplasm, within different tissues. Over the last decade, cancer research has gradually undergone a conceptual shift, where the complex and widely variable nature of cancers is being characterized by underlying and unifying concepts across different cancers (3). Termed the "Hallmarks of Cancer" by Hanahan and Weinberg, these principles provide a broad structural framework for approaching cancer research. As in the normal cell state, the genomic state of cells contributes to the neoplastic phenotype observed in cancers. In addition to single somatic point-mutations and chromosomal rearrangements observed in cells acquiring some of the cancer hallmarks, single-

event broad alterations of genomic structure have recently been observed in cancer development as well (4). These advances in our understanding of cancer have increasingly revealed the pivotal role of epigenetic alterations in the development and progression of cancers.

Breast Cancer

Breast cancer accounts for one quarter of all cancers excluding non-melanoma skin cancers in women (2). While the best known genetic causes for breast cancer in humans are mutations in the breast cancer susceptibility (BRCA) genes 1 and 2 (BRCA1 and BRCA2, respectively), these only account for less than 5% of known causes of breast cancer in women (5). Despite the characterization of breast cancers based on gene expression patterns, the functional role of these genes for the development and metastatic progression of breast cancer remains to be elucidated (6). Still, the processes involved in breast cancer progression represent an underlying set of characteristics in accord with the Hallmarks described by Hanahan and Weinberg.

As with all cancers, the underlying primary insult leading to a tumorigenic cell involves the expression of an oncogene (such as the RAS) or inactivation of a tumor suppressor to allow for uncontrolled cell division and avoidance of apoptotic signals (as in mutations in the PTEN tumor suppressor found in breast cancer) (7). During their growth, cells accumulate advantageous genetic changes that allow for the tumor to continue to grow and metastasize in the patient.

With increased growth tumors may be restricted by limited nutrient supply or face elimination by anititumor immunity. The expression of angiogenic factors (i.e. vascular endothelial growth factors) by tumors to increase blood supply to the growing tumor is well known and has been

studied extensively (3). Over the past decade more research has elucidated the role of immune cells in preventing and promoting cancer. The immune system plays key roles in the formation of cancers from the point of initiation to final dissemination and development of the metastatic state of the disease (8).

In the final stages of breast cancer, the cancer becomes systemic and spreads to other organs in the body. The most common metastatic sites are bone, lung, brain and liver (9). While less is known of the processes mediating late stage metastasis due to the complexity of steps involved in metastasis, many studies have looked at the processes mediating the initiation of metastasis. This has lead to the identification of a distinct set of genes and pathways thought to be involved in promoting metastasis, including inflammatory cytokines, a modified extracellular matrix (ECM) in the surrounding tumor environment, activation of epithilial-mesenchymal transition (EMT), and others (6). Even less is understood about the processes that mediate the colonization and formation of secondary tumor sites at distant organs. However, there are a number of useful animal models in use now that are allowing the study of the events involved in the formation of primary and metastasis of breast cancers.

Mouse Breast Cancer Model

Female BALB/cJ mice are among the most widely distributed mouse models in animal research (10). A strain of BALB/c mice fostering a C_3H virus have increased incidence in spontaneous mammary tumors, not typically seen in BALB/c mice. A sub-population of 5 tumor cell lines were derived from a BALB/c C_3H mouse, and were subsequently characterized for tumorigenic and metastatic propensities (11). The highest tumorigenic cell line, 67NR, is non-metastatic. The

two metastatic cell lines, 4T1 and 66cl4, are suspected to undergo metastasis via hematogenous and lymphogenous modes, respectively (12). Additionally, as with clinical observations, these two metastatic cell lines established secondary tumors with propensities for different organs. The 4T1 line was shown to preferentially metastasize to lung, and later to liver, bone, and brain, the primary organs affected by metastatic breast diseases in humans (13). However, 66cl4 cells did not appear to establish secondary tumors in the liver. The variability observed and the characterization of these cell lines provides a useful tool to study breast cancers in an animal model that recapitulates the phenotypic variability seen in the human disease, thus allowing for the elucidation of underlying processes involved in tumorigenesis and metastatic progression. The model is also useful for studying the role of the immune system in breast cancer because the cancers are syngenic to BALB/c mice.

Epigenetics and Cancer

Epigenetics describes heritable expression patterns that are not determined by underlying genetics, or DNA sequence. Epigenetic mechanisms are prevalent during normal development of cells and play critical roles in determining the gene expression profiles observed across differentiated cell lines. Unraveling epigenetic mechanisms has provided insight into how two distinct cells with the same genetic sequence can have drastically altered expression patterns giving divergent phenotypes. Perhaps the best understood epigenetic mechanism is the methylation of cytosine in CpG dinucleotides, in what are known as CpG islands to repress transcription. More recently, the roles of histone variants, non-coding RNAs, and ATP-dependent nucleosome remodelers in determining the epigenetic state of cells are coming into

light (14). The epigenetic contribution to cancer development is also being elucidated, revealing a large role for epigenetic regulators in the initiation and progression of cancers. For example, Berdasco and Esteller (15) provide a broad overview for the role of epigentics in cancer, including the hypermethylation of housekeeping genes and tumor suppressing miRNAs to inactivate their expression and aberrant histone modification in cancers. Although known to be critical in development, less understood is the role of nucleosome remodeling factors in the development of cancers.

ATP-Dependent nucleosome remodelers: Nuclesome Remodeling Factor (NURF)

There are currently four families of nucleosome remodeling factors: SWI/SNF, INO80, CHD, and ISWI, grouped by their ATP-ase homology (16). These remodelers are thought to alter DNA-transcription factor interaction through sliding or evicting nucleosomes away from or over transcription binding sequences. The Nucleosome Remodeling Factor (NURF) complex is the founding member of the ISWI family of nucleosome remodelers. NURF's effects on nucleosome positioning were initially characterized in *Drosphila melanogaster* (17). The NURF complex in *D. melanogaster* is composed of four subunits, NURF301 (Bptf), NURF140, NURF55, and NURF38. The NURF140 subunit revealed sequence homology with the ATPase SWI2/SNF2, but still maintained unique biochemical properties (18). Subsequent characterization of NURF's activity showed NURF slides nucleosomes in 10 bp increments in either direction with minimal unraveling of DNA (19). Purification and characterization of human NURF revealed the mammalian ortholog for *D. melanogaster* NURF. The mammalian NURF is composed of three subunits, the bromodomain PHD-finger-containing transcription factor (BPTF), the SNF2L

ATPase, and pRBAP46/48, representing the respective homologs for the NURF301, NURF140, and NURF55 found in *D. melanogaster* (20). The largest subunit, NURF 301, has been shown to be essential and unique to the *D. melanogaster* NURF complex, making it an ideal target for studying the function of NURF.

While *D*. *melanogaster* NURF has been well studied, characterized, and revealed to be essential for a number of biological pathways, much remains to be discovered in understanding NURF's functions *in vivo* in mammals (21; 22). Previous studies characterized roles for mammalian NURF in mouse embryogenesis (23) and immune thymocyte maturation (24).

MNase sensitivity experiments in wild-type and Bptf knockout mouse embryonic fibroblast (MEF), DP thymocytes, and embryonic stem cells (ESC) revealed Bptf-dependent nucleosome positions (Landry, unpublished data.) Unpublished microarray data revealed Bptf-dependent changes in the expression of cancer related genes, including E- and N-Cadherins, which are implicated in metastatic progression in cancers (25) and broad effects in the MHC genes involved in tumor evasion of the immune system (3).

Cancer Metastases and Bptf

Metastatic events account for more than 90% of cancer associated mortality (26). While primary tumors remain treatable with surgical intervention and subsequent chemo- and radio-therapy, the metastatic stage of the diseases has poorer prognosis and fatal due to its systemic and sometimes unpredictable course (27; 28). Valastyan and Weinberg outline the hypothesized cascade of biological steps involved in the metastatic progression of cancers, the series of steps a cancer cell is thought to undergo in order to escape the primary tumor site and

colonize a distance secondary site. These steps involve first the invasion of local ECM and survival in the local tumor microenvironment (escaping immune detection, for example.) The cells would need to intravasate into local vasculature and survive the biological and physical pressures of passing through the vasculature. Then extravasate at the distant organ, survive within the ECM, and subsequently re-establish a proliferative phase to form secondary tumors (27).

Given the challenges cancer cells face in the metastatic process it is unsurprising that only a small fraction of tumor cells survive up to any point in the metastatic cascade. Additionally, tumors exhibit cell population heterogeneity with only a subset of the tumor cells having metastatic propensity (29). Chaffer and Weinberg suggest that the subpopulation of metastatic cells are either intrinsically metastatic or develop metastatic ability through the activation of cellular programs. The activation of these pathways enable a non-metastatic cell to become metastatic, or a metastatic cancer stem cell.

One of the programs activated in the metastatic progression of cancers is EMT. Developmentally, EMT describes the process with which epithelial cells in the early embryo are internalized and differentiate into mesodermal germ layer. During this process cells lose their cell-to-cell adhesion markers, become more motile, and shift from having other epithelial cell properties to a more mesenchymal state (30). One surface marker distinguishing epithelial cells from mesenchymal cells is E-cadherin. In development, E-cadherin expression is lost during EMT. Cells undergoing EMT will concurrently gain N-Cadherin expression as they enter the mesenchymal state (31).

Several group's results implicate EMT in the metastatic progression of many cancers (30). Unpublished RT-qPCR has shown increased N-Cadherin expression in 66cl4 when Bptf is knocked down with shRNA (Landry, unpublished). Bptf knockdown in 4T1 resulted in decreased E-Cadherin expression. Both of these results are indicative of EMT, and thus suggestive of increased motility. Transwell migratory assays revealed increased cell motility in Bptf knocked down 4T1 and 66cl4 cells versus control. These results indicate a possible regulatory role for Bptf in cancer cell metastasis through affecting EMT and cell migration.However, Lou et al (32) show EMT markers are neither sufficient nor necessary for cancer metastasis. Their results show that the non-metastatic 67NR cell line has greater EMT marker expression *in vitro* than metastatic 4T1, whereas the 67NR cell line is known to be non-metastatic *in vivo*. Additionally, *in vitro* migration and invasion assays show the 4T1 cell line having greatest motility and invasion ability, as expected. In contrast to this, the metastatic 66cl4 cell line, which does express an EMT like state *in vivo*, has less migration and invasion ability *in vitro* than the non-metastatic EMT expressing 67NR cell line. Because of these results more work needs to be done to determine if Bptf does affects metastasis *in vivo*.

Cancer Immunosurveillance and Immunoediting

The road to understanding the interplay between the immune system and cancers has been arduous and often contentious. Despite the conception of cancer immunosurveillance early in the twenty first century (33), Hanahan and Weinberg do not mention in their 2000 review proposing the "hallmarks of cancer" the immune system as a common obstacle for tumorigenesis (34). Yet, in the intervening decade, researchers building on results from the

1990's continued to collect experimental evidence in support of a role for the immune system. These results prompted Schrieber and colleagues to propose the concept of "immunoediting", to explain the paradoxical interaction of the immune system to defend the host against tumors while at the same time shaping tumors for immune escape (33; 35). In recognition of the collective research work supporting the critical role of immune surveillance in tumorigenesis in cancers, "Evading Immune Detection" was proposed as an emerging hallmark of cancers in the 2011 follow up review by Hanahan and Weinberg.

Three stages of immunoediting were proposed by Schrieber et al (35): elimination, equilibrium, and escape. In the elimination phase, cells with newly acquired abnormal replicative capacities are recognized and eliminated by the immune system, with a few cells surviving this selection. The few surviving cells then enter an equilibrium with the immune system and gradually develop new mutations, some of which contribute to the other hallmarks of cancer, while others will allow the cell population to escape immune destruction, either by evading detection or resisting elimination by the immune system.

The immune system consists of two major subdivisions that are involved in immune surveillance and elimination, termed the innate and adaptive immune system. Cells of both systems are involved in antitumor immunity (36). Of the Innate immune system, NK cells are thought to play a central role in providing rapid response against forming tumors to eliminate cells as they become tumorigenic (37). Cytotoxic T-lymphocytes, which comprise a part of the adaptive immune system, are also able to induce death in tumor cells upon activation through the recognition of tumor antigens presented on MHC-I surface antigens (38). Both NK and CTL

cells induce tumor death by release of cytolytic granules containing perforin (39) and the stimulation of apoptotic signals in tumor cells through FAS signaling. In the early tumor, macrophages are among the first cells to infiltrate the microenvironment to attack the tumor (36). This releases antigens into the microenvironment that ultimately assists CTL cytotoxicity against tumors. Additionally, CD4 cells also assist in activating and maintaining the activity of CTL against tumors (40). Other cells of the immune system, including regulatory T cells and monoctyes, are also recruited to and infiltrate the tumor microenvironment to promote tumor growth and inhibit antitumor immunity (41), including the cytotoxic activity of NK and CTL against tumors.

While much remains to be discerned about tumor escape mechanisms, two proposed mechanisms include the proliferation of Myeloid-Derived Suppressor Cells (MDSC) (42) and dysregulation of MHC-I molecules in cancer (43).

MDSCs are a heterogeneous population of immature myeloid derived cells that normally function as anti-inflamatory cells and prevent auto-immunity (44). This cell population is greatly expanded in many cancers and has been shown to be a major regulator of immune suppression in cancers (45). These cells are found to be amplified to varying degrees in different murine tumor models, including the 4T1 cell line used in this study (46).

MHC-I antigens are expressed on all nucleated cells, and are used to present degraded cytosolic proteins, in effect displaying intracellular proteins on the cell's surface. MHC-I molecules are lost either completely or partially in a number of cancers, with metastatic cancers exhibiting MHC-I loss more commonly (36). MHC-I surface antigens are an integral part of $CD8^+$ cytotoxic

T-lymphcyte (CTL)-mediated immunity against cancers. CTLs have receptors that bind and can become activated in response to specific signals. Dysregulation of MHC-I in cancer cells plays an important role in enabling cancer cells to evade the immune system during metastatic progression (3; 47). The loss of MHC-I molecules on cancers essentially renders the adaptive immune system blind to the cancer cells, and thus incapable of mounting an effective immune response.

In addition to the activation of EMT for metastasis and any one of several programs to evade the immune system, a number of diverse signaling pathways may be altered in cancer cells to overcome the hurdles faced in tumorigenesis. Given our advancing knowledge of the epigenetic states of cancer and the broad roles elucidated for NURF across several signaling pathways (21), it is plausible for NURF to play a critical role at any point in cancer tumorigenesis and metastasis.

The aims of this study are to characterize the role of NURF *in vivo* on tumor formation and metastatic progression using a well-characterized mouse breast cancer model. **We hypothesize that Bptf knockdown in cancer cells will decrease their tumorigenic capacity and reduce their metastatic propensity.**

To address this hypothesis we proposed the following specific aims and experiments:

Aim 1) Subcutaneously inject the mouse breast cancer cells into syngenic BALB/c mice and measure tumor weight at 3-4 weeks to determine the effects of Bptf knockdown on *in vivo* tumor growth. Harvest and weigh spleens from mice challenged with 4T1 tumors as a measure of MDSC amplification. Subcutaneously inject the mouse breast cancer cell lines into NOD/SCID

immunocompromized mice and measure tumor weights at 3-4 weeks for comparison with BALB/c tumor weights to determine any role for the immune system.

Aim 2) Perform metastatic colony formation assays on the lungs of mice challenged with metastatic 66cl4 or 4T1 tumors to determine effects of Bptf knockdown on metastasis.

3) Perform an *in vitro* cytotoxicity assay using NK cells to determine susceptibility of the tumors to NK killing.

Chapter 2: Materials and Methods

Murine Breast Cancer Cell Culture and Preparation

The well characterized metastatic mouse breast cancer 4T1 and 66cl4 cell lines were used (11). The cells were graciously provided by Frank Miller.

Cell Culture

Cells were transfected using retroviral pSIREN-RetroQ (Clontech) retroviral vector to stably express an shRNA targeting Bptf for degradation.

Bptf knockdown was confirmed using Western Blot analysis. Cells were grown in 4T1 Medium (DMEM supplemented with 10% fetal bovine serum, 1x penicillin-streptomycin, 1x non-essential amino acids, and 2 mM glutamine) with 5 µg/mL puromycin for selection of transfected cells. 60 µM 6-Thioguanine supplemented medium was used to grow metastatic clonogenic assays as the 4T1 and 66cl4 cell lines are designed to be 6-Thioguanine resistant. 60 mM stock 6-thioguanine was prepared by dissolving 100 mg in minimal volume of 1 M NaOH and making the solution up to 10 mL.

Mouse injection preparation

Mice were injected with 1 x 10^4 viable 4T1 cells for all 4T1 analyses, and 1 x 10^4 66cl4 cells for tumor weight collection and 1 x 10^5 cells for metastasis assays. Viable cell counts were performed using 0.2% trypan blue and counted on Cellometer Auto T4 cell counter (Nexcelom). Cells were resuspended to 2 x 10^5 or 2 x 10^6 cells/ml in DMEM for injection, and 50 µL of the

single cell suspension was injected for 1 x 10^4 or 1 x 10^5 cells. Cells were injected subcutaneously into the mouse breast fat pad.

Mouse Model

BALB/cJ mice from Jackson Laboratories were used for the majority of the studies. Female mice were obtained between 6 to 8 weeks of age. 10-15 mice were used for each group. Syngenic murine breast cancer cells were subcutaneously inoculated into the mouse fat pad between 6 and 12 weeks of age. Mice were sacrificed 3 or 4 weeks post inoculation and blood, lung, tumor, liver, and spleens were collected for analysis. To evaluate the role of immune system, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (also referred to as NOD SCID Gamma, NSG) mice were obtained from Jackson laboratories. These mice are bred from a NOD background coupled with severe combined immunodeficiency (Prkdc^{scid}) and knockout of the IL-2 receptor gama (Il2rg) chain. The combined effect of this genotype is a mouse model completely void of mature B, T, and NK cells of the immune system. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University.

Bptf shRNA Knockdown

shRNA sequences targeting Bptf for knockdown were ligated into RNAi-Ready pSiren-RetroQ vector (clontech) MMLV transduction system. The control shRNA sequence is GTGCGTTGCTAGTACCAACTTTCAAGAG. shRNA 1 sequence for both 66cl4 and 4T1 is CGACGATGACTCGATTATT-TCAAGAG-AATAATCGGAGTCATCGTCG. shRNA 2 sequence for 66cl4 is GGCGAAAACCAAGAGTACAT-TCAAGAG-ATGTACTCTTGGTTTCGCC and for 4T1 is AGCACAGAGAAGACCATGAT-TCAAGAG-ATCATGGTCTTCTCTGTGCT.

This construct was transfected into PT67 packaging cell lines (Clontech) to package the construct into retroviral packages. Cell culture medium was added for 24-48 hours to PT67 cell lines before being removed and filter sterilized for cancer cell transduction. This virus containing medium was added to breast cancer cell lines for 48 hours. After 48 hours, the culture medium was replaced with 5 µg/mL puromycin containing medium to select for cells with integrated viral genome containing the shRNA sequence. 6 pools of tranductions were performed at a time and Bptf knockdown was assessed by Western Blot, with the two or three highest knocked down populations being selected for experiments.

Protein Extraction

All protein extracts were obtained using TRI® reagent (Sigma) following Sigma protocol. After washing cells with PBS, trizol reagent was added straight to the plates and the samples were incubated at 37° C for 5 minutes. The samples were homogenized by forceful pipetteing. 200 µl of chloroform was added and mixed by vortex for 15 seconds before incubating at room temperature for 15 minutes. Following this, the samples were cold centrifuged at high speed for 10 minutes prior to aspirating the upper aqueous and middle interphase layers. 1 ml of isopropanol was added to precipitate the protein and incubated at room temperature for 10 minutes before a second centrifugation to pellet the protein extract. The pellet was washed overnight in 0.3 M guanidine in 95% ethanol at 4° C before a second overnight 100% ethanol wash. Samples were then dissolved in appropriate volume of 8 M urea in 1% SDS and protein concentration quantified using Bio-Rad D_c protein assay. Samples were run on Ultrospec III

spectrophotometer (Pharmacia LKP) according to manufacturers protocol, and dissolved to 2 mg/ml concentration for Western Blot analysis.

Tumor protein extracts were obtained by mincing tumors and dissolving 0.5 mg of tumor into 1 ml of TRI reagant. The tumor was homogenized in the Trizol using T 10 Basic Disperser (IKA). The samples were processed following the same protocol described above.

Western Blot

Western blot experiments were performed using SDS-PAGE. Bptf, SNF2L, and pRBAP46/48 were separated 4% gel, 6%, and 15% gels, respectively, for one hour at 200 mV and 300 mA. The proteins were transferred to PVDF membrane in 1x N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (10 mM CAPS, 15% methanol, 2.5 mM dithiothreitol) for 17 hours overnight at 20 mV and 20 mA. After transfer, the membranes are blocked in 5% non-fat dry milk (NFDM) for one hour. Following blocking, 10 ml of fresh 5% NFDM was added to the membrane and incubated overnight with the appropriate antibody. pRBAP46/48 was probed with rabbit anti-mouse RbAp48 (1:5000, abcam, cat# 1765), SNF2L was probed with rabbit anti-mouse SMRCA-1 (1:5000, Abcam, cat# ab37003), rabbit anti-mouse Bptf was used to probe Bptf (1:10000, custom antibody) (23). After primary incubation membranes were washed 3 times with 10 ml PBST (1x PBS, 1% tween-twenty) for 5 minutes each. 10 ml of 5% NFDM was added again and secondary anti-rabbit peroxidase (1:10000, NA 9344VS) for 1 hour. Following this the membranes were washed 3 times with 10 ml PBST. Membranes were exposed using femto (Thermo Scientific, cat# 1859023).

Population Doubling Time

Bptf shRNA knockdown and control mouse breast cancer cell lines 4T1 and 66cl4 were seeded at 1 x 10^4 cells into each of 4 wells for each hairpin (2 knockdown and 1 control), into 12 well plates. 3 plates were prepared for each of 3 consecutive days. Starting at 24 hours post plating, and at 24 hour intervals thereafter, cells were counted using a hemacytometer and calculated at total number of cells/well for the volume of trypsin and culture medium used to release cells from the plate. Doubling times were calculated using <http://www.doubling-time.com/compute.php>.

Tissue Harvesting

Mice challenged with 4T1 tumors were anesthetized with 3% isofluroane at 1.5 ml/min then sacrificed by cervical dislocation. Mice challenged with 66cl4 tumors were sacrificed at 4 weeks. Tumors, lungs, and livers were collected from all mice. Blood and spleens were collected from mice inoculated with 4T1 tumors only. Tumors were frozen immediately following harvesting by submersion into liquid nitrogen.

Tumor and Spleen Weights

Tumors and spleens were weighed. Spleens were used for flow cytometry analysis for MDSC populations in mice challenged with 4T1 tumors. Tumors were frozen, Western Blotted to confirm Bptf expression status, sectioned for immunohistochemistry, and sent for microarray analysis. The immunohistochemistry and microarray profiling was performed by Aiman Alhazmi in partial requirement for a Master's thesis in the Human and Molecular Genetics Department at Virginia Commonwealth University.

Metastasis Assays

Metastatic clonogenic assays were performed as described previously (48). Following cervical dislocation, blood was collected into a heparin rinsed syringe containing 0.1 ml of heparin (0.625 mg/ml) solution until the point of resistance. The collected blood was dispensed into 10 ml of 1x Hank's balanced salt solution (HBSS). The blood was centrifuged at 500 x *g*, washed once with 10 ml of 1x HBSS followed by a second wash with 4T1 6-TG medium. Following the second wash, the pellet was resuspended in 10 ml of the culture medium and plated entirely and at 1:10 dilution and left undisturbed 10-14 days.

Livers were washed in 2 ml of 1x HBSS. Minced into pieces smaller than 1mm and transferred to 2.5 ml of 2mg/ml collagenase type I cocktail (Worthington Biochemical). 2.5 ml of 2 mg/ml hyaluronidase in 0.1% BSA were added to the livers, for a total working concentration of 1 mg/ml of enzyme. Samples were incubated at 37° C for 30 minutes. 1x HBSS was added to make up the sample to 10 ml before passing through 40 µm filter to obtain single cell suspension. The samples were then centrifuged at 500 x *g* for 5 minutes and the supernatant carefully removed. The cells were washed with 10 ml 1x HBSS followed by a second wash with 4T1 6-TG medium. The cells were then resuspended in 10 ml culture medium and plated at 1:4 dilutions and left undisturbed for 10-14 days.

Lungs were washed in 2 ml of 1x HBSS. Minced into pieces smaller than 1mm and transferred to 2.5 ml of 2mg/ml collagenase type IV and elastase cocktail (Sigma). 2.5 ml of 1x HBSS was added for a total working concentration of 1 mg/ml of enzyme cocktail. Samples were incubated at 4° C for 75 minutes. 1x HBSS was added to make up the sample to 10 ml before

filtering through 40 µm filter to obtain single cell suspension. The samples were then centrifuged at 400 x *g* for 8 minutes and the supernatant carefully removed. The cells were washed with 10 ml 1x HBSS followed by a second wash with 4T1 medium supplemented with 60 µM 6-thioguanine medium. The cells were then resuspended in 10 ml culture medium and plated neat and at 1:10 dilution, and left undisturbed for 10-14 days.

After 10-14 days, all plates were washed with 5 ml of 1x PBS and stained using 2% methylene blue in 50% methanol for 5 minutes before washing with water. Colonies were counted.

Natural Killer (NK) Cell Purification

The spleens of BALB/c mice were harvested for NK cytotoxicity assays. The NK cells were purified by negative selection using a magnetic separation MACS Column (Miltenyi Biotech, cat# 130-690-864.) The spleens were cut length wise and pressed gently to release the splenocytes. The splenoctytes were collected with 5 ml of MACS Column buffer (0.5% BSA, 2 mM EDTA in PBS, at pH 7.2) and passed through a 0.45 µm filter to create a single cell suspension. The cells were counted and centrifuged for 10 minutes at 300 x *g* before resuspending into 400 µl of buffer per 1 x 10^8 cells. 100 µL of a primary biotin-conjugated antibody cocktail (Miltenyi Biotec) was added per 1 x 10^8 cells and incubated on ice for 20 minutes. This cocktail binds all non-NK cells. 300 µL of buffer was subsequently added followed by 200 µL of secondary magnetic microbead-strepavidin antibody per 1 x 10^8 cells and incubated for 20-30 minutes on ice. The samples were then washed with 4 mL of buffer per 10^8 cells and centrifuged for 10 minutes at 300 x *g*. After carefully aspirating the supernatant, the

cells were resuspended into 500 µL of buffer per 1 x 10^8 cells and separated on MACS LS columns (Miltenyi Biotec).

The LS Columns were rinsed first with 3 mL of buffer before adding 500 µL of the prepared splenocyte suspension (1 x 10^8 cells are applied per column.) Following this, 3 mL of buffer was added to the column to elute out the unlabelled NK cells, while the column retained the biotin-labeled cell fractions. NK cells were eluted using 3 additions of buffer. The eluted NK cells were centrifuged for 10 minutes at 300 x *g* and resuspended into 2 mL of complete RPMI-10 medium (10% FBS, 2 mM glutamine, 1x penicillin-streptomycin, 50 µM 2-mercaptoethanol) and counted. 3 x 10^4 cells were removed for flow cytometry analysis to determine NK purity as described above.

After determining NK purity, the effective concentration of NK cells was determined in the sample and used for cytotoxicity assay.

Natural Killer (NK) Cell live/dead Cytotoxicity Assay

Cancer target cells were plated at 6,000 cells per well on a 96 well plate, with 12 wells per hairpin plated for triplicate counts. 4 additional wells were also plated for appropriate controls for flow cytometry detector compensation (unstained, PE-only, FITC-only, 7AAD-only) and 2 wells for cell counting. After 24 hour incubation, $DiOC_{18}$ (Invitrogen, cat# 17010) containing (1:4000) complete RPMI-10 medium was added to the cells and incubated overnight. The cells were then washed twice with 200 µL of complete RPMI-10 medium. The number of cells were counted per well. Following this, the appropriate number of effector NK cells was added to each well for Effect:Target (E:T) ratios of 0:1 (target only control), 1:1, 5:1, and 10:1. Effector

only control wells for each E:T ratio was plated as well (1:0, 5:0, 10:0). The cells were incubated for 48 hours to allow NK killing.

Percent cytolytic activity was calculated to = (percent dead at ***x***:1 ratio) - (percent dead at O:1 E:T ratio) / (percent dead at 0:1 ratio) x 100.

Flow Cytometry

Measuring Natural Killer Cell purity

After purifying NK cells as described, 3 x 10^4 cells were labeled with FITC-NKp46 (BD Pharmigen, cat# 560756) antibody, an NK cell marker, and PE-strepavidin (BD Pharmigen, cat# 554061) for 20 minutes in the dark on ice. Cells were washed with 1 mL of FACS (10% FBS, 1x Hank's Balanced Salt Solution) buffer and centrifuged at 300 x *g* for 10 minutes. The samples were then resuspended into 500 µL of FACS buffer with 7AAD (1µg/500µL) to test cell viability, and analyzed for NK purity.

All samples were run on BD FACScaliber (BD Biosciences). Analyses of data was performed using Cyflogic free software (CyFlo Ltd.)

Measuring NK Cytolytic Activity

To determine the cytolytic activity, the medium was collected off the cells after the 48 hour incubation. The cells were then trypsinized off the well and collected using the medium for each well. The cells were spun for 10 minutes at 300 x *g* at 4°C and the supernatant carefully aspirated. The cells were then resuspended in 130 µL of propridium iodide (Invitrogen, cat# 17010, 1:800) containing FACS buffer. The cells were incubated in the dark on ice for 20

minutes, followed by washing with 1 mL of FACS buffer. We then resuspended the cells into 500 µL of FACS buffer and the samples were ready for flow cytometry analysis. The $DiOC_{18}$ is a green fluorescent membrane stain that stains all target cells while the propidium iodide counterstain is a red fluorescent molecule that stains the nuclei of membrane compromised cells. Thus, the two stains allow for distinguishing live target cells (green only), dead target cells (green-red), live effector (no fluorescence), and dead effector cells (red only).

Statistical Analysis

Student two-tail paired T-test statistical analysis was performed on tumor weight data using Microsoft© Excel to determine significance.

Chapter 3: Results

Confirmation of NURF and a Mouse Model for Breast Cancer

For our study we chose a well characterized mouse model for breast cancer consisting of five cell lines, 66cl4, 4T1, 4T07, 168FARN, and 67NR. These cell lines were obtained through the sub-cloning out of a spontaneous mammary tumor from a BALB/c mouse by Fred Miller (Karmanos Cancer Institute, Detroit, MI). While these cell lines exhibit similar kinetics in tumor formation when transplanted into syngenic BALB/c mice, they have different metastatic potentials and mechanisms (12). We chose three of these cell lines, 67NR, 4T1, and 66cl4 for the focus of our study. The 67NR line is non-metastatic and does not intravasate or escape the primary tumor site when inoculated into the primary fat pad of mice. Thus it is not detected in either lymph nodes or blood of mice challenged with 67NR cells. The 66cl4 and 4T1 cell lines are both metastatic through lymphogenous and hematogenous routes, respectively, with mainly liver and lung target sites for their metastatic progression (12). To determine the suitability of using these cell lines for studying the role of the nucleosome remodeling factor (NURF) in tumorigenesis and metastasis, we set out to confirm the expression of NURF in these breast cancer cell lines. The mammalian NURF consists of three subunits, the SNF2L ATPase, pRB46/48 WD domain containing subunit, and the largest essential subunit, BPTF, which is known to associate uniquely with NURF (20). We used Western Blot to detect the presence of all three subunits of NURF, and thereby infer the presence of active NURF in the cells. We found all five cell lines in the breast cancer model express all of the subunits of NURF (figure 1A) as well as a panel of human breast cancer cells (Figure 1B). The work presented in this paper is focused on

the two metastatic cell lines, 4T1 and 66cl4. All 67NR experiments were performed by Aiman Alhazmi (data not shown).

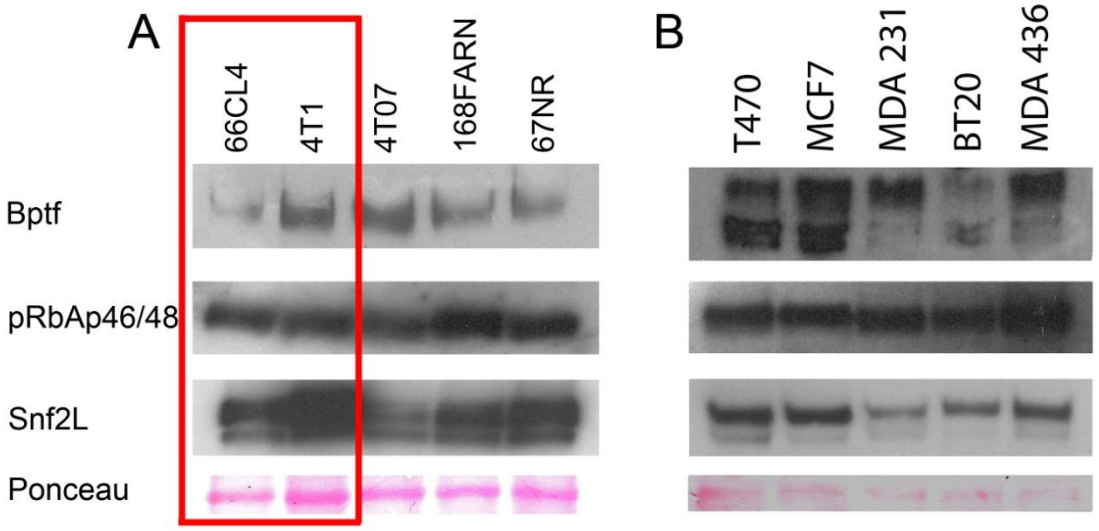


Figure 1. Western blot of all mouse breast cancer cell lines in the mouse model (A) and in a panel of human breast cancer (B) cells express the 3 subunits of the nucleosome remodeling factor (NURF), Bptf, pRbAp46/48, and Snf2L. Cells investigated in this study are highlighted within a red box.

Having confirmed the expression of all NURF subunits in the breast cancer cells, we proceeded to determine the effectiveness of knockdown of NURF using shRNA technology with shRNA sequences targeting the largest essential and unique subunit, Bptf. We used the PT67 packaging cell line to package 3 unique shRNA sequences targeting Bptf for knockdown, and a fourth packaging cell line to package control hairpin with no known targets, into retroviral particles for transduction of our cancer cells. The shRNA vector contains a puromycin resistance gene for selection. Following transduction, we selected the cells expressing the shRNA using puromycin containing medium. About 50% cells were transduced by shRNA containing virus as judged by resistance to puromycin selection. After selection and expansion of transduced cell lines, we confirmed Bptf knockdown using Western Blot. We were able to achieve stable knockdown of Bptf in each of our cell lines using 2 separate hairpins for each of the 66cl4 (Figure 2A) and 67NR (Alhazmi,data not shown) cell lines, and a single hairpin for the 4T1 (Figure 2B) cell line.

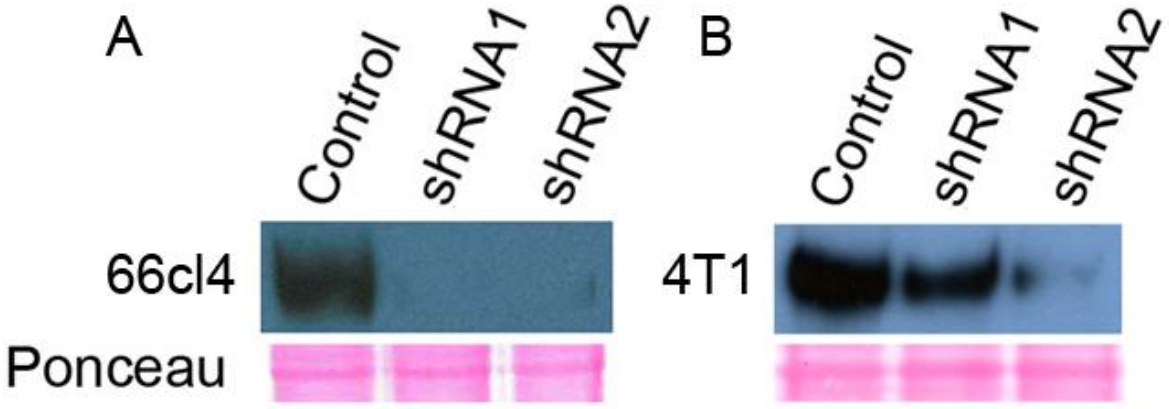


Figure 2. Western blot confirmation of Bptf knockdown with two shRNA in 66cl4 (A) and one shorthairpin in 4T1 (B) cells.

Bptf Knockdown Does not Affect Population Doubling Times of Mouse Breast Cancer Cells

qPCR analysis of 67NR and 4T1 cells revealed dysregulation of cyclin-D1 with Bptf knockdown (Landry, unpublished data). Additionally, Bptf has known roles in regulating embryonic development in mouse embryos with Bptf knockout ESC failing to form teratomas in NOD/SCID mice (23). These results suggest that Bptf has important roles that may affect proliferation and cell viability. Before moving into *in vivo* experiments with our mouse models, we set out to measure any effect Bptf knockdown may have on the proliferation of our cells *in vitro*. To this end, we seeded plates for population doubling time experiments. Using 3 independent knockdown cell line pools, we found no significant difference in doubling time for the 66cl4 cells with two distinct shRNA targeting Bptf when compared to cells tranduced with control hairpin (figure 3A). The 4T1 cell lines also did not have any difference in doubling times between the Bptf knockdown cell line and controls (Figure 3B).

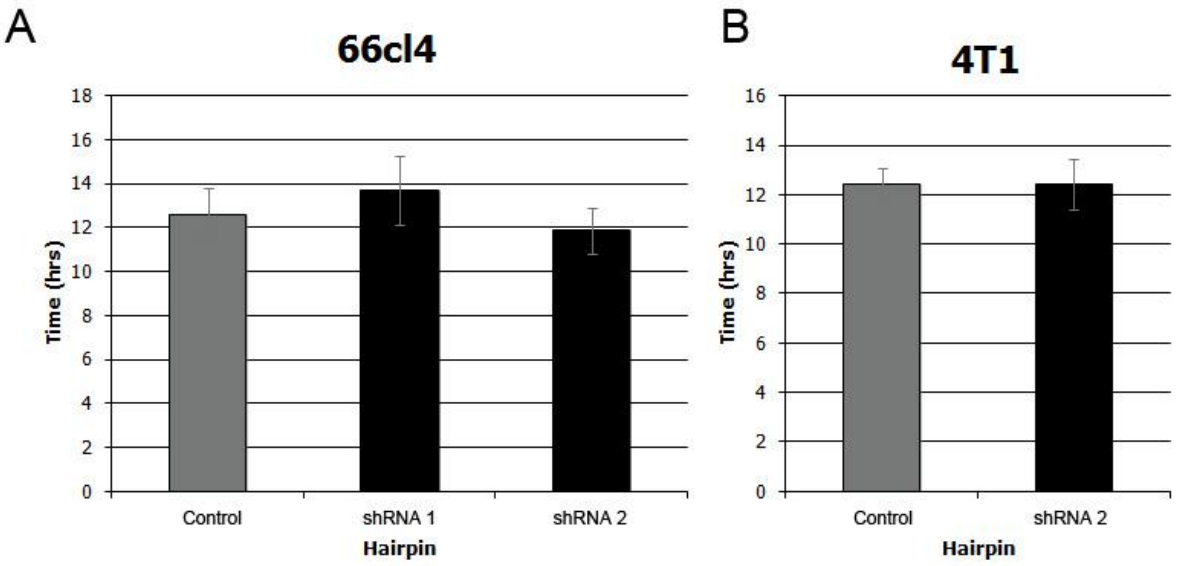


Figure 3. Doubling time experiments on 66cl4 (A) and 4T1 (B) reveal no significant difference between control and Bptf knockdown cell lines. Counts were performed in triplicates on each day and using 4 independent knockdown cell line pools (n=4).

Results for the 67NR cell lines revealed no difference in the non-metastatic cell line's doubling times (data not shown). Further, our population doubling times agreed with published doubling times for our cell lines (49). These results affirm that any observed difference in tumorigenesis observed *in vivo* with injection of the cell lines into syngenic mice is not due to an inherent effect of Bptf knockdown on cell proliferation.

Bptf Knockdown Increases Expression of Major Histocompatibility Class I genes in Metastatic Mouse Breast Cancer Cells

Microarray analysis of Bptf KO ESC revealed broad dysregulation of MHC-I genes (23). MNase hypersensitivity experiments in Bptf KO embryonic stem cells, embryonic fibroblasts, and double-positive thymocytes showed nucleosome positional changes clustering around MHC-I genes (Landry, data not shown). To confirm these results in our breast cancer cell model, we performed flow cytometry analysis on our cells to study the cell surface expression of MHC-I on the surface of cell lines with Bptf knockdown as compared to controls, using an H2-K^d conjugated fluorochrome. We also looked at the surface expression of a representative of MHC class II molecules, H2-A^d, in addition to the NK cell inhibitor MHC-I presented peptide Qa-1.

Our flow cytometry results show increased expression in the MHC-I molecules with Bptf knockdown in both 66cl4 and 4T1 cell lines (Figure 4A & B). MHC-II and Qa-1 expression

appeared to be unaffected in both 4T1 and 66cl4 cell lines (data not shown).

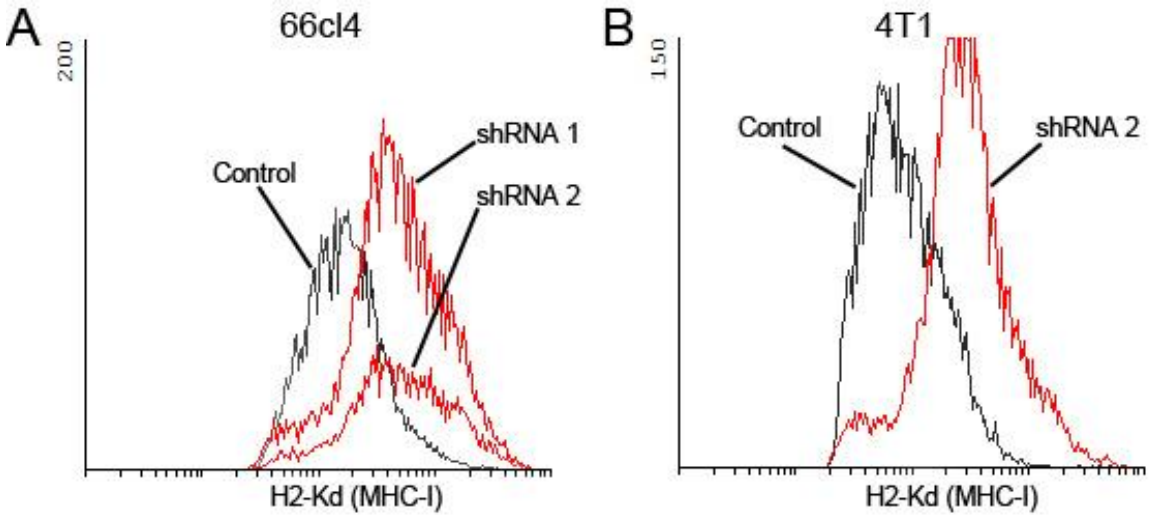


Figure 4. Flow cytometry analysis of MHC-I surface antigens on 66cl4 (A) and 4T1 (B) revealed increased MHC-I expression with Bptf knockdown versus control. Three separate transduction pools were analyzed for each hairpin, shown here is a representative sample.

MHC-I dysregulation is known to be involved in cancer evasion of immune surveillance, with recent immunotherapy approaches targeting MHC-I molecules for re-enabling the adaptive immune system to detect and destroy cancer cells (43). Therefore, our results suggest that Bptf knockdown in cancer cells may affect their ability to escape immune detection and subsequently affect tumorigenesis *in vivo*.

Bptf Knockdown Decreases *in vivo* Tumor Growth in Some Mouse Breast Cancer Cell Lines

To determine if the preceding results would translate into an observable phenotype on tumor growth *in vivo* we injected the mouse breast cancer cells into the fat pad of syngenic BALB/c mice in accordance with previously established protocols (50). We hypothesized that knockdown of Bptf would decrease tumor growth *in vivo*. Following innoculation of 1 x 10^4 (4T1,

66cl4) or 1 x 10^5 (67NR) into mice, we let tumors grow for 21 (4T1, 67NR) to 28 (66cl4) days. Mice were sacrificed using approved institutional standards and tumors were harvested and freshly frozen by submersion into liquid nitrogen. To assess *in vivo* tumor growth we weighed the tumors following freezing. 66cl4 tumor weights were significantly lower with Bptf knockdown versus control (n=12, $p \leq 0.01$) (Figure 5A) for both shRNAs. However, 4T1 tumor weights did not yield any difference between the knockdown tumors and control (Figure 5B).

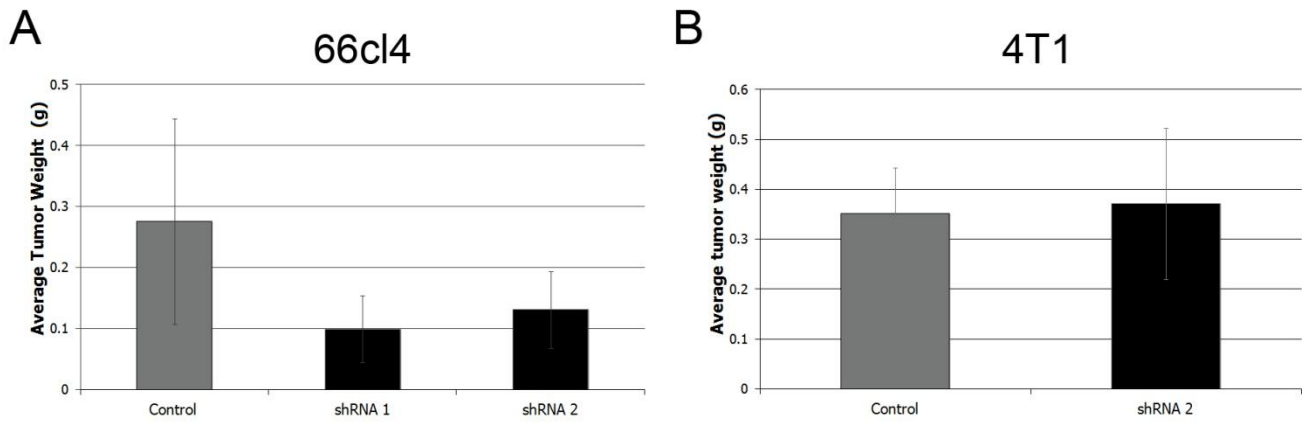


Figure 5. Tumor weights (g) harvested from Balb/c mice challenged with control or Bptf knockdown 66cl4 (A) and 4T1 (B) cells show significantly lower tumor growth with Bptf knockdown in 66cl4 but not 4T1 tumors. $n \geq 12$, $p \leq 0.01$

The 4T1 tumors were about 300 mg when harvesting at 21 weeks, which matches previously reported results for this model (12).The 67NR cell line results showed the most robust phenotype, with less than 30% tumors forming with Bptf knockdown, versus more than 90% tumors forming in mice challenged with control cell lines (n=12, $p \leq 0.005$) (Alhazmi, A. data not shown).

While the observed tumor weight difference in 66cl4 and 67NR lend support to our hypothesis, the 4T1 tumors weights did not. We suspected this may be due to the well known phenomenon of 4T1 tumors signaling the amplification of MDSC population, which is known to inhibit antitumor immunity (42).

Bptf Knockdown Does Not Affect Myeloid-Derived Suppressor Cell Amplification in 4T1 Tumors

4T1 tumors have been extensively studied in tumor immunology for their signaling of MDSC proliferation in mice at the highest frequency of any other mouse cancer model (51). These cells infiltrate into tumors and allow increased tumor growth through inhibition of antitumor immunity. Additionally, in 4T1 tumors these cells accumulate in the spleen and result in splenomegaly in 4T1 tumor bearing mice (51). We suspect that this cell population may account for the lack of an observable difference in 4T1 tumor weights between Bptf knockdown tumors and controls. To determine this possibility, we harvested spleens from mice challenged with 4T1 tumors and weighed them. Our results show a linear distribution between spleen weight versus tumor weight (figure 6), suggesting that as tumors increase in size, MDSC proliferation is increased, allowing for subsequent tumor growth followed by increased signaling for MDSC proliferation.

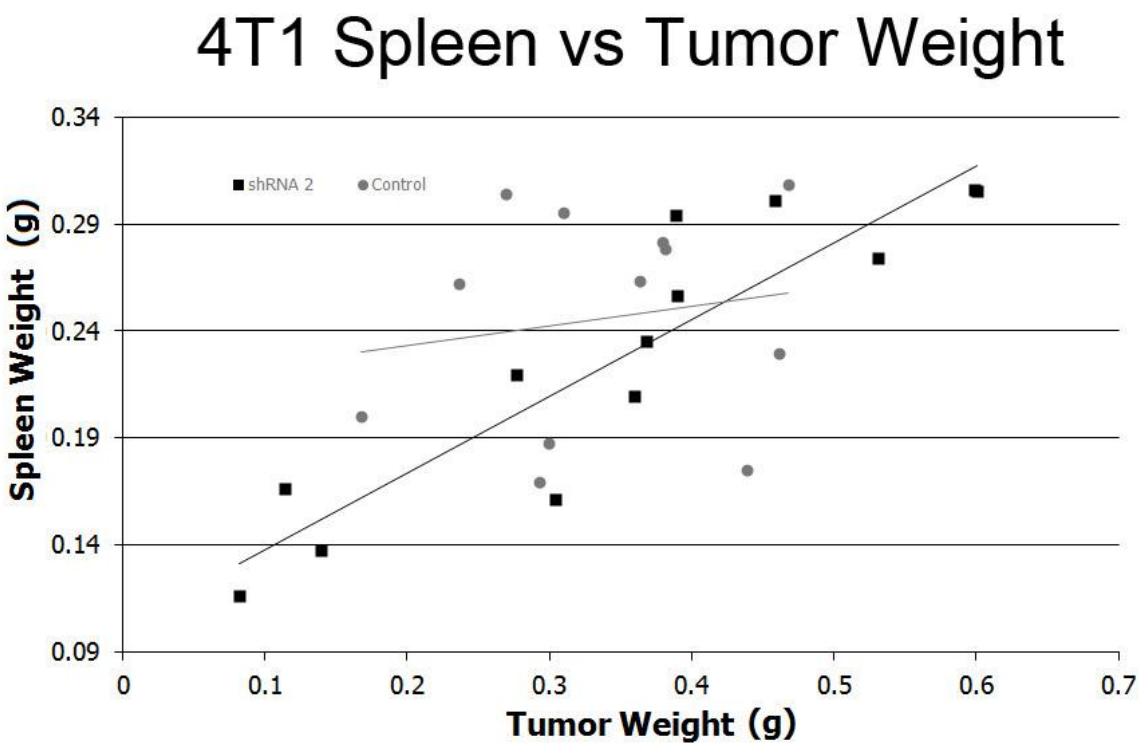


Figure 6. Plotting spleen weight (g) versus tumor weight (g) from mice challenged with 4T1 tumors reveals a proportional relationship betweem the two. n = 13.

These data support the notion that the MDSC population may indeed account for the lack of a difference in tumor weight between Bptf knockdown and control with the 4T1 cell lines.

Bptf Knockdown and Control Tumors Grow Similarly in the Absence of an Intact Immune System

Given the *in vitro* confirmation for the dysregulation of MHC-I molecules, we suspected a role for the immune system in mediating differences in Bptf dependent effects on tumor growth *in vivo*. In support of this, microarray analyses performed on our tumors from BALB/c mice revealed differential expression of immune-specific genes, further implicating the immune system (Alhazmi, A. Data not shown). We were therefore confident in testing tumor growth in the absence of an immune system. To do this, we chose one of the most complete

immunodeficient mouse models, termed NOD SCID Gamma, or NSG. This mouse model lacks both innate and adaptive immunity, in addition to a targeted mutation of the IL-2 receptor gamma chain required for the cytokine signaling in the innate arm of the immune system.

We selected the 66cl4 and 67NR cells for injection into NSG mice due to the differences observed in tumor weights between Bptf knockdown and controls in BALB/c mice. We used the same cell lines as those inoculated into the BALB/c mice at the same concentrations and allowed the tumors to incubate for the same duration in the NSG mice. We then harvested the tumors for weighing at the predetermine time points. The tumor weights were not significantly different between the Bptf knockdown tumors and controls in the NSG mice for both 66cl4 (n=9, Figure 7) and 67NR tumors (Alhazmi, A. Data not shown).

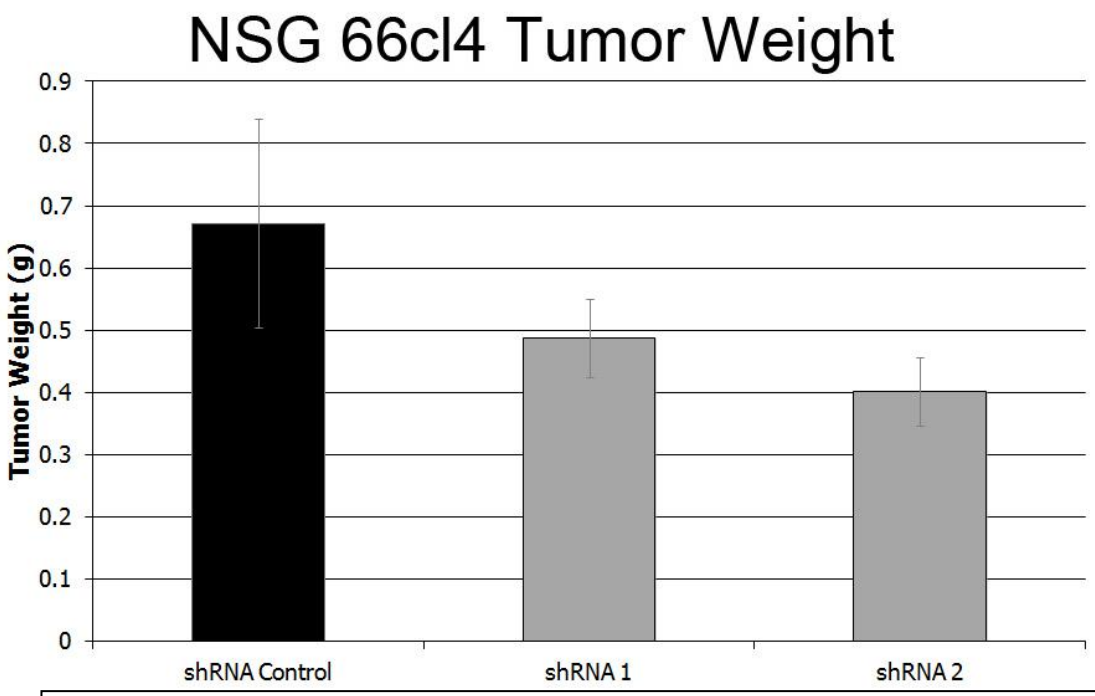


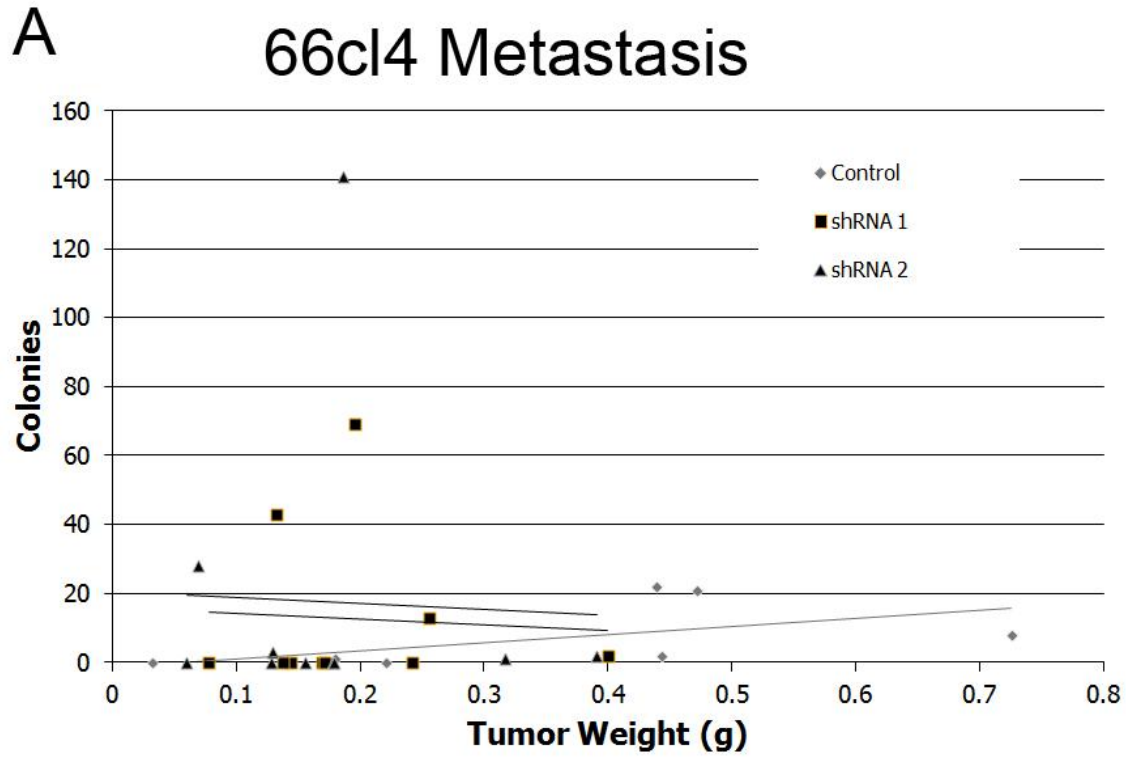
Figure 7. Tumors harvested from NSG mice challenged with 66cl4 tumors yield tumors of similar size with no significant decrease in tumor weight in Bptf knockdown tumors, n=9/hairpin.

These results support our hypothesis that the immune system plays a critical role mediating the observed difference in tumor growth between Bptf knockdown and control cells. However, further studies are required to tease out which effector cells (NK, CD8, CD4, macrophage, etc) of the immune system are specifically causing this difference.

Bptf Knockdown Decreases Metastasis in 4T1 Tumors but May Have No Effect on 66cl4 Metastasis

Microarray analysis of Bptf KO mouse embryonic stem cells showed dysregulation of cadherin molecules (23). This prompted the testing of N- and E-cadherin expression in 66cl4 and 4T1 cell lines with Bptf knockdown. RT-qPCR analysis revealed decreased E-cadherin expression in 4T1 cells and increased N-cadherin expression in 66cl4 cells (data not shown). These results suggested to us the knockdown of Bptf may have an effect on the metastatic propensity of the cancers. We therefore wanted to measure the number of metastatic events in our cancers to detect any changes in the metastatic propensity of our cells.

Our results show decreased metastasis to the lung of mice challenged with Bptf knockdown 4T1 tumors (n=13, Figure 8A). Metastasis assays on the lungs of 66cl4 tumors were suggestive of no difference in the metastasis of Bptf knockdown tumors versus control (n=9, Figure 8B).



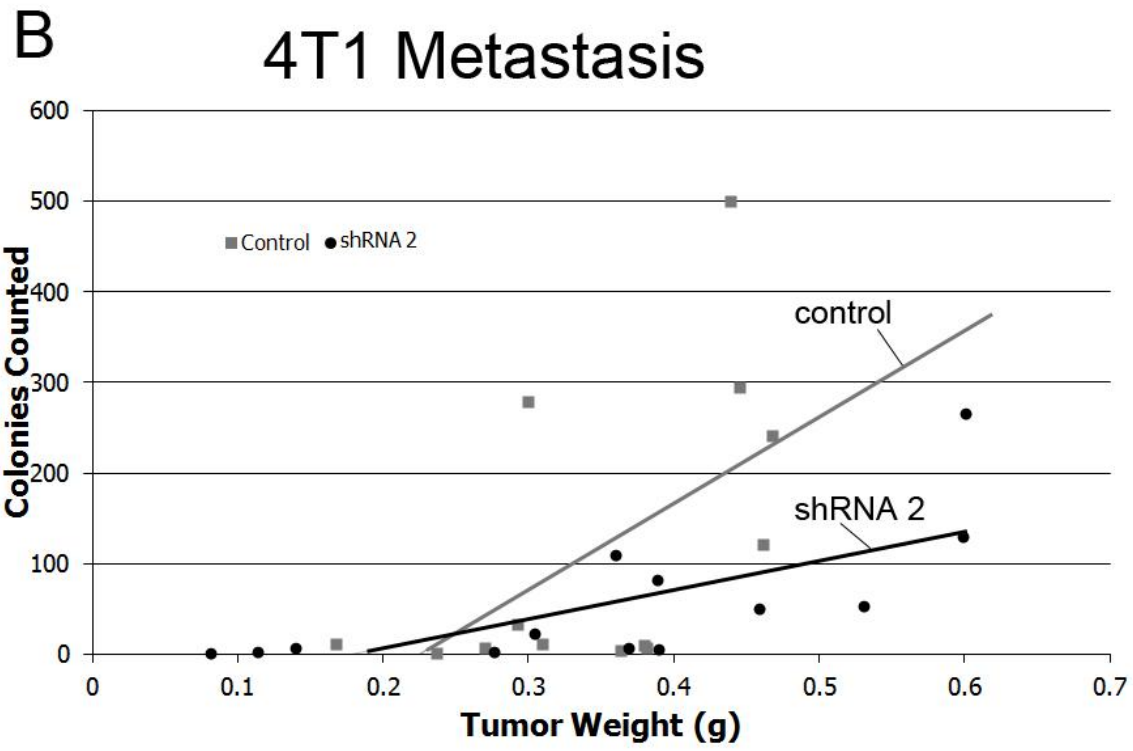


Figure 8. Metastatic colony formation assays comparing number of colonies counted versus primary tumor weight show no difference in metastasis of 66cl4 (A) tumors to the lungs and decreased metastasis to the lungs with Bptf knockdown in 4T1 (B) tumors.

Studies are currently underway to determine if the observed differences in 4T1 metastasis are mediated by the immune system. Additional experiments with the 66cl4 cell line would need to be performed to determine the reproducibility of our current data due to the high amount of biological variability inherent in the model and methods.

Analysis of Primary and Metastatic Bptf Knockdown Tumors

We wanted to determine the Bptf status of the primary and metastatic tumors colonizing the lungs of our mice. Following the harvesting and weighing of the tumors, we performed a protein Trizol® extraction on tumor tissue. Western Blotting was performed on the extracts to determine the expression of Bptf in the tumors. The primary tumors formed from cell lines transduced with shRNA targeting Bptf for knockdown remained knockdown after *in vivo* growth (Figure 9A) in the 66cl4 tumors.

Prior to counting the number of colonies formed in our metastatsis assay, we cloned out three colonies for each of the shRNA transduced cell lines. These were grown to sub-confluence and the protein extracted from them for Western Blotting. Our results show that the 4T1 cells that metastasize to the lung express increased Bptf to levels comparable with controls (Figure 9B).

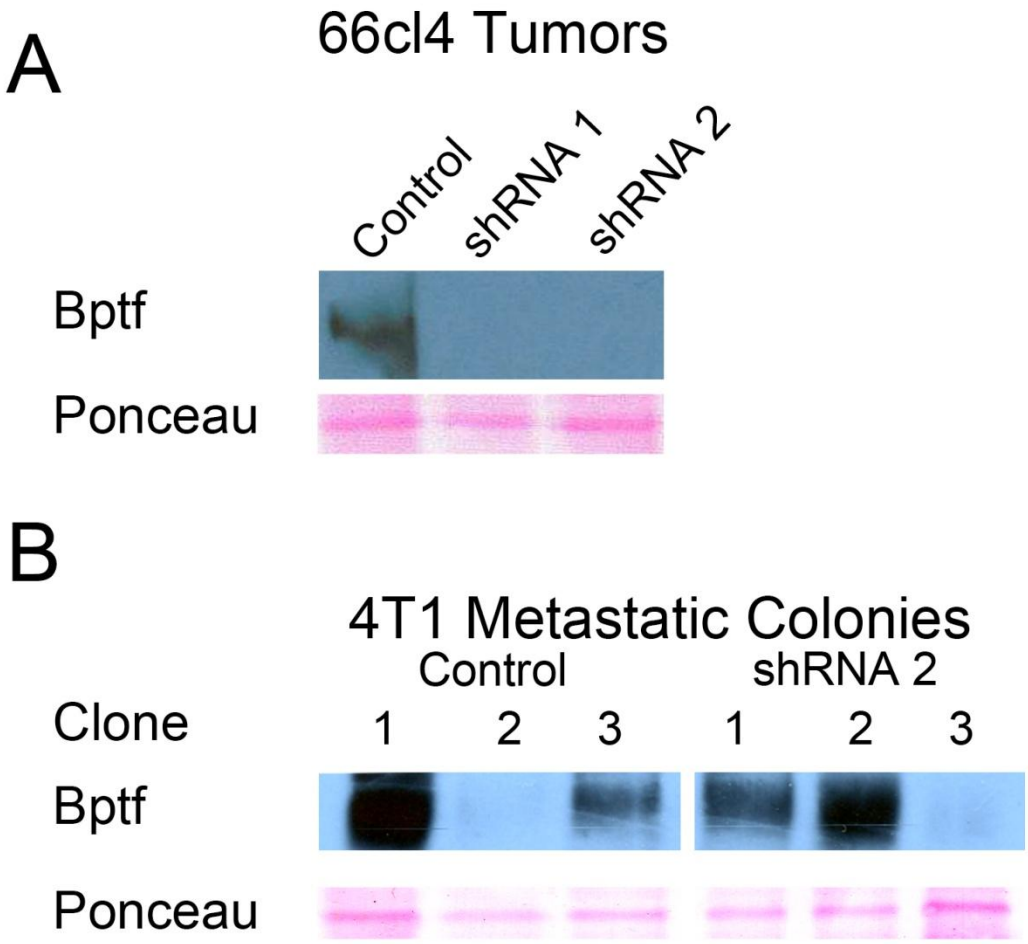


Figure 9. (A) Western Blot of tumors harvested from mice challenged with control and Bptf knockdown 66cl4 cell lines shows that tumors Bptf remains stably knocked down. (B) Western Blot analysis of 4T1 metastatic colonies shows some re-expression of Bptf in knockdown tumors.

These results need to be validated for the 4T1 cell line tumors and the metastatic colonies out of mice challenged with 66cl4 tumors. These experiments are underway in our lab.

Bptf Knockdown Increases Natural Killer Cytotoxicity Against 66cl4 Cells *in vitro*

While we observed decreased tumor weight with Bptf knockdown in BALB/c mice, the same tumors grew similar to control tumors in mice lacking a functional immune system. These observations lead us to test the ability of NK cells to mount an immune response against our

66cl4 cells *in vitro*. NK cells were obtained from wild type BALB/c mice and purified by negative selection. These were then added at various Effector:Target ratios and incubated for 48 hours. We measured cytolytic activity using flow cytometry and found that Bptf knockdown 66cl4 cancer cells are more susceptible to NK activity versus controls (figure 10).

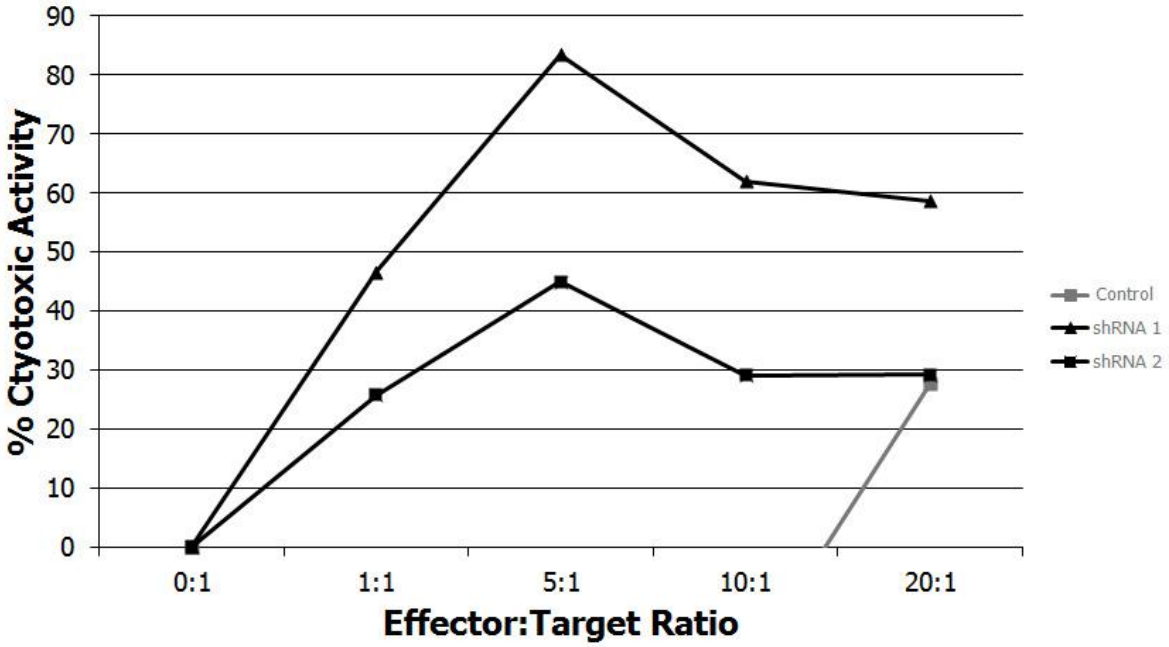


Figure 10. Natural Killer cell *in vitro* cytotoxicity assay on 66cl4 tumors shows increased susceptibility of Bptf knockdown tumors to NK killing versus control, n = 2.

These data are in preliminary stages and biological replicates performed in triplicates are underway. Additionally, these experiments are required for 4T1 and 67NR cell lines.

Chapter 4: Discussion and Future Directions

Our interest in studying the roles of NURF tumorigenesis was guided by previous studies in ESC indicating important regulatory functions for Bptf in mouse embryonic development, and the failure of Bptf KO ESC to form tumors in NOD/SCID mice versus wild-type ESC (23). Additionally, microarray expression profiles of mouse ESC, MEF, and DP thymocytes revealed broad dysregulation of MHC-I genes, which are important in immune system surveillance, and of genes in the cadherin superfamily (23). The cadherin superfamily of surface markers is important in cancer metastasis progression. Dysregulation of MHC-I and cadherins has been observed in many cancers and provide some of the underlying mechanisms by which cancers develop and progress (3). Collectively, these results suggest NURF may play a role in the processes governing cancer formation and progression, providing rationale for our proposed studies.

To determine the potential role for NURF in cancer tumorigenesis and metastasis we chose to use a well characterized mouse model of breast cancer. This model includes several cell lines that exhibit varying degrees of tumorigenic and metastatic phenotypes, of which we chose the two metastatic, 66cl4 and 4T1, cell lines and the non-metastatic 67NR cell line for our study. We set out to first confirm the MHC-I and cadherin dysregulation observed in the ESC, MEF, and DP thymocyte microarray experiments. To this end, a set of shRNA sequences that are able to appreciably knockdown Bptf expression, and thus knockdown NURF, in the breast cancer cell lines were identified so that two separate shRNA sequences were available for each cell line. Additionally, a control shRNA sequence without any known targets was used as control. We

used a packaging cell line to package the shRNA sequence with a puromycin selectable marker into integrating non-replicating retroviral packages for transduction into our cell lines. This technique provides a stable knockdown model to study the effects of Bptf knockdown in our cancer model.

Having identified shRNA sequences to target Bptf for knockdown, we confirmed the knockdown of our cell lines up to 2 weeks following transduction. We then used our knockdown cell lines to study the expression of MHC-I surface antigens using flow cytometry. We chose to use fluorochrome conjugated H2-K^d antibody belonging to the MHC class I. In accord with microarray results in ESC, MEF, and DP thymoctye, flow cytometry revealed increased expression of MHC-I in 4T1 and 66cl4 cancer cells with Bptf knockdown. Additionally, we measured the expression of MHC-II using fluorochrome conjugated H2-A^d antibody and the specific MHC-I NK inhibitor Qa-1 surface antigen. These revealed no specific MHC-II expression, as expected. MHC-II molecules are characteristic of antigen-presenting cells. There was slight expression of Qa-1 in our cancer cells, but this expression was not found to be significantly altered with Bptf knockdown. At first glance, these results might suggest Bptf knockdown of cancer cells may increase the cancer cell's susceptibility to CTLs. CTL are the primary effectors of the adaptive immune system's antitumor cytolytic activity and recognize tumor-associated antigens as presented on MHC-I molecules (36). At the same time, MHC-I antigens typically have inhibitory effects on NK cells. These cells mediate the innate immune responses antitumor effects. However, the precise signaling and activation mechanisms of NK cells are still being discovered. NK cells express a balance of activating and inhibitory receptors, and it's thought the balance of these receptors determines the activation state of NK cells (52). So while our

results would suggest a possible inhibition of NK cells, it is possible that NURF knockdown alters a number of other signals that may bring the balance of activating signals in favor of improving NK cell response against the tumors. If this is the case, additional studies would be required to elucidate the markers and underlying molecular mechanisms mediating the observed increase to NK cytotoxicity associated with Bptf knockdown.

To confirm the cadherin dysregulation that was observed with ESC, MEF, and DP thymoctyes, RT-qPCR was performed to determine the effects of Bptf knockdown on the expression of N- and E- cadherins in the two metastatic cancer cell lines, 66cl4 and 4T1. These experiments revealed decreased E-cadherin expression in the 4T1 cell lines and increased N-cadherin expression in the 66cl4 cells (data not shown). While decreased E-cadherin with concurrent increased N-cadherin is characteristic of an epithelial-mesenchyman transition, a program thought to be initiated with the onset of metastasis, at least one group has shown the expression of EMT markers is not a sufficient indicator for metastatic propensity (32). Still, the expression of N- and E-cadherins are largely regarded as important molecules in the metastatic progression of a number of cancers. This data supports our hypothesis that Bptf knockdown will affect the metastatic potential in our model, and give further rationale to initiating *in vivo* experiments to determine the physiological effect of Bptf knockdown on cancer tumorigenesis and metastasis.

To this end, we proposed injecting the cancer cells into the mammary fat pad of BALB/c mice which are syngenic to the cell lines. This would allow for future study of the immune system. Following established protocols for the model, we chose to weigh tumors at 3 or 4 week end

points to determine tumor growth. We also harvested spleens from 4T1 challenged mice to determine spleen weight as a measure of proliferation of the MDSC that are known to play a critical role in allowing 4T1 tumors to evade immune surveillance and elimination. Metastatic propensity was determined by harvesting and plating lungs from mice challenged with metastatic tumors, 66cl4 and 4T1, in medium supplemented with a 6-thioguanine (6-TG) to select for the 6-TG resistant 66cl4 and 4T1 cells.

Tumor weight measurements of primary tumors formed from 66cl4 cell lines showed significant decrease in the Bptf knockdown tumors versus control. While all the Bptf knockdown 66cl4 tumors formed tumors of a significantly smaller weight, the results out of the nonmetastatic 67NR tumors were much more profound with only 10-30% of Bptf knockdown tumors forming. To determine the effects of Bptf knockdown mediating the observed decrease in tumor weight, we performed *in vitro* doubling time experiments to rule out the possibility of an inherent effect of Bptf knockdown on cell proliferation. The doubling time results did not account for the observed difference in tumor weight, and reassuringly agree with previously published data on the *in vitro* doubling time for our cell lines.

We then injected our cell lines into a unique mouse model lacking its entire immune system. Termed NOD SCID Gamma (NSG), these mice come from the non-obsese diabetic genetic background and lack the protein kinase DNA activated polypeptide, resulting in a severe combined immunodeficiency phenotype. Additionally, these mice have a targeted knockout mutation in the IL-2 receptor gamma gene, effectively removing any cytokine signalling involved in the differentiation of most innate immune cells and in their cytokine signaling. If the

immune system played a critical role in the lower tumor weight with Bptf knockdown in BALB/c mice with intact immunity, we hypothesized that the Bptf knockdown tumors would grow similarly to control tumors in NSG mice. The tumor weights in these mice supported this hypothesis, showing no significant difference in tumor weight between knockdown and control.

The results of our 4T1 showed no difference in tumor weight out of the BALB/c mice. We suspect this result may be due to MDSC population that is amplified. We hypothesize that 4T1 tumors in BALB/c mice would exhibit a similar phenotype to that observed in the 66cl4 and 67NR cell lines, yielding decreased tumor weight with Bptf knockdown if MDSCs are removed. Additional experiments need to be performed to test this hypothesis. One proposed experiment is the administration of drugs to restrict MDSC proliferation and activity prior to challenging mice with the 4T1 Bptf knockdown and control cells, and administering the drug regularly throughout the duration of the experiment. To lend support to this experiment, we looked at the spleen weights harvested from the mice given 4T1 tumors. Analysis of the spleen weights revealed a proportional relationship between spleen weight and tumor weights. Although not presented in this paper, flow cytometry experiments were performed showing a directly proportional relationship of spleen weight with the percent MDSC composition in the spleens as observed by other groups previously. These results give support to our working hypothesis that Bptf knockdown may make cancer cells more susceptible to antitumor immunity. In the 4T1 cells, the immune system is unable to mediate this response due to the suppressive effect of the MDSC population which causes an enlargement of the spleen.

Interestingly the results of our metastasis assay in 4T1 cells did show a Bptf dependent effect on the metastatic potential, with Bptf knockdown, while not affecting 66cl4 metastasis. The decreased metastasis observed in 4T1 tumors may be due to an effect of Bptf knockdown on molecular mechanisms involved at any stage of the metastatic cascade, such as N- and E-cadherins, matrix metalloproteinases, for extravasation at distant sites, or for the colonization and formation of secondary tumors. Alternatively, this observed decrease in metastasis may be mediated by the immune system. To determine this, we have begun experiments in NSG mice with the hypothesis that Bptf knockdown 4T1 tumors will metastasize similar to control in the absence of an intact immune system. If the data do not support this hypothesis, the breast cancer model we chose consists of 2 other cell lines with the ability to metastasize to different end points in the metastasis cascade, which can be used to plan experiments to determine the point at which Bptf knockdown may affect the cancer's ability to metastasize.

It is not entirely surprising to have a different outcome with 66cl4 tumors. The 4T1 tumors metastasize through a hematogenous route, while 66cl4 tumors utilize a lymphogenous route for metastasis that has not been as well characterized. Due to the inherently different routes by which these cell lines metastasize, it is entirely plausible that the signaling pathways activated in 66cl4 tumors are not relevant in a Bptf dependent context whereas those in 4T1 are. However, the results for the 66cl4 cells come from a smaller sample number which make the results inconclusive given the variability present in the system. Additional metastasis assays will be performed to provide more conclusive results.

We used the *in* vitro NK cytotoxicity assay to begin elucidating the underlying immune mechanism determining the decreased tumor weight in BALB/c mice. A little unexpectedly, we found the Bptf knockdown 66cl4 cells to be more susceptible to NK cytotoxicity versus control. This result requires follow up experiments to reevaluate surface markers presented on 66cl4 cells to determine what activation markers are upregulated or inhibitory markers downregulated with Bptf knockdown to determine these effects. Additionally, we hypothesize *in vivo* experiments with a mouse model lacking NK cells will result in identical tumor weights between Bptf knockdown and control cells. While it is possible that NK cells represent the main arm of the immune system mediating our observation, it is still worthwhile to consider CTL cytotoxicity assays to determine or rule out a role for CTLs. These experiments would also need to be performed with the 4T1 and 67NR cell line to determine if the same mechanisms might be present across all three cell lines.

We have shown here using a mouse model for breast cancer consisting of various cell lines that express distinct metastatic potentials a role for Bptf in the formation of grafted syngenic tumors and in their metastatic progression. Using a mouse model lacking entirely an intact immune system, our data support a role for Bptf in cancer immunity. Our results reveal Bptf as a promising candidate for future studies to elucidate the machanisms by which Bptf knockdown results in decreased tumor weight in immune competent mice while having no effect on tumor weight in immune compromised mice. Additionally, Bptf knockdown appears to affect metastatic propensity of at least one cell line that undergoes metastasis through a hematogenous route, but not another cell line that utilizes a lymphogenous route. Additional

experiments are required to tease out the mechanisms through which Bptf exerts the observed phenotypes.

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