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# **Investigating the Effects of Nucleosome Remodeling Factor Knockdown on Anti-Tumor Immunity**

**A thesis submitted in partial fulfillment of the requirements for the Master of  
Science degree in Human and Molecular Genetics at Virginia Commonwealth  
University.**

**By**

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**April 2016**

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## List of Abbreviations

**BALB/C**- Bagg albino (inbred mouse strain)

**BPTF**- Bromodomain PHD-finger containing transcription factor

**BSA**- Bovine Serum Albumin

**cm**- Centimeter

**CTL**- Cytotoxic T-lymphocytes

**DNA**- Deoxyribonucleic acid

**ELISA**- Enzyme-Linked Immunoabsorbent Assay

**FACS**- Fluorescence-activated cell sorting

**FBS**- Fetal Bovine Serum

**g**- gravity, specifically in relation to the force of Earth's gravity

**HBSS**- Hank's balanced salt solution

**HEK**- Human Embryonic Kidney

**IFN $\gamma$** - Interferon gamma

**kb**- Kilobase

**KD**- Knockdown

**LB**- Lysogeny broth

**LDH-** Lactate dehydrogenase

**mA-** Milliamps

**MDSC-** Myeloid Derived Suppressor Cells

**MHC-** Major Histocompatibility Complex

**NK-** Natural Killer

**NSG-** NOG/SCID, IFNg2r <sup>-/-</sup> mouse strain

**NURF-** Nucleosome Remodeling Factor

**OD-** Optical Density

**PFU-** Plaque Forming Unit, or one infectious viral particle

**PVDF-** Polyvinylidene fluoride

**rAd-** Recombinant Adenovirus

**SDS-** Sodium dodecyl sulfate

**TIL-** Tumor infiltrating lymphocyte

**uL-** Microliter

**V-** Volts

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# Abstract

## INVESTIGATING THE EFFECT OF NUCLEOSOME REMODELING FACTOR KNOCKDOWN ON ANTI-TUMOR IMMUNITY

by Mark G. Roberts, B.S.

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

Virginia Commonwealth University, 2016

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The nucleosome remodeling factor (NURF) is a chromatin remodeling complex involved in early animal development and is implicated in a number of cancers. In previous work, knockdown of NURF's largest subunit, BPTF, resulted in diminished tumor growth in mouse cancer cell lines. Other studies in our lab demonstrated increased activation of T-lymphocytes into BPTF KD tumors. In order to examine if this approach has any therapeutic potential, this work investigates the effects of BPTF knockdown in established tumors by using recombinant adenoviruses (rAd), as well as observe the way the immune system interacts with BPTF knockdown cells, both *in vivo* by flow cytometry and in culture with cytotoxicity assays.

## Chapter 1: Introduction

### **Immunoediting of Cancer Cells**

Immunoediting is the phrase used to describe the role that the immune system plays in the initial prevention and later shaping (“editing”) the immunogenic features of neoplastic cells (Dunn, 2004). Immunoediting is comprised of three parts: elimination, equilibrium, and escape.

During elimination, emerging cancer cells which have acquired new mutations that lend a proliferative advantage are killed in a similar manner to that of foreign pathogens. Incomplete elimination results in inactive tumor cells or cancer cells which are changing their immunogenicity to survive, such as altering surface antigens. This dormant phase in tumorigenesis is the second part of immunoediting, known as equilibrium. In equilibrium, tumor cells are selected by the immune system with some cells being killed and others changing their antigenic profiles to survive. Eventually, some of the cells that survive gain enough of an immunological advantage to move on to the final phase of immunoediting. During the final phase of immunoediting, the escape phase, a tumor begins to form. Note that at all three steps, a failure of the immune system to recognize tumor cells is required to move on to the next stage (Swann 2007).

In the last 20 years there has been a surge of interest in the role of lymphocytes in tumor development. When researchers showed that tumors in mice treated with monoclonal antibodies for IFN gamma (IFN-  $\gamma$ ) grew faster (Dighe et al, 1994) and that mice without competent immune cells formed tumors more than controls, scientists

began looking at the role of the immune system in cancer. Since then, two types of lymphocytes have been found at the frontline of the human body's battle with cancer: T-cells and NK cells.

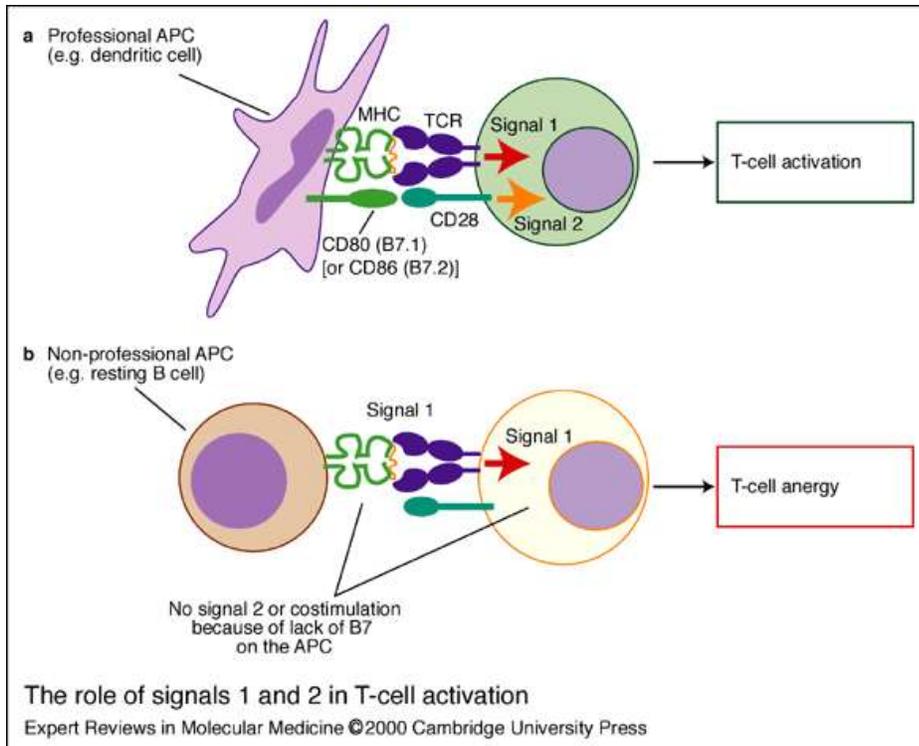
### **T-cells and NK cells: Tumor Infiltrating Lymphocytes**

Lymphocytes, commonly known as white blood cells, are so called because they are found in the lymph nodes and lymphatic system, a filtration system of the blood. They include NK cells, B-cells, and T-cells. Although there is a subset of B cells (CD20+ B-cells) that have been shown to infiltrate tumors (Nelson, 2010), the focus of this thesis will be on the better characterized TILs of NK and T-cells.

T-cells are a part of the adaptive or acquired immune system. Originating in the bone marrow from hematopoietic stem cells (Shortman and Wu, 1996), they mature in the thymus, where they are exposed to antigens presented by the major histocompatibility complex (MHC). The function of the MHC is to take fragments of foreign pathogens that have been killed by professional phagocytes and present them on the cell surface to T-cells (Janeway 2001). Once in the thymus, T-cells are selected for their affinity for either MHC class I or II. MHC I is found on all nucleated cells, and typically presents cytosolic or viral peptide fragments from infected or stressed cells (such as tumor cells) to cytotoxic T-cells to elicit a response (Tortorella et al, 2000). MHC class II typically presents extracellular antigens such as cell surface proteins or soluble antigens (Cresswell, 1994). T-cells which bind MHC I with high affinity express an MHC class I restricted TCR along with a co-receptor known as CD8, which is needed

to activate the cell in the presence of an MHC-I antigen (Rudolph et al 2006). T-cells which bind MHC II express CD4, and are known as helper T-cells, due to their role in activating other effector cells such as B-cells, rather than displaying their own cytolytic activity (Mosman et al 1986, Zhu et al 2008).

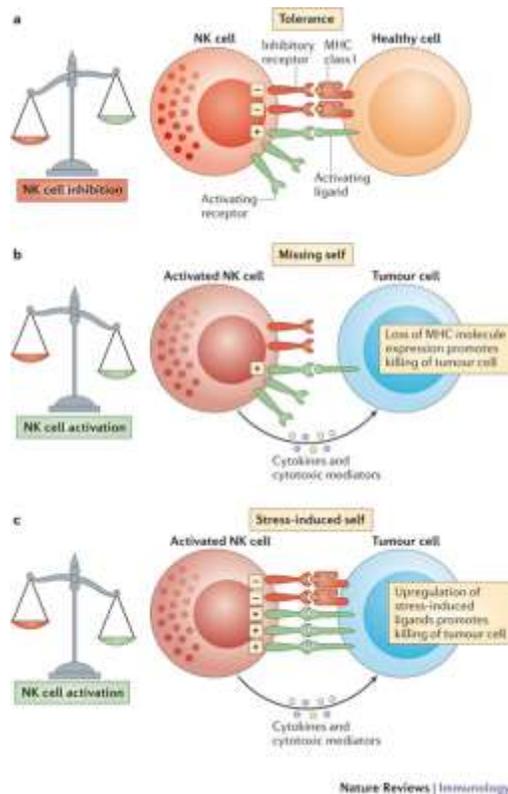
CD8+ CTLs operate by recognizing antigens presented by MHC I, which binds to their TCR. At the same time, CD8 binds the MHC I molecule itself (Janeway 1992.) Following MHC/CD8 binding, a second stimulatory molecule, such as B7, binds to either CD28 or CTLA-4 on the CTL cell surface. This second step leads to activation of the antigen-specific T-cell, which expands in a clonal manner to address the antigen presented to it (Kolumam et al 2005). In the absence of these stimulatory signals, T-cells die and a few memory cells remain in an anergic state, in case the antigen should be encountered again (Williams & Bevan, 2006).



**Figure 1. A cartoon representation of the 2 signal process of T-cell activation.** A) A professional antigen presenting cell brings antigenic fragments via MHC to a T-cell. As the MHC binds the TCR, co-stimulatory CD80 on the APC binds CD28, leading to activation. B) Lack of the second signal from binding of CD28 leads to anergy of the T-cell. (Harber et al. 2000).

NK cells belong to the innate or passive immune system. They were originally named “natural” killer cells due to their observed ability to kill targets without antigen driven priming, as seen in T-cells (Caligiuri, 2008). NK cells are covered with activating and inhibitory receptors, and the status of the cell’s activation is determined by the ratio of those receptors being bound (Moretta et al, 2001). Essentially this means that when there are more activating receptors than inhibitory receptors bound, then NK cells will kill the target. NK cells kill by what’s known as “the missing self hypothesis”(Kärre et al, 1986). NK cells are inhibited when they come in contact with self-MHC class I, sparing healthy cells with normal levels of MHC I on their surface. When they come in contact

with a cell with activating ligands (for instance, MICA on tumors (Bauer et al, 1999)) that do not express the inhibitory MHC I, they kill the cell by releasing perforin and granzyme, lysing the cells (Smythe et al,1999). An example of NK cell activation by tumor cells is provided in Figure 2.



**Figure 2. Cartoon representation showing three modes of NK cell activity:** a.) in the case of healthy cells, NK cells will recognize their MHC class I on the surface, causing inhibition. b.) In tumor cells, MHC I is sometimes lost, which triggers activation of the NK cell due to the lack of binding to inhibitory receptors (“missing self hypothesis”). c.) Finally a tumor cell can present stress-induced proteins and glycoproteins on the surface, which NK cells recognize and are activated by to kill the tumor cell. (Vivier et al, 2012).

Both NK cells and CTL’s are known to infiltrate tumors (Coca et al, 1997, Ishigami et al 2000, Naito et al 1998). In fact, in mice which lacked NK cells, tumors

grew more rapidly and aggressively (Dunn et al 2004, Gorelik et al 1982). Additionally, increased infiltration is a good indicator for survival (Coca et al, Ishigami et al, Gooden et al, 2011).

## **Immunotherapy**

Immunotherapy is a developing field of cancer treatments (cancer.org). Its goal is either to enhance or suppress an immune response, resulting in a reduction of tumor growth. Given the role the immune system is understood to play in the immunoediting of tumor cells, this represents a significant opportunity for the exploration of new therapeutics for cancer.

Currently, there are a number of FDA-approved monoclonal antibody drugs to treat cancer. These drugs bind to antigens present on the surface of cancer cells, allowing them to be recognized by either cytotoxic T cells or B cells and are subsequently killed (Scott et al, 2012). Another approach which is currently being investigated is known as chimeric antigen receptor therapy (CAR-T). In this treatment, blood is taken from the patient and his or her lymphocytes, specifically T-cells are isolated. These then are given receptors specific to the patient's cancer, and then reintroduced into the blood of the patient. These new chimeric T-cells are able to recognize and kill the tumor cells in the patient (Porter et al, 2011).

More recently, targeted NK cells, "taNKs" are being developed for more individualized cancer treatments (Klinngemann et al, 2016). These cells, like their CAR-T cousins, would be given receptors for an antigen found on the surface of a patient's

tumor cells. Given the ability to easily transfect NK cells and grow them, this is an exciting development.

## **Epigenetics**

Epigenetics, which means “above the gene”, is the process which changes expression of genes in a heritable manner but without changing the basic genomic DNA sequence (Dupont, 2009). Epigenetic mechanisms are crucial during embryogenesis (Li et al, 1992), X-chromosome inactivation (Brinkman, 2006), and is also involved in imprinted gene disorders such as Prader Willi and Angelman’s Syndromes (Ohta et al, 1999). Abnormal epigenetic function leading to altered gene expression has been found almost universally in cancer (Sharma et al 2010).

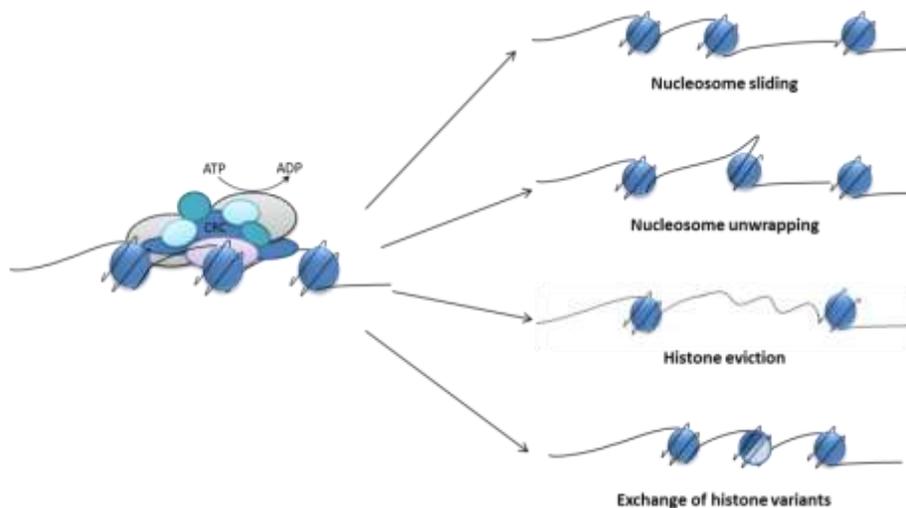
There are four common epigenetic modifications to DNA: DNA methylation, modifications to histones, RNA interference, and chromatin remodeling (Kim et al, 2009, Grewal & Moazed 2003).

## **Chromatin Remodeling Complexes**

DNA, which encodes the instructions for life, is roughly six billion base pairs long in humans. When linearized this is roughly six feet of DNA inside each cell. In order for it to fit within the nucleus it must be condensed by proteins called histones. This DNA-protein complex is known as chromatin (Fig. 3). Chromatin is divided into two classes: open and accessible euchromatin, and closed and inaccessible heterochromatin.



There are four main classes of ATP driven chromatin remodelers: SWI/SNF, ISWI, CHD and INO80. The SWI/SNF (or switch/sucrose non-fermentable) family is able to slide the nucleosome or evict it from the DNA entirely (Priya Sudarsanam, 2000). ISWI (imitation switch) is similar to SWI/SNF in that it also slides the nucleosome, but does not evict it. The CHD (chromodomain) class of remodelers can slide or eject nucleosomes, while other CHD remodelers have been known to repress transcription by interacting with deacetylase enzymes to condense chromatin (Clapier 2008). Finally, the INO80 (inositol requiring 80) group is defined by a split ATPase domain, and are known for removing and replacing two histone proteins to remodel the nucleosome (Clapier 2008). A summary of these mechanisms can be seen in Figure 4.



**Figure 4. Chromatin remodeling complexes, which modify the position of nucleosomes to make accessible the DNA they contain, display 4 main modes of ATP-dependent remodeling.** ATP is hydrolyzed and ADP is released creating energy for the remodeling complex to: slide the nucleosome as seen in SWI/SNF, ISWI and CHD remodelers, unwrap DNA from the nucleosome, evict histones as seen in SWI/SNF and INO80, and exchange histone variants which has been observed INO80 (Xu et al, 2013).

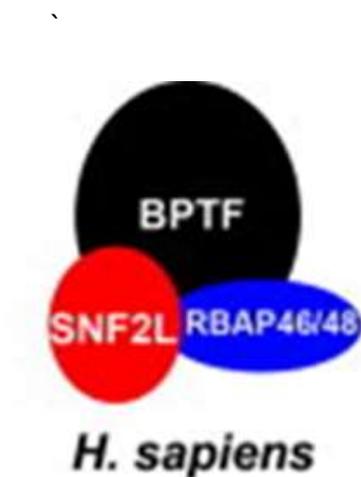
## **Nucleosome Remodeling Factor (NURF)**

The nucleosome remodeling factor, or NURF (Fig. 5) , is a member of the ISWI family. Composed of BPTF (bromodomain PHD finger transcription factor), an ATPase subunit SNF2L, and pRBAP46/48 subunits in mammals (Barak et al 2003). NURF slides nucleosomes bi-directionally in 10 base pair intervals (Schwanbeck 2004). The PHD finger binds H3K4me3, while the bromodomain has been found to bind H4K16ac. Both of these modified histones are associated with active transcription sites (Zhang et al 2015, Gelbart et al 2009, Taylor et al 2013).

In *Drosophila*, where it was first characterized, NURF is involved in embryogenesis. Specifically, the gene *Ubx*, which aids in the development of the thorax, abdomen and wings, requires NURF for transcription (Badenhorst, 2002). In *D. melanogaster*, mutants for NURF301 (the homolog to human BPTF) exhibit abnormal expression of heat shock, JAK/STAT, and homeobox genes (Badenhorst 2002, Badenhorst 2005, Kwon 2008). In mice, BPTF knockout mice fail to gastrulate, due to the need for BPTF by embryonic mouse stem cells for mesoderm and endoderm differentiation (Landry et al 2008). BPTF is involved in thymocyte development in both mice and humans, (Landry et al, 2011).

In humans, BPTF is located on the distal arm of chromosome 17q24.3 (Jones et al, 1999). This region has been found to be amplified in a number of neoplastic tissues, including liver, lung, brain, prostate, bladder and breast cancers (Buganim et al, 2010. Kallioniemi et al, 1994, Choi et al, 2006, Sun et al 2008, Raidl et al 2003). These

amplifications suggest BPTF may play a role in the development of tumors. Knockdown of BPTF in mouse melanoma tumors led to a significant reduction in tumor growth in a study by Dar et al. Additionally, BPTF has recently been shown to associate with c-Myc, a well-known oncogene found in a diverse array of cancer types (Richart, 2016). Finally, high BPTF expression in lung cancer and hepatocellular carcinoma is linked to poor survival (Xiao et al, 2014, Dai et al, 2015). For these reasons, it is a target for investigation, as these studies indicate a causal relationship between BPTF and tumorigenesis.



**Figure 5. The components of NURF as seen in humans.** BPTF, the largest subunit is essential for the function of NURF. SNF2L is the human homolog to the *Drosophila* ISWI, and RBAP46/48, a WD repeat protein (Alkhatib & Landry, 2011).

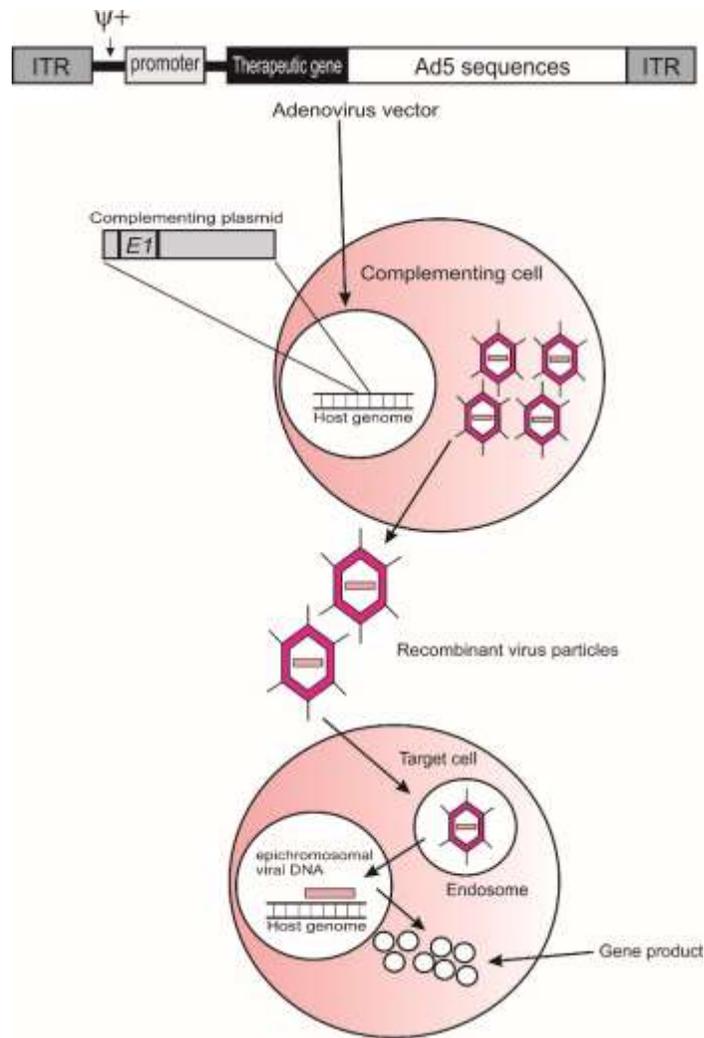
### **Adenovirus**

Human adenoviruses are DNA viruses, and are nearly ubiquitous in humans. Belonging to the genus Mastadenovirus (Davison et al, 2003), there are 51 recognized serotypes. Upon infection of the host cell, adenoviruses quickly begin the process of

transcribing their early genes, E1-E4. These genes encode viral replication proteins as well as some virulence factors needed for evasion of the host immune response.

Recombinant adenoviruses can be made from kits that come with strains ready made for insertion of a given DNA sequence (Fig. 6) . Most strains provided have deleted E1 and E3 sites. Deletion of E1 genes allows not only for substitution with desired transcripts (up to roughly 8 kilobases), but also renders the virus replication deficient. As such it can only grow in cells which contain E1 genes, for example, HEK 293 cells. Deletion of E3 genes, as has become more common in addition to E1 deleted genomes, results in a virus that is devoid of proteins which are normally used for evasion of the immune response (He et al, 1998).

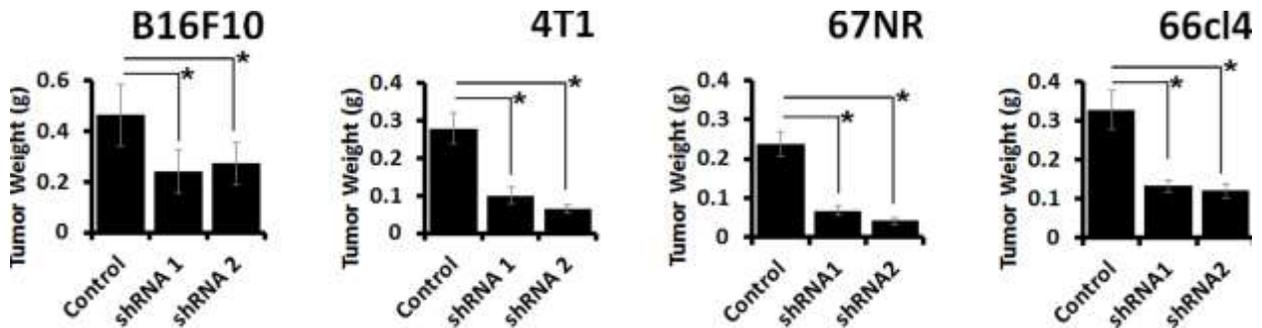
Adenoviruses are advantageous as vectors because of their large cassette capacity, their ability to infect a wide range of cell types, and because of their extremely high delivery efficiency, which approaches 100% (Wagner et al, 1992). Finally, and perhaps most importantly, adenoviruses do not integrate their genome into that of the host (Salahuddin, 2011). This is crucial from a gene therapy perspective because this means there will be no disruption of host DNA, which has been known to lead to uncontrolled cell proliferation if the disrupted gene in question is one that regulates the cell cycle (Hacien-Bey-Abina et al, 2003).



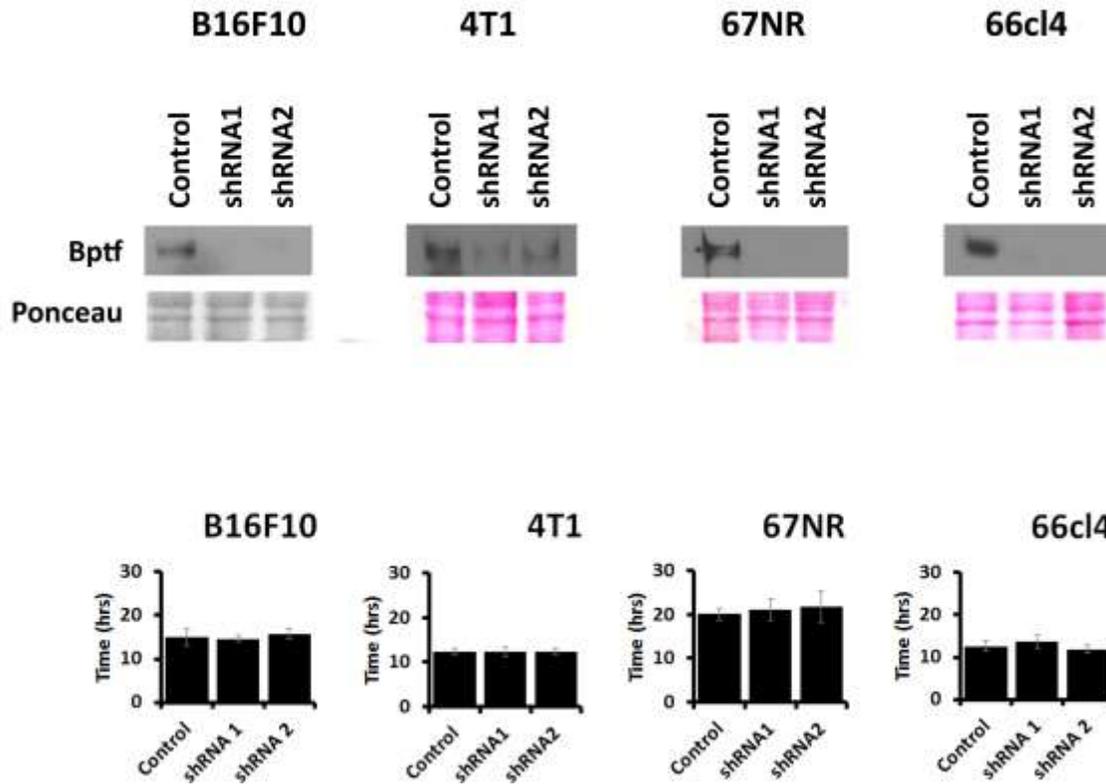
**Figure 6. A cartoon representation of the lifecycle of a recombinant adenovirus.** A recombinant adenovirus contains a desired gene flanked by the adenovirus genome and its associated promoters and inverted terminal repeat (ITR) regions, which promote transcription. In a complementary cell, the host provides the E1 genes needed for replication to produce recombinant virus. When the virus infects a target cell lacking E1, the result is expression of the inserted gene but without viral replication (Salahuddin, 2011).

## Previous Work in the Landry Lab and Rationale for Study

Previous work in the Landry lab originally focused on studying tumor growth upon KD of BPTF by short hairpin RNA via retrovirus. It was found that KD inhibited tumor growth in syngeneic implanted tumors, but did not affect doubling times in culture (Fig. 7-8, currently under revision for Cancer Research). This suggested that the BPTF KD cells were likely not killed directly by BPTF KD, as viability was not affected. Rather, it was hypothesized that the immune system mediated the reduced growth of the tumors.



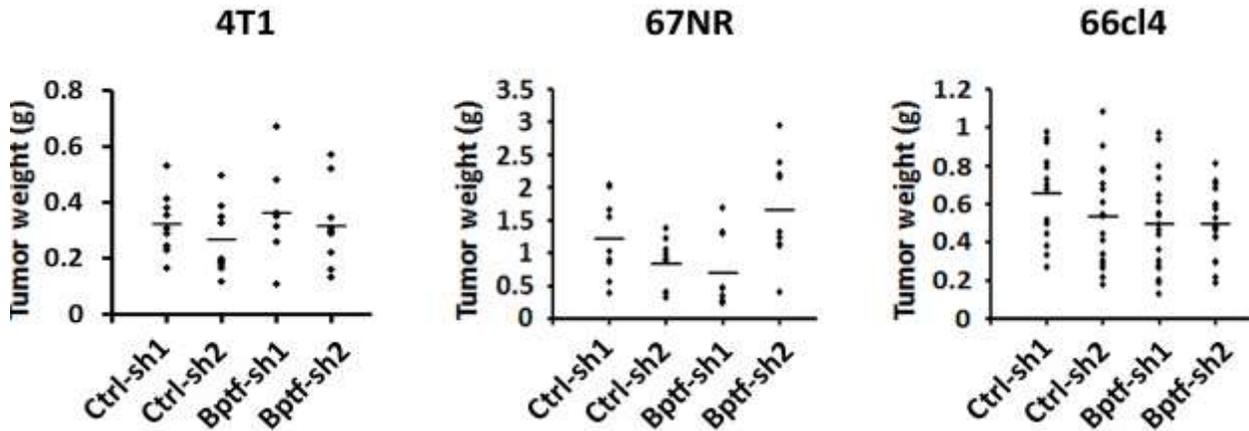
**Figure 7. BPTF KD in multiple cell lines reduces tumor cell growth.** The murine breast cancer lines 4T1, 67NR, and 66CL4 were treated either with control or BPTF short hairpins and implanted into BALB/C mice. A mouse melanoma line, B16F10, was also used in syngeneic C57BL/6 mice to assess effects of BPTF KD on tumor growth. Tumor weights in the KD lines were significantly reduced compared to controls.



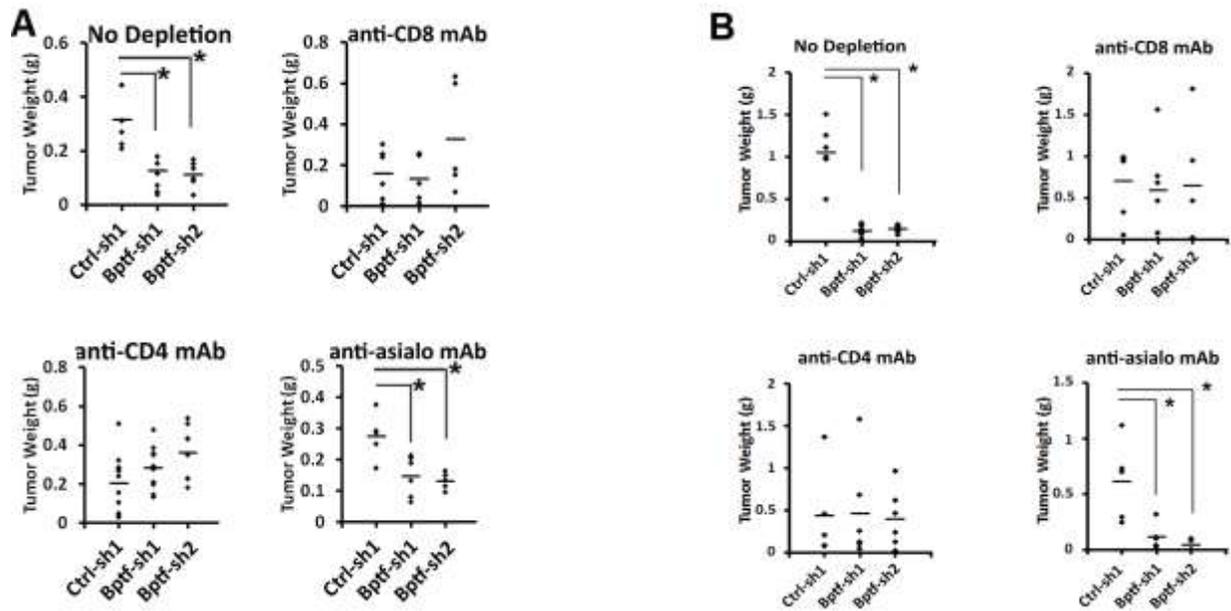
**Figure 8. Doubling times of multiple tumor cell lines unaffected by BPTF KD.** BPTF KD and control cells of mouse cell lines B16F10, 4T1, 67NR and 66CL4 were plated and were counted at 24, 48 and 74 hours to calculate doubling time. No significant change in doubling time was observed between KD and controls.

To test whether the immune system mediated tumor reduction, two approaches were used. First, control and KD tumors would be implanted in NSG mice. NSG mice lack B cells, T cells, and NK cells. Tumors grown in NSG mouse had no significant difference in size (Fig. 9). Next, the type of lymphocyte responsible for the reduced growth was sought. Monoclonal antibodies were used to deplete CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, and NK cells. These depletion studies showed that cytotoxic CD8<sup>+</sup> T-cells (CTL) were likely responsible for reduced tumor size in 4T1 and B16F10 tumors (Fig. 10), and

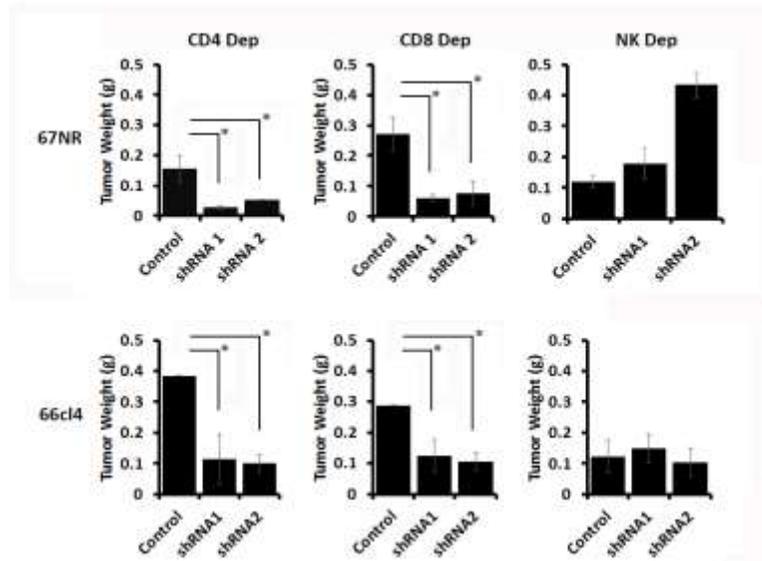
that NK cells were responsible in 66CL4 and 67NR cells (Fig. 11).



**Figure 9. Tumor weight in 4T1, 67NR and 66CL4 control and KD tumors in NSG mice.** Murine breast cancer lines 4T1, 66CL4 and 67NR, as well as a mouse melanoma line, B16F10, were treated with either control of BPTF KD shRNA. When these lines were transplanted into NSG mice lacking major immune system components, tumors showed no difference in weight between KD and control in all three lines, indicating an immune system involvement.

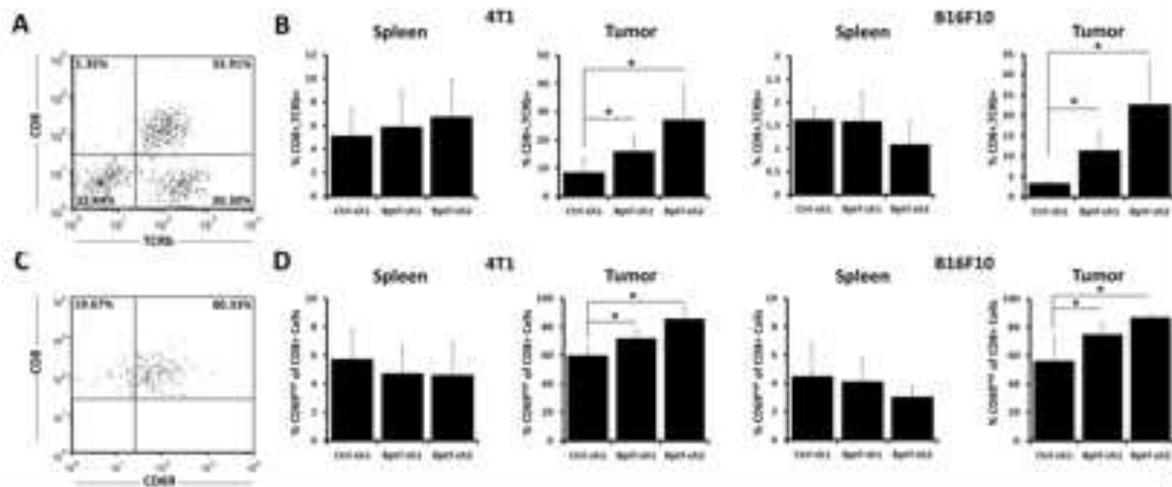


**Figure 10. Monoclonal antibody depletion of lymphocytes identifies T-cells as important for enhanced anti-tumor immunity to BPTF KD in 4T1 and B16F10 tumors.** Mice with 4T1 (A) and B16F10 (B) tumors were treated with monoclonal antibodies for CD4, CD8 and Asialo-GM1 to deplete helper T-cells, cytotoxic T-lymphocytes, and NK cells respectively. The results show no observable difference in tumor weights between no depletion and NK cell (anti-Asialo-GM1) depletion. Depletion of T-cells resulted in a rescue of tumor weights between controls and BPTF KD, indicating a role of T cells in BPTF KD mediated tumor inhibition.



**Figure 11. Monoclonal antibody depletion of lymphocytes identifies NK cells as important for enhanced anti-tumor immunity to BPTF KD in 66CL4 and 67NR.** Control and BPTF KD tumors of 66CL4 and 67NR mouse breast cancer lines were treated with antibodies specific for CD4, CD8, and Asialo-GM1, an NK cell surface molecule and tumor weights were assessed. Anti-asialo-GM1 antibody depletion of NK cells in 67NR and 66CL4 demonstrated a rescue of tumor weight in BPTF KD.

To address whether or not CTLs were actually infiltrating BPTF KD tumors in greater numbers, flow cytometry was used to count and characterize CD8+ T-cells within 4T1 and B16F10 tumors. Upon assaying the tumors, it was found that T-cells in KD tumors were not only more numerous, but more active as well (Fig. 12). This effect was not seen in the spleen, however, which suggested that indeed there was an immunogenic component to the KD tumors themselves.



**Figure 12. Increased infiltration and activation of CTLs seen in BPTF KD tumors but not spleen of mice with 4T1 and B16F10 lines.** 4T1 and B16F10 control and BPTF KD tumors were assessed by flow cytometry. A) Anti-TCRb and anti-CD8 antibodies were used to select cytotoxic T-lymphocyte population in order to quantify level of infiltration. B) Percent of lymphocytes which were positive for CD8 and TCRb (CTLs) were compared between spleens and tumor populations in 4T1 and B16F10. C) Anti-CD8 and anti-CD69 antibodies allow for quantification of activation in CD8 positive cells. D.) Percentages of active CD8 cells were compared in spleen vs tumor populations, with significant increase in activation found in the tumors of both 4T1 and B16F10 BPTF KD, but not spleens.

These results suggest that: 1.) BPTF KD results in the upregulation of a stimulatory molecule or the downregulation of an inhibitory antigen on the surface of the cell, inducing an immune response, 2.) this immune response is mediated by NK and T-cells, 3.) BPTF KD results in an increase in anti-tumor activity. From this, we *hypothesize that 1.) BPTF KD in established tumors will have a similar effect as seen in syngeneic KD tumors, thereby imitating a therapeutic agent, and 2.) NK cells will be more active and preferentially kill BPTF KD cells in vitro and in vivo.*

## **Specific Aims**

**Aim 1:** To determine whether KD of BPTF using recombinant adenoviruses will result in reduced growth of established tumors.

Hypothesis: Knockdown of BPTF in established tumors will result in reduced tumor growth compared to controls.

**Aim 2:** To characterize NK cell infiltration and activation in 66CL4 and 67NR knockdown tumors in comparison to controls.

Hypothesis: NK cell infiltration and activation will be greater in BPTF KD tumors.

**Aim 3:** To determine if BPTF KD renders human breast cancer cells more susceptible to NK cell cytotoxicity in vivo.

Hypothesis: BPTF KD human cancer cell lines will be more susceptible to human NK-92 cytotoxicity.

## Chapter 2: Methods and Materials

### **Cell Culture**

4T1, 66CL4, and 67NR tumor lines were all grown in DMEM supplemented with 10% FBS, 1X non-essential amino acids (NEAA), 2mM glutamine, 100 units/mL of penicillin and 100mcg /mL of streptomycin at 37°C and 5% CO<sub>2</sub>. For the purpose of growing tumors for analysis by flow cytometry, confluent cells were counted and diluted to the following cell counts: 6 x 10<sup>6</sup> cells/mL for 4T1, 2 x 10<sup>6</sup> cell/mL for 67NR, and 2 x 10<sup>5</sup> cells/mL for 66CL4. These dilutions would be used to transplant the tumors into mice. The tumors would grow for 3 weeks for 67NR flow cytometry purposes, 4 weeks for 66CL4 for flow cytometry, and 7-10 days for 4T1 adenoviral experiments, or whenever first palpable.

Previously retrovirus treated BPTF knock down human tumor cell lines T47D and MDA-MB-436 were grown in DMEM supplemented with 10% FBS, 1X NEAA, 2 mM glutamine, 100 units/mL of penicillin, 100 mcg/mL of streptomycin, 10mcg/mL bovine insulin, and 5 mg/5 mL puromycin at 37°C and 5% CO<sub>2</sub>.

NK-92 cells, an immortalized line of human natural killer cells, were used in the LDH assay to assess killing in BPTF knockdown human cancer cell lines. NK-92 cells were grown in Alpha Minimum Essential Media (Alpha MEM) without ribonucleosides or deoxynucleosides, supplemented with 12.5% FBS, 12.5% horse serum, 1.5 grams sodium bicarbonate/L, 0.2mM inositol, 0.1mM beta mercaptoethanol, .02mM folic acid, 100 units/mL penicillin, 100mcg/mL streptomycin, and 200 Units/mL of IL-2.

## Creation of rAd Containing Control or BPTF shRNAs

Using plasmid pE1.2 (containing the early 1 gene backbone for adenovirus) cloned with either BPTF knockdown short hairpin (shRNA) 28 or a shRNA to luciferase, pE1.2 plus insert was digested with DraIII restriction enzyme, as was pE3.1. The 300 base pair fragment from pE3.1 (Early 3 gene backbone) and the 1.8kb fragment from pE1.2 were then gel purified using ethanol precipitation. These were then ligated with pAd388, which contained the adenovirus genes, using T4 ligase. Ligation mixture was incubated at 16 degrees Celsius overnight.

To package this ligation into competent bacteria, the Gigapack III Gold Packaging Extract from Agilent Technologies was used. This kit contained a lambda phage extract, which would preferentially package DNA of around 40kb. Thus it was ideal for use in our plasmid, which was 38kb. Two microliters of DNA containing either the pE3.1+pE1.2 with luciferase, or pE3.1+pE1.2 with shRNA28, was added to the extract and allowed to incubate for two hours at room temperature. At this point, 500  $\mu$ L of SM buffer, a lambda phage storage buffer, and 20  $\mu$ L of chloroform were added to the tubes. Tubes were spun down to sediment the debris, and the supernatant containing the phage was decanted into a new tube and stored at 4 degrees until use.

VCS257 strain of *E. coli* from the Gigapack kit was plated overnight on LB agar. A mini-prep from the resulting colonies was performed the next day in LB broth containing 10 mM MgSO<sub>4</sub> and 0.2% (w/v) maltose. This was grown at 37°C in a shaker for six hours at 200 rpm. The bacteria were then pelleted at 500 x g for ten minutes and resuspended to an OD<sub>600</sub> of 0.5 with sterile 10 mM MgSO<sub>4</sub> solution. 25  $\mu$ L of each

packaging reaction were added to 25  $\mu$ L of cell suspension and incubated at room temperature for 30 minutes. 200  $\mu$ L of LB broth was then added to each sample, which were then incubated for one hour at 37 degrees in a water bath to allow for antibiotic resistance expression. Finally, these cells were spun down and resuspended in 50  $\mu$ L of LB broth. They were then plates on LB+Kanamycin and Ampicillin agar plates and grown overnight.

Two colonies of each cosmid were observed the next day. A mini-prep for each of these was performed using LB+kanamycin and ampicillin broth. The cosmids from the cells were then purified using the Qiagen Midi Plasmid Purification kit. Analytical digest using EcoRV as well as MluI enzymes on the purified cosmids confirmed the correct identity of the cosmid.

## **Production and Recovery of rAd Virus**

HEK 293 cells were grown on 6 cm cell culture dishes in 10 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1X non-essential amino acids (NEAA), 2 mM glutamine, 100 units/mL of penicillin and 100  $\mu$ g/mL of streptomycin at 37°C and 5% CO<sub>2</sub>. The cells were grown to 90% confluence before the transfection.

In order to ensure proper adenovirus replication inside the HEK 293 cells, the Luciferase and short hairpin 28 cosmids were digested with SwaI restriction enzyme. SwaI digestion was used to linearize the cosmid for transfection, as adenovirus

genomes can only be replicated in linear form. Three micrograms of DNA were digested with 3 units of enzyme for one hour.

To transfect the cells with the purified digested cosmid, we used Lipofectamine 2000 from Life Technologies. First, 15  $\mu$ L of lipofectamine were added to 250  $\mu$ L of antibiotic-free DMEM, and in a separate 1.5 mL centrifuge tube, 3  $\mu$ g of cosmid DNA were added to 250  $\mu$ L of DMEM. These were allowed to sit for five minutes at room temperature. Then they were combined, and incubated at room temperature for 30 minutes. During the incubation, the antibiotic containing media was removed from the cell culture dishes. After incubation, the lipofectamine-DNA mixture was added to the HEK 293 cells. 4 mL of antibiotic free DMEM was then added to the cells.

### **Purification of High Concentration Virus**

Plates were monitored daily for signs of viral infection. In the case of adenovirus, the hallmarks of infection (known as cytopathic effect, or CPE), include a refractive edge, rounding, detachment and clumping into grape-like clusters. After 13 days, the shRNA luciferase and BPTF shRNA lines showed CPE. When more than 30% of cells had detached from their plates, the media and all cells were collected into a 15 mL conical tube and spun down at 300 x g for 10 minutes. Roughly 20% of the media was removed, and the pellet was resuspended in the remaining DMEM. The tubes were then frozen in liquid nitrogen, and thawed at room temperature. This was repeated three times for a total of four freeze-thaw cycles to release the virus from the cells. Finally, the tubes were spun again to pellet cell debris and collect viral supernatant.

The viral supernatant from the primary transfection was plated dropwise onto a newly confluent 6 cm dish. This plate grew until CPE was observed and 30-50% of cells detached. Then the viral supernatant was harvested again and plated onto a 10 cm dish. The virus from the 10 cm dish was then plated onto three 10 cm dishes for a final round of amplification.

The resulting cells were spun down, and resuspended in 3 mL of solution of PBS with 10% glycerol. They were then frozen and thawed three times, spun down and the supernatant containing the virus was aliquoted and frozen. This virus suspension was to be used for intratumoral injections in mice.

### **Determination of Viral Concentration**

Using a protocol obtained from OD260 (Boise, Idaho), the virus was assayed with a Tissue Culture Infectious Dose 50 assay (TCID<sub>50</sub>). For each virus, one 10 cm plate was seeded with HEK-HEK 293 cells in penicillin/streptomycin 10% FBS DMEM. The confluent plates were trypsinized and collected into a 50 mL tube. This was spun down at 300 x g for 10 minutes and the culture media was discarded. The pellet was resuspended in 30 mL of DMEM.

Serial ten-fold dilutions were made of virus per Table 1.

Virus Dilution	10 <sup>1</sup>	10 <sup>2</sup>	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>7</sup>	10 <sup>8</sup>	10 <sup>9</sup>	10 <sup>10</sup>	10 <sup>11</sup>
DMEM (μL)	90	90	90	90	90	90	180	1080	1080	1080	1080
Add:	10 μL virus stock	10 μL from 10 <sup>1</sup> dilution	10 μL from 10 <sup>2</sup> dilution	10 μL from 10 <sup>3</sup> dilution	10 μL from 10 <sup>4</sup> dilution	10 μL from 10 <sup>5</sup> dilution	20 μL from 10 <sup>6</sup> dilution	120 μL from 10 <sup>7</sup> dilution	120 μL from 10 <sup>8</sup> dilution	120 μL from 10 <sup>9</sup> dilution	120 μL from 10 <sup>10</sup> dilution
Mix:	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 90 μL	Pipet in/out 4 x 180 μL	Pipet in/out 4 x 1000 μL			

**Table 1.** Dilution scheme for TCID50 assay to determine viral titer. 10 μL of raw virus was serially diluted by ten fold to achieve 10<sup>-8</sup>, 10<sup>-9</sup>, 10<sup>-10</sup> and 10<sup>-11</sup> concentrations of the undiluted virus. These were plated with HEK 293 cells in replicates of 10 and observed for cytopathic effect.

Using two 24 well plates for each virus, 10 replicates each of the 10<sup>-8</sup>, 10<sup>-9</sup>, 10<sup>-10</sup> and 10<sup>-11</sup> dilutions were added to each plate with two negative controls for each dilution. 100 μL of each viral dilution were added to each well, and 150 μL of HEK-HEK 293 cell suspension were also added. To the negative controls wells 100 μL of DMEM were added. After seven days the plates were monitored daily for CPE, which was noted as a “positive” well. When the number of wells with signs of virus stabilized, the assay was considered complete. The number of positive wells was entered into a spreadsheet which used a formula given to us by OD260. The output of the formula was in

TCID50/mL units, which correlates to roughly 1 plaque forming unit (PFU) per 0.69 TCID50. This was used to calculate virus concentration.

### **Confirmation of rAd Delivered shRNA KD Efficacy**

In order to confirm that the created virus would knock down BPTF in mice, an in vitro experiment was performed. 66CL4, 67NR, and 4T1 cells were plated at 20% confluency on 6 cm dishes and 1 mL of concentrated viral supernatant were added and allowed to incubate for three days. After three days, the plates were split 1:2 and again 1 mL of virus was added. One week after initial infection, the cells were collected with Tri Reagent. 0.5 mL of Tri Reagent was added to each plate and the lysate was collected into 1.5 mL microcentrifuge tubes. 100  $\mu$ L of chloroform were added to each tube of Tri Reagent and cells remnants. The tubes were spun at 21,000 x g for 5 minutes and the organic layer was transferred to a new tube. The proteins in the organic layer were precipitated with 750  $\mu$ L of isopropanol. The protein was pelleted by centrifugation and resuspended in 0.3 M guanidine hydrochloride/95% ethanol solution and allowed to wash overnight at 4°C. The next day the guanidine was removed and replaced with 200 proof ethanol, and the proteins were again washed overnight. The purified protein was washed with 70% ethanol and placed in 8M urea/1% SDS solution at 65°C until dissolved.

The dissolved proteins were tested for concentration using a DC protein assay and diluted to 2 mg/mL. 50  $\mu$ g of protein from each cell line were loaded into 4% SDS

gel. The proteins were transferred from the gel to a PVDF membrane by electrophoresis at 20 V/ 30 mA for 17 hours. The membrane was blocked with 5% non-fat dry milk for one hour, before anti-BPTF rabbit antibody was added at 1:5000 dilution and incubated overnight at 4°C. The next day the membrane was washed once with PBST and then placed in non-fat dry milk again, and incubated at room temperature for one hour with horseradish peroxidase (HRP) linked anti-rabbit IgG at a dilution of 1:10,000. The membrane was washed three times at room temperature on a rocker for 20 minutes each. After washing the membrane was rinsed with luminol/ECL and exposed in a dark room on film. Successful knock down was observed as a BPTF band of reduced intensity.

### **Injection of rAd into Established Tumors**

Mice were placed under 3% isoflurane and on a warm pad to maintain body temperature for all injections. 4T1 cells were injected at  $3 \times 10^5$  cells per 50  $\mu$ L into the 4<sup>th</sup> mammary fat pad of BALB/C mice. After 5 days, mice were injected with 1.2 mg gemcitabine intra-peritoneally as per previously established lab protocols, and thereafter given gemcitabine once a week. Mice were monitored three times a week for tumor development. When the tumors reached 5mm in any direction, injections were initiated.

$1 \times 10^8$  PFU of either virus were injected per day per mouse, three times a week for three weeks. On injection days, tumors were measured by caliper before injection, and tumor size was recorded.

After sacrificing the mice, tumors were excised. These were then weighed, and

then ground up in a Trizol solution, and allowed to sit at room temperature for an hour. To the Trizol suspension, 200  $\mu$ L of chloroform was added for every mL of Trizol. After vortexing, the suspension was spun at 12,000 x g for 15 minutes, and the red organic layer was extracted. Proteins were precipitated with 1.5mL isopropanol for every mL of Trizol, and the pellets were placed in 95% ethanol/guanidine hydrochloride overnight at 4°C. The next day the pellets were spun down and resuspended in 100% ethanol, and once again placed on a rocker at 4°C overnight. The next day, pellets were washed in 70% ethanol and then placed in a 8M urea/1%SDS solution and heated at 65°C overnight to dissolve the protein pellet.

Proteins were assayed by DC protein assay, and a Western Blot was performed as described above to assess levels of BPTF in both the Luciferase and BPTF shRNA treated tumors.

### **Isolation of Lymphocytes for Analysis by Flow Cytometry**

At the end of their previously described incubation times, mice were sacrificed and tumors were harvested. After weighing tumors and taking measurements, tumors were sliced into 1 mm<sup>3</sup> pieces. An enzyme mix consisting of 5 to 10 mL (5 mL for tumors up to 100 mg, 10 mL for greater than 100 mg) HBSS salt solution, 1 mg/mL collagenase type IV, 2% FBS and 0.4 mg/mL DNaseI was made, and the tumor slices were placed in the mix. The digest was run for 30 minutes at 37°C.

After digestion, tumor digests were run through a 40  $\mu$ m mesh cell strainer (Life Technologies), and the resulting solution was spun down at 300 x g for 10 minutes. The

cells were resuspended in 40% Percoll, and a 70% Percoll layer was placed below them. This solution was centrifuged at 3000 x g for 30 minutes. At the interface, lymphocytes were collected and washed with PBS. The cells were spun down, and the supernatant was discarded. The resulting pellet was resuspended in FACS buffer. The cells were then split evenly into tubes for staining.

### **Flow Cytometry and Analysis of Tumor Infiltrating Lymphocytes**

Lymphocytes isolated from the Percoll gradient were split into two tubes. One tube contained 0.25 µg anti-NKp46 (FITC) and 0.1 µg anti-CD69 (PE), the other tube contained 0.25 µg anti-NKp46 (FITC) and 0.25 µg anti-CD3e (PE). Cells were incubated on ice for 20 minutes in the dark with the antibodies, then washed twice with FACS buffer. Finally, all test samples were suspended in FACS buffer containing 0.5 µg of the viability dye 7AAD.

For compensation controls, splenocytes were treated with red blood cell lysis buffer for five minutes and then washed in order to obtain lymphocytes. These were divided into four tubes; one unstained, one stained with 0.25 µg anti-CD8 (FITC), one with 0.25 µg anti-CD3e (PE), and a tube containing 1 mg/mL 7AAD viability dye. These were used to eliminate overlap in wavelengths between the two fluorescent signals of FITC and PE, and any overlap between PE and 7AAD.

T-cells isolated from 4T1 adenovirus treated tumors were stained (per sample) with 0.25 µg anti-CD8 (FITC) antibodies to identify T-cell populations, 0.1 µg anti-CD69 (PE) to measure activation, and 0.5 µg 7AAD to separate live from dead cells.

## **IFN- $\gamma$ Sample Preparation and ELISA**

The night before harvesting, a 96 well plate was coated with capture antibody for IFN $\gamma$  provided by the IFN $\gamma$  kit from Affymetrix. This was washed three times for one minute each in PBST. Tumors from adenovirus injected mice were harvested the next day. The tumors were minced into  $>1\text{mm}^2$  pieces and placed into an appropriate volume of HBSS plus protease inhibitor. They were then frozen and thawed three times and spun down at  $21,000 \times g$  for 15 minutes at  $4^\circ\text{C}$ . The supernatant was taken and the protein concentration was measured by DC protein assay. Using a BSA standard curve, all tumor samples were diluted to match the concentration of the least concentrated tumor sample.  $100 \mu\text{L}$  were loaded in triplicate along with an IFN $\gamma$  standard curve in triplicate and allowed to incubate overnight at  $4^\circ\text{C}$ . The following day, the plate was washed and blocked for one hour with assay diluent. Following this, it was washed again and incubated with detection antibody. After an incubation of 1 hour at room temperature, the plate was washed and then incubated with Avidin-HRP for 30 minutes. Following the avidin step, the plate was washed seven times and  $100 \mu\text{L}$  of TMB solution was added for 15 minutes. Finally, stop buffer was added and the plate was read at  $450 \text{ nm}$  for IFN $\gamma$  concentration by a spectrophotometer (Synergy H1 Multi-Mode Plate Reader, BioTek).

## **LDH Cytotoxicity Assay**

Human tumor cells, both control and BPTF KD, were grown in a 6 well dish until confluent. The day before the assay, these target cells were trypsinized, counted three times for accuracy, and plated in a 96 well V-bottom dish at 5,000 cells per well, in replicates of three per E:T ratio in experimental wells. In addition, wells were set aside to compensate for maximum killing values and spontaneous release of LDH from targets as well as effector cells.

The next day, NK-92 cells were added to targets at effector to target ratios of 2.5:1, 5:1, and 10:1. Once effector cells were placed on target cells, the plate was spun down at 300 x g for five minutes and incubated at 37°C for four hours. 45 minutes before adding substrate mix, lysis buffer was added to the maximum killing wells. At the end of the four hour incubation, 25 µL of media was removed from each well, and to it was added 25 µL of substrate mix. The plate was placed in the dark for 30 minutes at room temperature. After incubating, 25 µL of stop solution was added to end the reaction. The plate was then placed on a plate reader and the wells were read at 490nm absorbance for LDH release by spectrophotometer (Synergy H1 Multi-Mode Plate Reader, BioTek).

## **Statistical Analysis**

Two tailed T-tests for significance between control and KD tumor weights were performed on Microsoft Excel.

## Chapter 3: Results

### **Adenovirus Containing shBPTF Effectively Knocks Down BPTF in Established Tumors; Tumor Growth is Inhibited.**

Previous work in the Landry lab had shown that when cells were infected with a short hairpin containing Lentivirus specific for BPTF, those cells grew less when implanted in mice compared to controls. It was hypothesized that this anti-tumor effect could be mirrored in an established tumor if an adenovirus were used to deliver the BPTF shRNA.

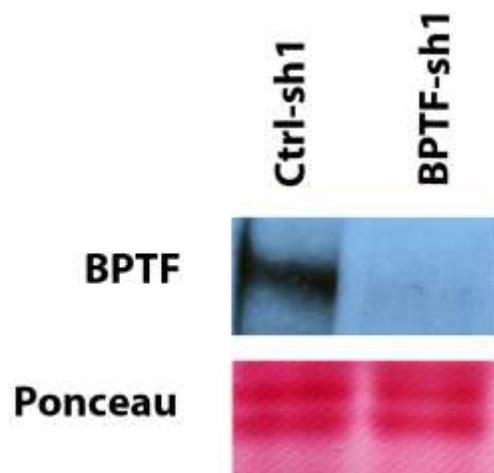
In culture, BPTF was successfully knocked down in 4T1 (Fig. 13). For reasons unknown, 67NR cells died after infection of rAd and thus could not be used for study. After completing the production of the virus as described, mouse breast cancer line 4T1 was chosen as our model tumors based on the efficacy of the knock down in culture. Throughout the 3 week growth period for 4T1, caliper measurements showed decreased tumor volume in the knockdowns compared to controls (Fig. 14). After excising them, tumor weights confirmed this trend. The knockdown virus in 4T1 tumors resulted in a decrease of roughly 51% compared to luciferase hairpin containing virus injected tumors (Fig. 15). Mean tumor weight for controls was .196 grams, compared to .096 grams in knockdowns. Knockdown within the tumors was verified by Western Blot (Fig. 16).

To confirm previous findings of increased T-cell activation and infiltration in BPTF KD tumors, another set of mice (n=6) was used to investigate the T-cell infiltration and activation of the 4T1 tumors by flow cytometry. Our results show that T-cells, while not

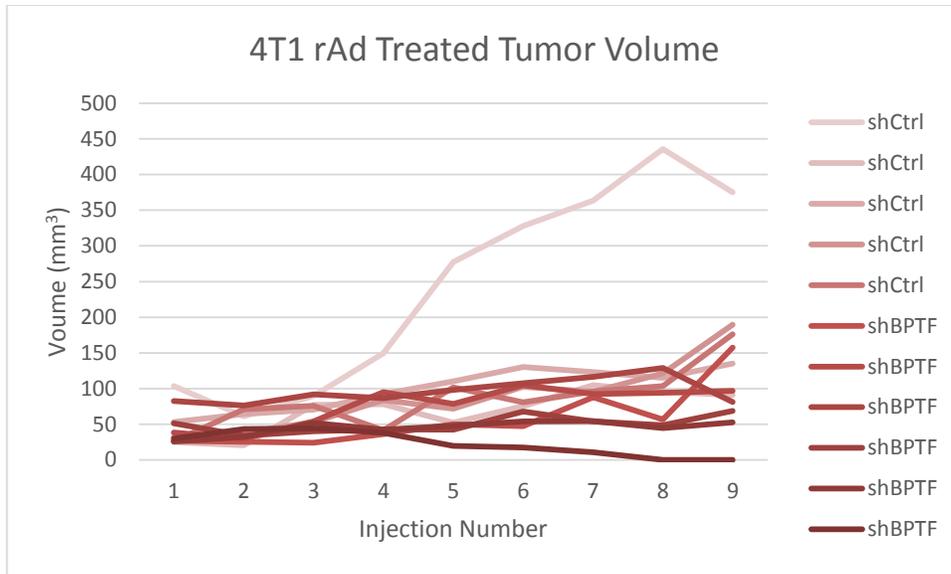
more numerous, are more active in tumors treated with BPTF KD virus than controls (Fig. 17).

### **BPTF KD Results in Increased IFN $\gamma$ Within the Tumor Microenvironment**

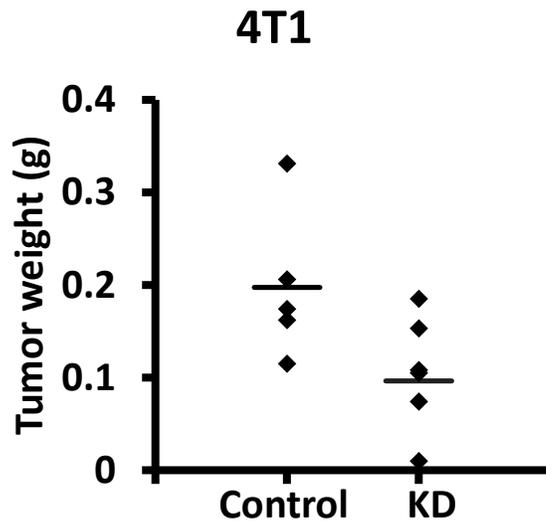
Interferon gamma is a cytokine that has broad activity in activating lymphocytes, and IFN $\gamma$  knockout mice have been shown to be highly vulnerable to carcinogenesis, both in transplantable and endogenous cancers (Ikeda et al, 2002). To bolster our hypothesis that lymphocytes are more active in BPTF KD tumors, we investigated rAd treated tumors using the Affymetrix IFN $\gamma$  ELISA kit. As seen in Figure 18, a noticeable (though not significant,  $p=.22$ ) increase in IFN $\gamma$  was observed in the KD tumors, with an average value of 632 pg/mL compared to 518 pg/mL in controls.



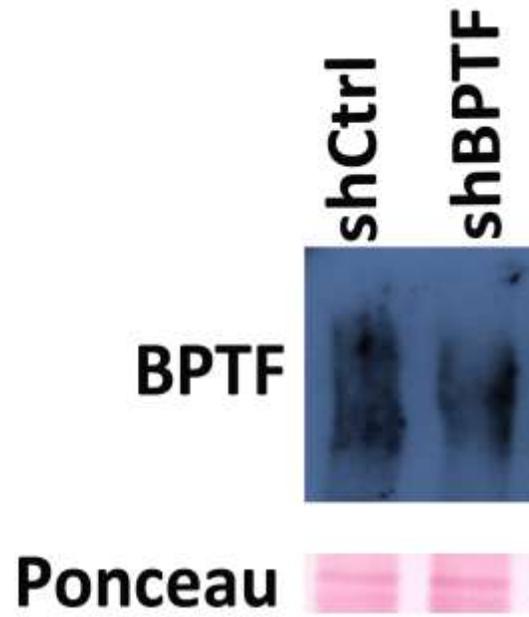
**Figure 13. Western blot of control vs BPTF shRNA treated 4T1 cells in culture.** 4T1 cells were plated in 10 cm dishes and treated with either control or BPTF shRNA rAd for one week. Protein was extracted using Tri Reagent and tested for BPTF levels using a Western Blot. Ponceau S stain shows equal loading.



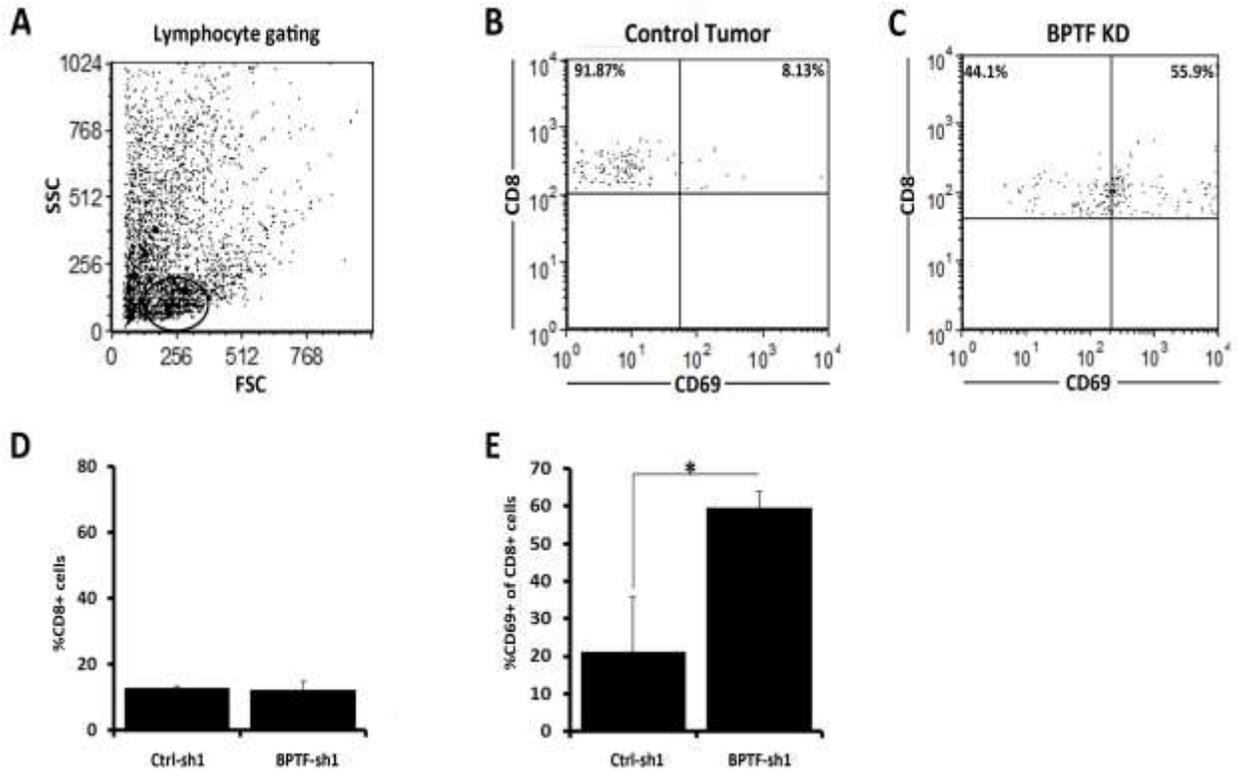
**Figure 14. Representative plot showing progression of tumor volume changes during rAd treatment.** 4T1 tumors that were treated with either control or BPTF shRNA virus were measured with calipers on every injection day. Tumor volumes were calculated using  $V=\pi/6 (a*b^2)$  where  $a$  is the longest measurement and  $b$  is the shortest.



**Figure 15. BPTF KD in 4T1 results in reduction of tumor mass.** 4T1 tumors treated with either control (n=5) or BPTF KD (n=6) rAd were harvested and weighed. The mean weight of control tumors was .196 g, compared to a mean weight of .096 g for KD.

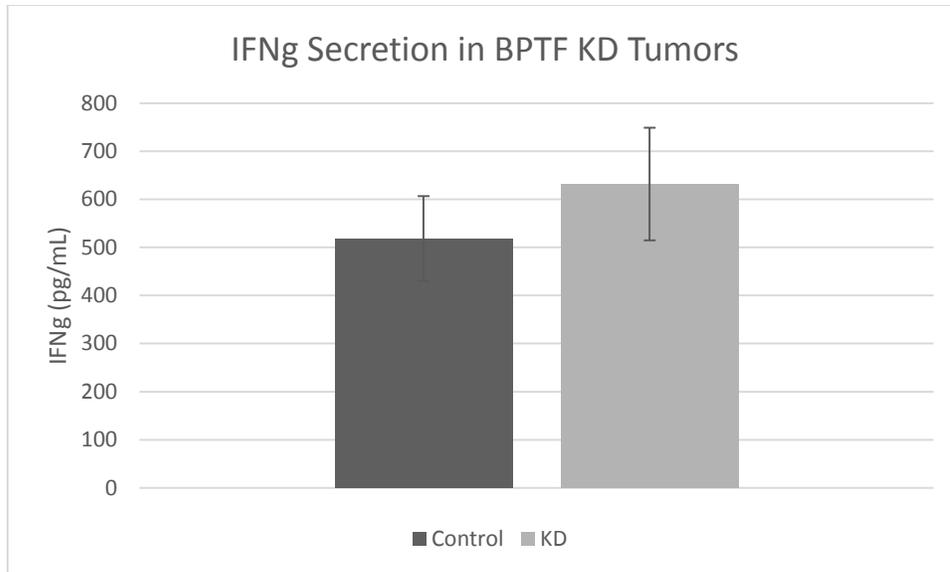


**Figure 16. BPTF is knocked down by rAd in established tumors.** Western Blot showing BPTF knockdown in tumors injected with shBPTF rAd. Ponceau S stain confirmed equal loading.



**Figure 17. Flow cytometry shows an increase in T-cell activation in BPTF KD tumors.**

Flow cytometry was performed on four (two control, two KD) 4T1 tumors treated with recombinant adenovirus. A.) Gating strategy shows the lymphocyte population from which T-cells were selected. B.) Representative plot from a control tumor showing CD8 vs CD69 T-cell activation. C.) Percentages of TIL CD8<sup>+</sup> population in control and KD. D.) Significant ( $p < .05$ ) increase in active T-cells in BPTF KD tumors.



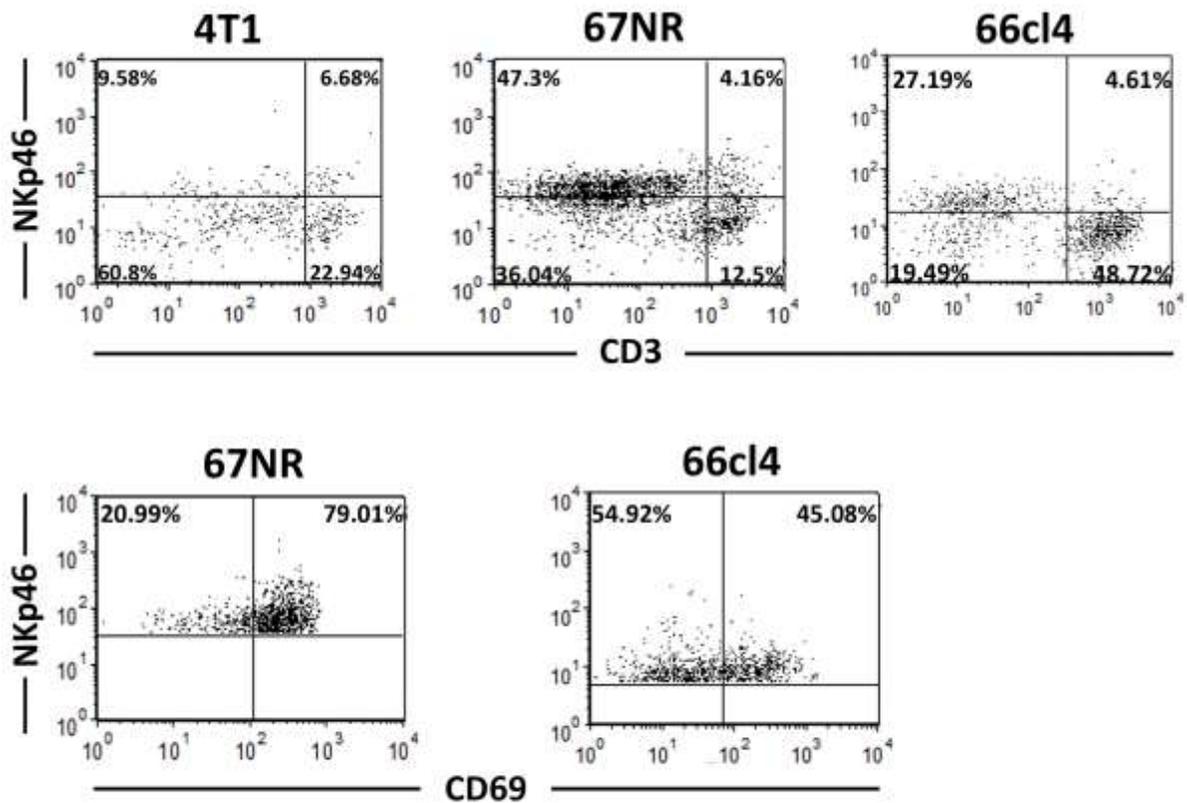
**Figure 18. BPTF KD Tumors Show a Slight Increase in IFN $\gamma$  Secretion.** An increase was seen in IFN $\gamma$  secretion from BPTF KD tumors (n=4), with an average of 632 pg/mL compared to controls (n=3), which had an average of 518 pg/mL.

### **NK Cells in 66CL4 and 67NR are More Active in BPTF KD Tumors**

Our previous studies showed increased NK cell mediated anti-tumor immunity to BPTF KD 66CL4 and 67NR BPTF KD tumors. We next sought to confirm whether there was increased NK cell infiltration or activity in these tumors. Towards this end, lymphocytes harvested from 66CL4 and 67NR tumors were assayed by flow cytometry to measure NK infiltration and activation. To measure infiltration, CD3 and NKp46 antibodies were used. CD3 is found on T-lymphocytes and is required for T-cell activation. It is not found on NK cells. Conversely, NKp46 is an activating receptor found exclusively on NK cells. Using these two stains allowed us to separate T cells from NK

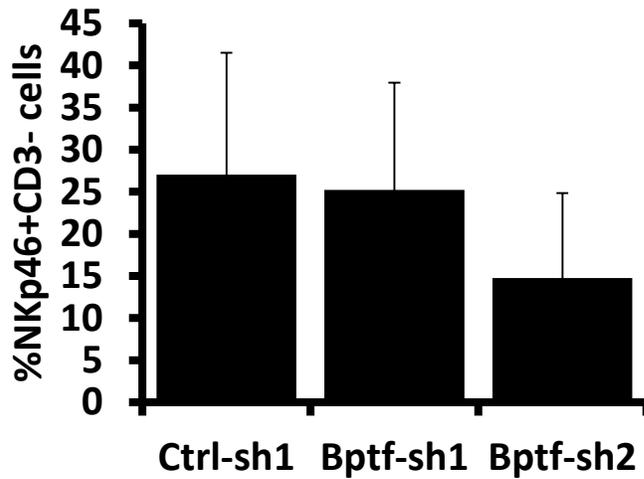
cells, as seen in our gating strategy (Fig. 19). We found no significant difference between control and KD in NK infiltration (Fig. 20).

To measure activation, we once again employed anti-NKp46 to identify NK cells, as well as anti-CD69 staining to measure activity. CD69 is an early activation marker found on T-cells and NK cells. We found that there was a slight increase in the activation of infiltrating NK cells in 67NR KD tumors. There was, however, a statistically significant increase ( $p < .05$ ) in activation of infiltrating NK cells in BPTF KD in 66CL4 tumor lines (Fig. 21). On average, 66CL4 BPTF KD cells had 48 and 44% active NK cells for short hairpins 1 and 2, respectively. This is 19 to 23 percent greater than the control, which had an average of 25% activated NK cells.

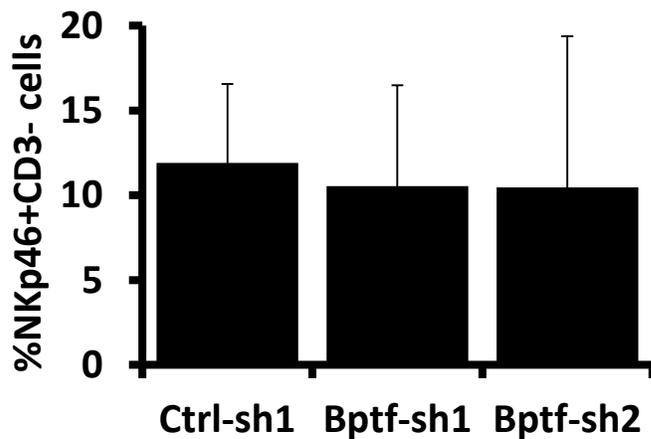


**Figure 19. Gating strategy for 66CL4 and 67NR flow cytometry.** CD3<sup>Neg</sup>,NKp46<sup>+</sup> populations (above) were gated to measure activation (below) in 67NR and 66CL4 using CD69 as an activation marker. 4T1 tumors were used as a control for the staining method.

### Infiltration of NK cells in 67NR

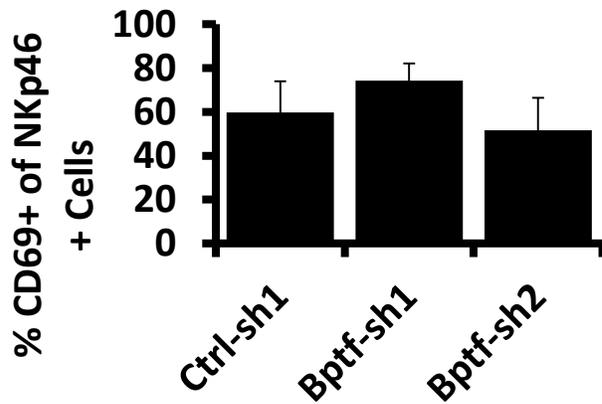


### Infiltration of NK cells in 66CL4

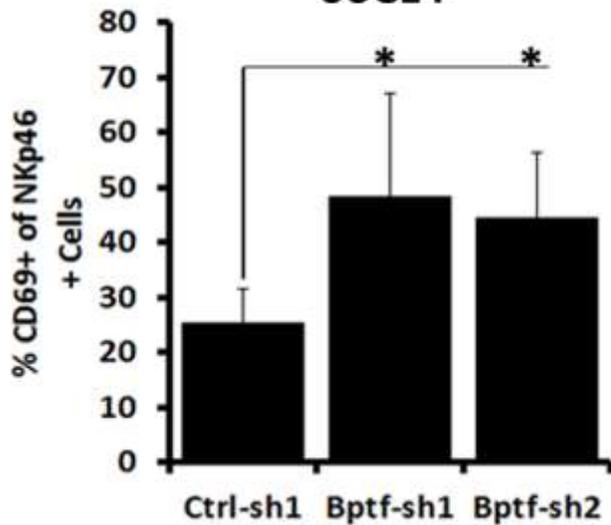


**Figure 20.** Flow cytometry results show no difference in NK cell infiltration between control and BPTF KD tumors. Level of infiltration was assessed by flow cytometry using NK cell specific anti-NKp46 (FITC) and T-cell specific anti-CD3 (PE) antibodies. Infiltration of NK cells in 66CL4 and 67NR tumors showed no significant difference between control and KD.

## Activation of NK Cells in 67NR



## Activation of NK Cells in 66CL4



**Figure 21. BPTF KD show increased T-cell activation by flow cytometry.** Anti-NKp46 (FITC) and anti-CD69 (PE) stains were used to measure activation of T-cells in 66CL4 and 67NR tumors. Greater activation of NK cells was seen in 66CL4 to a statistically significant degree KD lines. Statistical significance was not met for the increase in 67NR. \* = p-value <.05

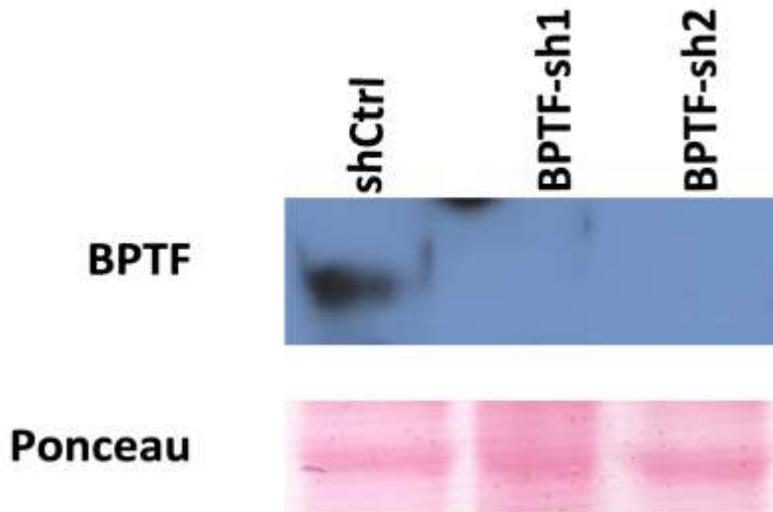
## **NK-92 Cells Preferentially Kill BPTF KD Cells in T47D and MDA-436 Lines**

Our hypothesis as to how BPTF KD aids in tumor clearance involves either an upregulation of cell stress (stimulatory) surface ligands, or a downregulation of inhibitory ligands. Therefore, it followed that NK cells would recognize these cell surface changes and kill BPTF KD cells more effectively than controls. To test this, we measured for increased killing of BPTF KD tumor cells compared to controls by NK cells using Promega's Cytotoxicity kit. This assay involves plating a number of target cells, then incubating them with NK effector cells and then assaying for released LDH. The amount of LDH correlates with cell death.

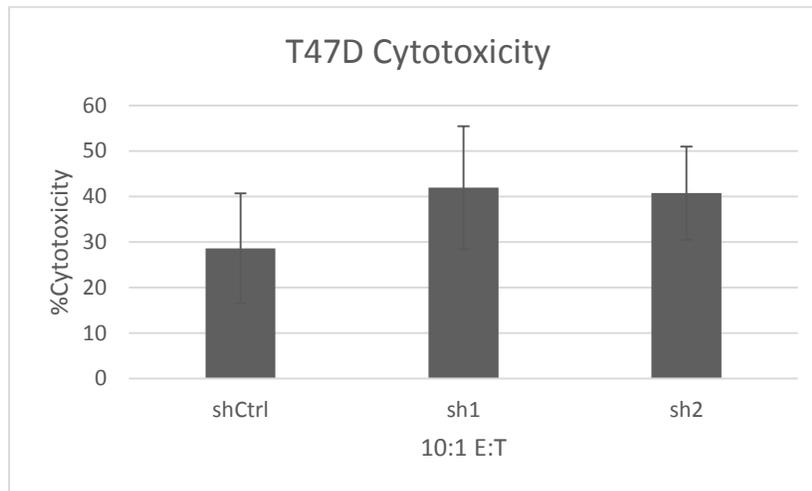
The NK cells used in these studies were an immortalized NK cell line known as NK-92. These cells are derived from a patient who had non-Hodgkins lymphoma. They are known for their high cytotoxicity compared to other NK lines and can be grown easily in a lab to great numbers (Tam et al, 2003). Additionally, they are being investigated for clinical use in cancer patients (Cheng et al, 2013), so their use in these studies lends further credibility to the therapeutic applications of BPTF KD.

For targets, we chose two cell lines that had previously been treated with a short hairpin containing retrovirus to KD BPTF; T47D (Figure 22), a human breast cancer cell line, and MDA-MB-436, another human breast cancer line. We used a 10:1 effector-to-target (E:T) ratio, as below 10:1 killing could not be observed. Our results show at 10:1 ratio T47D KD cells were killed in greater numbers than control cells (Fig. 23). The negative percent cytotoxicity from the control line can be attributed to little cell death but spontaneous release of LDH creating background. MDA-436 showed increased killing

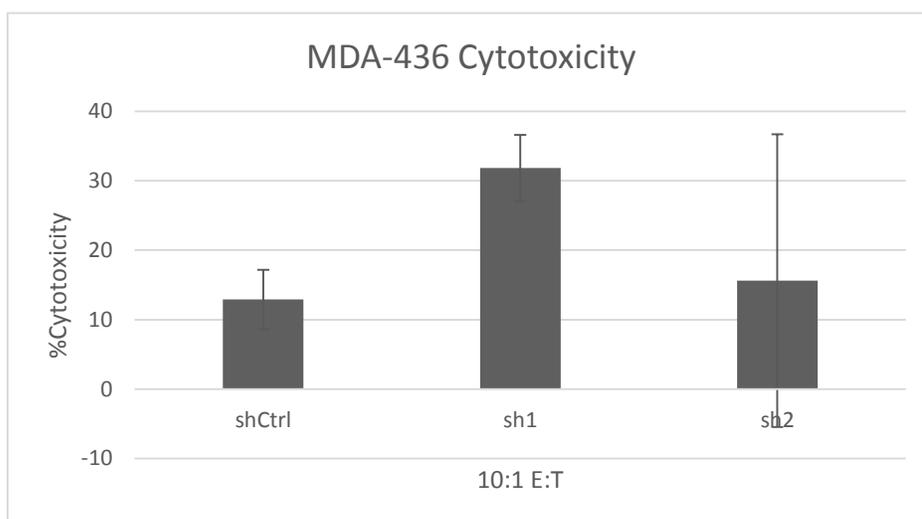
as well, however the second BPTF short hairpin was highly variable (Fig. 24).



**Figure 22. Western Blot of BPTF KD human breast cancer line T47D.** Human breast cancer line T47D was used to test effect of BPTF KD on human NK cell cytotoxicity. Lentivirus was used to introduce either a control shRNA or BPTF shRNA into T47D cells. Western blot confirmed KD of BPTF with both hairpins. Ponceau S stain was used to assess equal loading.



**Figure 23. BPTF KD T47D cells are killed in greater numbers than controls.** Using NK-92 effector cells at a ratio of 10:1 to target cells, cytotoxicity assay results show BPTF KD cells are killed more effectively than control.



**Figure 24. BPTF KD MDA-MB-436 cells are also more readily killed by human NK-92 cells.**

Preliminary data from cytotoxicity assay using human breast cancer cell line MDA-436 at an E:T ratio of 10:1. BPTF KD lines were killed in greater numbers ( $p=.053$  for sh1,  $p=.87$  sh2) than the control.

## Chapter 4: Discussion

Epigenetic abnormalities can be found in every type of cancer (Jones & Baylin, 2002). Among them, disrupted chromatin organization is found in virtually every known cancer type (Jones, Baylin 2007). For instance, the well-known tumor suppressor protein Rb (retinoblastoma) works to deactivate cell cycle genes by recruiting enzymes which deacetylate histones, rendering promoters inaccessible (Wolffe, 2001). When Rb is mutated, these genes go unchecked and malignancy ensues.

Mounting evidence supports the role that ATP-dependent chromatin remodeling complexes play in tumorigenesis (Wang et al, 2007. Bochar et al, 2000.) Given that chromatin reactions are reversible, they make ideal targets for therapy, as this would theoretically yield a better safety profile than traditional chemotherapeutics (Mayes et al, 2014).

### **BPTF KD Results in Dramatic Tumor Reduction in Established Tumors**

The results from my adenoviral studies showed that in established tumors of equal initial size, BPTF knockdown resulted in a near significant ( $p=.06$ ) reduction of mass by half, and more replicates will likely bring this result to statistical significance. It should be noted that most studies using intratumoral recombinant adenovirus use titers between three and tenfold higher or more than the one used in this work (Miller et al, 2004. Jung et al 2014. Chao et al, 2014). Dose response studies using higher viral concentrations may well reveal a more dramatic effect on tumor size in the future.

As the studies presented here show, the immune system is intimately involved in the clearance of BPTF KD cells. Previous work in the Landry lab indicated an upregulation of Lmp and Tap genes in 4T1 BPTF KD tumors (Mayes et al, in revision, Cancer Research). These genes are responsible for the creation of antigen processing molecules, which transport MHC class I molecules to the cell surface. This is important in the context of CTL mediated cancer cell killing. Cells which are able to avoid CTL killing often downregulate their Tap and Lmp expression, in order to limit T-cell recognition by MHC-I. Therefore when BPTF KD cells upregulate the delivery of MHC class I antigens to their cell surface, they become more antigenic, which leads to greater activation of lymphocytes and production of IFN-gamma. These results are consistent with the observations from each of my studies.

### **Increased IFN $\gamma$ in the Tumor Microenvironment of BPTF KD Tumors**

My results suggest that IFN $\gamma$  is increased in BPTF KD tumors relative to controls, although more experiments will be needed to achieve statistical significance. IFN $\gamma$  is a cytokine that regulates both innate and adaptive immune responses (Young and Hardy, 1995). Produced by NK cells and T-cells (and NK-T cells) it has been shown to be closely involved in the anti-tumor response from lymphocytes. In a study by Kaplan et al, mice which had had their receptors to IFN $\gamma$  knocked out grew tumors more rapidly and aggressively than their immunocompetent wild-type counterparts. Other studies have shown that increased intratumoral IFN $\gamma$  was associated with a reduction in tumor mass (Nastala et al, 1994).

When IFN $\gamma$  is secreted, it has a number of effects on immune cell function and differentiation. IFN $\gamma$  has been found to directly affect the differentiation of CD4 cells by promoting T<sub>H</sub>1 helper T cell development and inhibiting T<sub>H</sub>2 development (Bradley et al, 1996.) The consequences of increased T<sub>H</sub>1 differentiation is increased IFN $\gamma$  IL-2 secretion, recruitment of macrophage cells, and elevated IL-12 production (Montovani et al, 2002). IL-12 could further stimulate NK cell, CTL, and antigen presenting cell (APC) activity, as well as proliferation of said cells, which in turn would produce IFN-g (Trinchieri 1995). Taken together, this forms a feedback system where more anti-tumor effector cells are activated in the tumor which potentially inhibit tumor growth.

One of the downsides of increased production of IFN $\gamma$  is that it also promotes infiltration by myeloid derived suppressor cells, or MDSCs (Zaidi et al 2011). These cells suppress T-cell activity by secreting reactive oxygen species, arginase and nitrous oxides (Gabrilovich et al 2010.) It is possible that in our studies with 4T1, the disadvantages of increased IFN $\gamma$  associated with MDSC infiltration were eliminated by our use of gemcitabine, a drug used to deplete MDSC growth (Le et al, 2009). My studies on 66CL4 and 67NR tumors by flow cytometry indicate a possible granulocyte population (data not shown), which contrasts the distinct lack of granulocytic cells in flow cytometry results in 4T1. One reason that tumor growth inhibition in 66CL4 and 67NR is mediated by NK cells and not by CTLs could be because of the presence of an MDSC population. In future studies concerning 67NR and 66CL4, an ELISA based assessment of IFN $\gamma$  levels in BPTF KD tumors will likely find that IFN $\gamma$  is elevated. MDSC populations would then be identified by flow cytometry using anti-Gr1 and anti-CD11b staining as done by Youn et al. Once it has been established that there is a

population of MDSC in 67NR and 66CL4, MDSC depletion studies using gemcitabine could potentially increase the anti-tumor effects of BPTF KD in 67NR and 66CL4, and should be investigated further.

## **Flow Cytometry Reveals Role of Immune System in BPTF KD Tumor Growth Inhibition**

The increase in IFN $\gamma$  in BPTF KD 4T1 tumors suggests that KD of NURF is a driver of heightened lymphocyte activation. Flow cytometry results provide support for this idea by showing a repeated trend of increased activation in both NK cells and T-cells. NK cell infiltration, however, was not elevated in 66CL4 and 67NR BPTF KD tumors as we had previously seen with the T-cell studies using 4T1 and B16F10. A possible explanation for this is the difference in clonal expansion and proliferation between NK cells and T-cells. Once activated in the spleen, different T-cell populations which are primed by multiple antigens will expand 1,000 fold over the week following their activation. By contrast, studies have shown that NK-cells, which do not need to be activated by specific antigens, increase in number by only 10-15 fold (Raulet, 2004). Therefore a difference in infiltration does not necessarily diminish the repeated and significant findings of increased activation of lymphocytes in BPTF KD tumors.

These results confirm that something within the NURF KD tumor microenvironment is eliciting a heightened response from NK cells and CTLs, as seen from multiple studies on different tumor lines. It is likely that BPTF KD causes a downregulation of inhibitory signals on the cell surface, or an upregulation of stimulatory

ligands to these effector cells. It is more probable, however, that upregulation of stimulatory ligands are responsible for tumor inhibition, as our LDH studies indicate. This in turn leads to greater recognition and killing of BPTF KD cells by lymphocytes.

### **Cytotoxicity Results with Human Cells Yield Glimpse of Therapeutic Benefit**

To test whether the advantage BPTF KD provides in killing tumors is conserved in humans, the human cell lines T47D and MDA-MB-436, both breast cancer lines, were assayed using NK-92 cells. NK-92, an immortalized NK cell line, is very useful in cytotoxicity assays on tumors because it has high killing capabilities. To affect these responses it has very few inhibitory receptors, but maintains virtually all of the native activating receptors found on NK cells (Tonn et al, 2001). Given that there are almost no inhibitory receptors on NK-92 cells, the mechanism by which BPTF KD cells promote NK cell activation likely lies in the improved presentation of stimulatory ligands. This limits the recognition of BPTF KD cells to a handful of NK receptors: NKG2D, NKG2E, 2B4, NKp30, NKp46, and CD28 (Tonn, 2001). Many of these receptors are well studied and their ligands are known and largely characterized.

To characterize the effects of a NURF KD on anti-tumor immunity, we must first identify by which receptor NK cells are activated to kill BPTF KD tumor cells. Thanks to the well-characterized nature of the NK-92 receptors, the search for likely NK receptors important for enhanced killing of BPTF KD cells has narrowed considerably. To do this, there are several tools at our disposal: antibody based blocking of the receptors (Perussia et al, 1983. Bauer et al, 1999), competitive inhibition of the substrate

(Bloushtain et al 2004), or shRNA knockout of the receptors in NK-92. An shRNA knockdown is feasible, albeit more time consuming. The easiest of these strategies is likely a competitive inhibition based approach. Using known ligands of each NK receptor in the presence of both BPTF KD and control, we could then assess the effect on killing by cytotoxicity assay. If a specific ligand for an NK receptor is found to reduce BPTF KD killing, an antibody blocking experiment should be used to confirm the identity of the receptor.

Once a receptor has been identified, the hunt then begins for a ligand. Many of the activating receptors on NK cells have known and well characterized ligands (Lanier 1998. Bottino et al, 2005). Given that NURF is known to occupy promoter sites for transcriptional activation, it is quite plausible that in cancer, amplification of NURF leads to overexpression of a protein that functions to inhibit the cell surface presentation of an stimulatory NK-cell ligand. In the future, microarray data could be used to see changes in expression patterns of known NK cell receptor ligands.

Our studies have shown in the preliminary cytotoxicity data that the method of killing seen in the mouse model is potentially conserved in humans. The LDH assay used is prone to high intra-assay variability due to the background that is given off by these cell lines. More experiments will be needed before a definitive interpretation of the data is established. If further studies confirm these initial LDH data in human cells, it would represent a step forward in making the case for a new class of drug, as animal models alone often do not translate to clinical success (Pound and Bracken, 2014.)

## Therapeutic Potential of BPTF Targeting

The Holy Grail of cancer treatment is a targeted drug agent which limits collateral damage to healthy cells such as the kind seen in traditional chemotherapy and radiation regimens, while selectively killing tumor cells. The work presented in this thesis represents a proof of concept for a new drug. Adenoviruses are not a viable delivery vehicle for therapeutic molecules. They elicit too strong an immune response and can return to a replicative state, as seen in a patient who died after gene therapy in 1999 (Marshall 1999), which cuts into idea of a safer treatment than conventional cancer treatment. It may be possible for the results in this paper to translate to a gene therapy using adeno-associated virus. This is a vector which in recent years has seen increased use due to its low pathogenic potential but high rate of delivery for interfering RNA (Daya et al, 2008). However, the last 15 years have given way to a new class of anti-tumor medication as small molecule inhibitors have emerged as a viable option for targeted cancer treatment. Small molecule inhibitors have been used extensively in recent years to combat cancer.

The most well-known example of a small molecule inhibitor is imatinib, a tyrosine kinase inhibitor used to treat chronic myelogenous leukemia (Hoelder et al, 2012). Tyrosine kinases deregulated in cancer cause unchecked cell growth and proliferation (Paul et al, 2004). For reasons unclear, a mutation in these patients causes constitutive expression of TKs, leading to malignancy.

TK inhibitors success lies in the fact that so many cancers de-regulate tyrosine kinase activity. Likewise, BPTF has been found to be highly expressed many different

tumor tissues (Buganim et al, 2010.) As we have shown here, unlike TKs which give a proliferative advantage, BPTF represents a different tumor growth tactic: evasion.

A small molecule inhibitor of BPTF, perhaps one that competes for its SNF2L domain with ATP (taking cue from TK inhibitors), would allow the patient's own immune system to take over. It would also help as a second line treatment for kinase inhibitor resistant diseases, which due to the adaptive nature of cancer cells, may require combination drug therapy (Weinstein and Joe, 2006. Holohan et al, 2013). Future studies on BPTF knockdown by virus should not only utilize dose-response experiments as mentioned above, but also consider adenoviral treatment plus epigenetic based cancer drugs such as histone deacetylase inhibitors to observe the extent of remission that can be achieved.

The work presented in this thesis is the first to show that knockdown of BPTF in established primary tumors can reduce tumor growth. Along with the future studies suggested in this paper, a strong case can be put forth to develop a NURF inhibitor as a new class of cancer treatment.

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