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Role of Nucleosome Remodeling Factor (NURF) in Tumorigenesis Using a Breast Cancer Mouse Model

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

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List of Abbreviations

APCs	Antigen Presenting Cells
APM	Antigens Presenting Machinery
ATP	Adenosine-5'-Triphosphate
BPTF	Bromodomain and PHD-finger Transcription Factor
BSA	Bovine Serum Albumin
β2m	Beta-2 microglobulin
Ccl1	Chemokine (C-C motif) ligand 1
CD4+	Cluster of Differentiation 4
CD8+	Cluster of Differentiation 8
CD25+	Cluster of Differentiation 25
CD11b	Cluster of Differentiation molecule 11b
CD8a	Cluster of Differentiation 8a
CHD	Chromodomain helicase DNA binding
CTCF	CCCTC-Binding Factor
CTLs	Cytotoxic T-cells
Cxcl16	Chemokine (C-X-C motif) Ligand 16
Cxcl9	Chemokine (C-X-C motif) Ligand 9
DAVID	The Database for Annotation, Visualization and Integrated Discovery
DCs	Dendritic Cells
DDT	DNA Binding Homeobox and Different Transcription Factors
DMEM	Dulbecco’s Modified Eagle Medium
dNURF	Drosophila NURF
ESCs	Embryonic Stem Cells
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum

FDR	False Discovery Rate
FITC	Fluorescein Isothiocyanate
Fox3+	Fork-head Box Protein3
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H-60	Histocompatibility 60
H2-D1	Histocompatibility 2, D Region Locus 1
H3K4me3	Histone 3 Lysine 4 tri Methyl
H2-DMa	Histocompatibility 2, Class II, Locus DMa
H2-DMb2	Histocompatibility 2, Class II, Locus Mb2
H2-L	Histocompatibility 2, D Region Locus L
HSP70	70 Kilodalton Heat Shock Proteins
IgG	Immunoglobulin G
IL-2	Interleukin-2
Il2rg	Interleukin 2 Receptor Gamma
IL-10	Interleukin 10
INF-γ	Interferon Gamma
INO80	Inositol Requiring 80
iNOS	Inducible Nitric Oxide
ISWI	Nonfermenting Imitation Switch
JAK	Janus Kinase
KD	Knockdown
KIR	Killer cell immunoglobin-like receptor
KO	Knockout
MCA	Methylcholnthrene
MDSCs	Myeloid derived suppressor cells
MEFs	Mouse Embryonic Fibroblasts
MHC-I	Major histocompatibility Class I

MICA/B	MHC Class I Chain-related Proteins A and B
MMTV	Mouse Mammary Tumor Virus
NURF	Nucleosome Remodeling Factor
NaCl	Sodium Chloride
NCR	Natural Killer Receptor
NKs	Natural Killer Cells
NKp46	Natural killer Cell p46-related Protein
NOD/SCID	Non-Obese Diabetic /Severe Combined Immunodeficiency
NSG	NOD scid gamma
O.C.T compound	Optimal Cutting Temperature
ONOO-	Peroxynitrite
Rae-1	RNA Export 1 Homolog
RAG-2	Recombinase Activating Gene-2
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffer Saline With 0.1% Tween 20
PHD	Plant Homeo Domain
PMNs	Polymorphonuclear Granyolocyte
pRbAp46/48	Retinoblastoma-Associated Protein 46 and 48
Prkdc	Protein Kinase, DNA-activated, Catalytic Polypeptide
PR	Progesterone Receptor PR
Psmb8/9	Proteasome Subunit beta Type-8 and Type-9
PVDF	Polyvinylidene Fluoride
Rcf	Relative Centrifugal Force
SDS	Sodium Dodecyl Sulfate
SNF2L	Sucrose Non-Fermenting 2 Like
STAT	Signal Transducer and Activator of Transcription
SWI/SNF	Switching Defective/Sucrose

TAAs	Tumor Associated Antigens
TAP1/2	Antigen Peptide Transporter-1 and -2
Tapbp	Tapasin
TGF-β	Tumor Growth Factor beta
Th-1/2	T-helper Lymphocyte-1 and 2
TNF	Tumor Necrosis Factors
TRAIL	TNF related apoptosis induced ligand
Xcl1	Chemokine (C motif) Ligand

Abstract

Role of Nucleosome Remodeling Factor (NURF) in Tumorigenesis Using a Breast Cancer Mouse Model

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Understanding the impact of epigenetic mechanisms on tumorigenesis is essential, as epigenetic alterations are associated with tumor initiation and progression. Because epigenetic changes are reversible, they are potential targets for cancer therapy. Nucleosome Remodeling Factor (NURF) is a chromatin-remodeling complex that regulates gene expression by changing nucleosome positioning along the DNA sequence. Previous studies have shown a role for NURF in embryonic development as well as regulating genes involved in tumor progression. In this work we investigated the impact of eliminating NURF function in tumorigenesis *in vivo.* BALB/c mice challenged with syngeneic 67NR breast cancer cell lines, injected into the mammary fat pad, lacking NURF, due to knockdown of its essential subunits Bptf, showed reduction in tumor

growth comparing to control tumors. The observed reduction in tumor growth was abrogated in immunodeficient mice lacking a functional immune system. Bptf KD and control 67NR cells grew at similar rates *in vitro*. Similar findings were observed in our lab using 66cl4 breast cancer cell lines. Using immunofluorescence staining, no significant difference in CD8+, CD4+, NK and MDSC cells infiltrations into the tumor microenvironment was observed in 66cl4 tumors. Preliminary results from 67NR tumors suggested more CD4+ and CD8+ cells. Gene expression profile of tumor tissues from BALB/c mice injected with 67NR and 66cl4 cell lines showed enrichment of genes associated with immune response. Our findings suggested a role of the immune system in targeting tumor cells lacking Bptf *in vivo*.

1- Introduction

1.1- Epigenetics:

During embryonic development cells undergo transitions from the pluripotent stage to more specialized and lineage committed stages. This process is achieved by changing gene expression at different stages to ensure the availability of essential proteins for each cell type (Berdasco & Esteller, 2010). The control of gene expression in the cells is a highly regulated process that ensures normal growth, differentiation, function and life span of the cell. One important level of controlling gene expression within the cells is epigenetic regulation of the genome.

Epigenetics is defined as heritable regulation in a gene expression pattern that is not due to alteration in the DNA sequence. These regulations modify DNA or chromatin structure (Moazed, 2011). Epigenetic regulation of the genome is important in maintaining the normal gene expression, and account for different biological mechanisms in eukaryotic cells, including X chromosome inactivation and genomic imprinting. In addition, many pathological conditions are due to abnormal alterations in the epigenome including Angelman's syndrome, Prader-Willi Syndrome and different types of cancers (Berdasco & Esteller, 2010) (Egger, et al., 2004).

As alterations of the epigenetic mechanisms have been found in many malignant cells, it has been proposed that epigenetic abnormalities are involved in disease etiology and progression. Unlike alterations in DNA sequence,

epigenetic modifications are reversible. Because they are reversed, they are excellent therapeutic targets. We must understand the role of these mechanisms in the pathological conditions to design therapeutic approaches that reverse the abnormal changes (Sharma, et al., 2010).

1.1.1 - Epigenetic Mechanisms:

There are four epigenetic mechanisms involved in regulating gene expression, and interactions between these mechanisms ensure stable expression of the genome. These mechanisms are DNA methylation, histone modification, chromatin remodeling complexes and microRNA (Kim, et al., 2009).

1.1.2- Chromatin Structure:

In eukaryotic cells, genomic material is compacted and localized within the nucleus in the form of chromatin. Chromatin is composed of an interaction between DNA and proteins in which 147 base pairs of DNA is wrapped around histones H2A, H2B, H3 and H4 (Luger, et al., 1997). This level of compacting affects exposure of the DNA sequence and can hinder direct interaction with DNA binding factors such as transcription factors.

1.1.1.2-Chromatin Remodeling Complexes:

The need for chromatin remodeling complexes arises from the fact that the highly compacted chromatin within the nucleus requires mechanisms to rearrange nucleosome positions. This makes DNA element accessible for

different proteins that promote DNA replication, gene expression, and DNA repair mechanisms (Wang, et al., 2007). ATP-dependent chromatin-remodeling families include switching defective/sucrose (SWI/SNF) nonfermenting, imitation switch (ISWI), chromodomain helicase DNA binding (CHD) and inositol requiring 80 (INO80) function to rearrange nucleosomes positions. These families share an ATPase domain, and individual complex in each family has different subunits that account for its functions (Clapier & Cairns, 2009).

1.1.1.2.1-Nucleosome Remodeling Factor NURF:

NURF complex was discovered in *D.melanogaster* (Tsukiyama & Wu, 1995), and subsequently its homolog has been isolated and found conserved in *H.sapiens* indicating the importance of this complex (Barak, et al., 2003). NURF is a member of ISWI family of chromatin remodeling complexes that share a conserved ATPase domain (Clapier & Cairns, 2009).

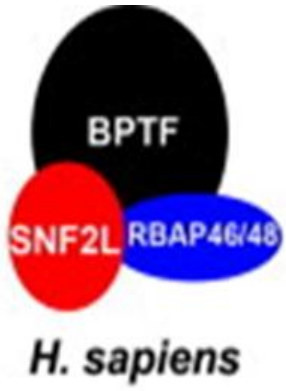
1.1.1.2.1.1- Structure:

Mammalian NURF is composed of three subunits; BPTF (Bromodomain and PHD-finger Transcription Factor), SNF2L (Sucrose Non-Fermenting 2 Like) and RBAP46/48 (Barak, et al., 2003) **(Figure 1)**. Studies in *Drosophila* NURF (*d*NURF) found that the Bptf homolog NURF301 and the Snf2l homolog IWSI are essential for the complex function (Xiao, et al., 2001). BPTF is the largest and exclusive subunit to mammalian NURF (Barak, et al., 2003). It is a large protein (~311Kda) that has important domains including; acidic batch, DDT and PHD

finger domains in the N-terminus, and poly-glutamate repeats, PHD-domain and bromodomain in the C-terminus. The second essential subunit is SNF2L which have character features of the ISWI family of chromatin remodeling include the ATPase domain, HAND, SANT and SLIDE domains (Alkhatib & Landry, 2011).

Figure 1)

a)



b)

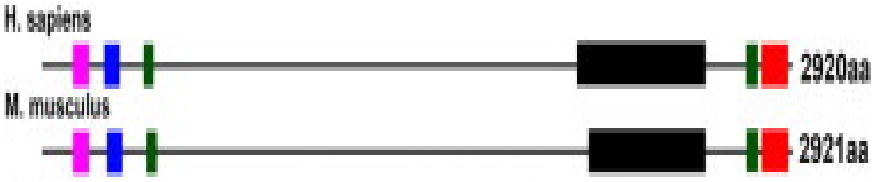


Figure 1: Nucleosome Remodeling Factor NURF's Subunits.

a) The three subunits of the NURF complex. Black is BPTF (Bromodomain and PHD-finger Transcription Factor subunit), Red SNF2L (Sucrose Non-Fermenting 2 Like subunit) and Blue is pRBAP46/48 (Retinoblastoma-associated Protein 46 and 48). **b)** Diagram shows the conserved domains in BPTF subunit between *H.sapiens* and *M.musculus*. Red, Green, Black, Blue and pink represent bromodomain, PHD finger, polyglutamate repeats, DDT and acidic patch domain respectively. The figure adapted from (Alkhatib & Landry, 2011).

1.1.1.2.1.2- Function:

The role of NURF as a chromatin remodeler is dependent on a nucleosome (Tsukiyama & Wu, 1995). Localization of NURF to its target sequences can be through; 1- Interaction with transcription factors as is the case for GAGA factor and progesterone receptor PR that subject NURF to HSP70 and MMTV promoters respectively (Badenhorst, et al., 2002) (Di Croce, et al., 1999). 2 - Recognition of histone modifications. e.g. binding of the PHD finger in the Bptf C-terminal with histone 3-lysine 4 trimethyl (H3K4me3) (Wysocka, et al., 2006). 3

- Presence of a specific DNA binding domain. However, a specific DNA binding domain for NURF has not been identified yet, its presence is not unexpected since DNA binding sequence has been identified for Bptf related protein Fetal Alz-50-reactive clone 1 (FAC1) (Jordan Sciutto, et al., 1999). 4- Recognition of histone variants. The NURF essential subunit BPTF is localized in nucleosome with H2A.Z variant (Goldman, et al., 2010). Once recruited, NURF utilizes ATP to slide the nucleosome position in both directions and expose the DNA sequence to different regulatory proteins (Hamiche, et al., 1999) (Badenhorst, et al., 2002).

Many studies have shown that NURF is involved in a number of important developmental and signaling pathways including TGFβ/Smad, JAK/STAT and Heat Shock (Badenhorst, et al., 2002) (Landry, et al., 2008) (Kwon, et al., 2008). In agreement with these findings, eliminating NURF function in a mouse model by knockout of its largest subunit Bptf is lethal which demonstrates the requirement of NURF in embryonic development through regulating important pathways such as Nodal/Smad signaling pathway (Landry, et al., 2008). Moreover, the same work showed that Bptf knockout in mouse embryonic stem cells (ESCs) prevents their ability to develop and form teratomas after injection into NOD/SCID (Non-obese diabetic /Severe combined immunodeficiency). Collectively, these findings demonstrate the requirement of NURF in cell development and differentiation *in vivo*. In addition, gene expression profile from embryonic stem cells (ESCs), mouse embryonic fibroblasts (MEFs) and double

positive DP thymocytes identify a number of genes that are involved in different aspects of carcinogenesis as Bptf dependent genes (Landry, et al., 2008) (Landry, et al., 2011) these include adherence genes (E-Cadherin, N-Cadherin, Vimentin and Fibronectin) (Makrilia, et al., 2009) and a group of major histocompatibility class I (MHC-I) and class II (MHC-II) genes (Campoli & Ferrone, 2008). Moreover, in the level of chromatin structure, KD of Bptf leads to alterations in nucleosome occupancy localized with DNA binding site for CTCF an important chromatin regulator at important sites such as promoters and insulators (Millau & Gaudreau, 2011).

Together, these findings suggested that NURF, as an epigenetic mechanism, might have a role in tumorigenesis.

1.2- Cancer:

Cancer is a term for multiple diseases that share common characteristics responsible for the associated malignant phenotype. According to the world health organization, cancer is the main cause of mortality in the world (Ferlay , et al., 2010). Although cancer is a focus of extensive amount of research worldwide, and much improvement has occurred in terms of detection and treatment of tumor lesions, the number of new cases and death rates are still high. In the United States, where cancer is the second cause of death, the estimated number of new cancer cases in 2012 is around 1.6 million cases, and

1,500 cancer patients die every day according to 2012 American Cancer Society's report (American Cancer Society. , 2012).

Malignant tumors generate from set of cells that undergo uncontrolled cellular division. As malignant cells develop, they progressively accumulate more alterations in the genomic and epigenomic levels that lead to profound changes in their gene expression profile and growth advantage (Sharma, et al., 2010). Although malignant tumors can be developed from different tissues and organs, all tumor types share common capabilities that ensure a tumor's survival (Hanahan & Weinnerg, 2011). One of these capabilities is the ability of tumor cells to escape the effect of the host immune system.

1.2.1- Role of the Immune System in Cancer:

1.2.1.1- The Immune System:

The mammalian immune system is divided into the innate immune system and the adaptive immune system; both systems are occupied by cellular and molecular components, and are activated against microbial pathogen, infection and tumor cells. The innate immune system's cellular component includes macrophages, dendritic cells (DCs), polymorphonuclear granyolocyte (PMNs) and natural killer cells (NKs), while the adaptive immune system is mainly composed of T-lymphocyte and B-lymphocytes (Medzhitov, 2007). Both systems differ in terms of onset of response and the level of specificity against pathogens. While the innate immune system responds faster and is less specific against

foreign or non-self antigens, the adaptive immune system has a slower but more pronounced and specific response. Cells from both systems are involved in mediating response against tumor cells. Natural killer cells, as a part of the innate immune response, and cytotoxic T-cells, as a part of the adaptive immune response, are the two main cell types that have cytotoxic activity against tumor cells (Russell & Ley, 2002).

1.2.1.1.1- Natural Killer Cells:

Natural killer cells, as the name depicts, mediate cytotoxic activity upon activation against target cells (Kiessling, et al., 1975). They have a lymphocytic origin similar to T- and B-lymphocyte, but they are considered an innate immune response, as they don't undergo clonal selection for specific antigens like in T-lymphocyte and B-lymphocyte receptors (Biron , et al., 1987). The cytotoxic activity of the NK cells can be mediated through secretion of cytotoxic granules containing perforin and granzyme (Russell & Ley, 2002) or through death receptors of TNF (Tumor Necrosis Factors) family ligands including TRAIL (TNF related apoptosis induced ligand) and FasL that are expressed in the surface of the NK cells (Zamai, et al., 1998). NK cells express two types of cell membrane receptors; inhibitory receptors include KIR (killer cell immunoglobin-like receptor) in human, CD94/NKG2A in human and mice, and Ly49 in mice; and activating receptors e.g. NKG2D and NCR (Natural Killer Receptor). These receptors have an important role in distinguishing target cells from host cells, and proper signaling through both receptors ensures the normal function of NK cells

(Langers, et al., 2012). Ligands for the inhibitory receptors include a set of MHC-I molecules that are normally express on surface of nucleated cells and therefore they inhibit the NK cells from targeting these cells. Down regulation of these molecules can trigger the NK cells response against these cells as missing self-signal. Ligands for the activating receptors include Rae-1 and H-60 in mice and MICA/B in human, which are non-classical MHC-I molecules found to be overexpressed in malignant cells (Groh, et al., 1999) (Diefenbach, et al., 2001). Studies have shown that NK cells infiltration into tumor tissues associated with favorable prognosis in cancer patients (Ishigami, et al., 2000) (Coca, et al., 1997).

1.2.1.1.2- Cytotoxic T Cells:

Cytotoxic T-cells (CTLs) or CD8+ T-lymphocytes are important cellular components of the adaptive immune system that are responsible to mediate cytotoxic activity against infected and tumor cells. Upon activation, CTLs mediate killing of target cells through two mechanisms, similar to NK cell, perforin mediated cytotoxicity and Fas/FasL pathway (Russell & Ley, 2002). CTLs recognize MHC-I molecules, which are expressed on all nucleated cells. Malignant cells express tumor-associated antigens (TAAs) that can be presented in the cell surface by the MHC-I through the antigens presenting machinery APM. TAAs include peptides of mutated genes or germ line genes that are abnormally expressed in transformed somatic cells (Restifo, et al., 2012). Infiltration of CTLs

into tumor tissues is associated with favorable outcomes in cancer patients (Naito, et al., 1998).

Antigens loading on the MHC-I are a sequential mechanism that starts by degradation of ubiqutinated proteins in the cytoplasm through immunoproteosom, which contains Psmb9 and Psmb8 catalytic subunits (Angeles, et al., 2012). The resulted peptides are transported to the endoplasmic reticulum (ER) through heterodimer of two transporter proteins TAP1 and TAP2 (ATP-binding cassette-1 and 2). Inside the ER the peptides loaded into MHC-I molecule, which is composed of α chain and β2m, in the ER membrane through tapasin (Tapbp) (Seliger, et al., 2000). The resultant complex, MHC-I and the peptide, is then transport to the cell membrane.

1.2.1.1.3- T-helper Cells:

The second cellular component of the adaptive immune system is T-helper cells. This set of cells characterized by expressing CD4 co-receptor on the cell surface and recognizing antigens such as TAAs that are presented on MHC-II molecules, which express in the antigen presenting cells APCs (macrophage, dendritic cells and B-cells) (Pieters, 1997). The T-helper cells further subdivided into Th-1 and Th-2 based on the cytokines expression (Kidd, 2003). In context of antitumor response, Th-1 is known to augment the CTLs activity and enhance the antitumor response through promote APCs activation and secretion cytokines such as INF-γ (Yu & Fu, 2006), which induce expression of MHC-I molecules in tumor cells. (Ikeda, et al., 2002). Th-2 has a pro-inflammatory role and studies

have shown that these cells might promote tumor growth in some type of cancers (Kidd, 2003).

1.2.1.2- Cancer Immunosurveillance:

Cancer immunosurveillance is defined as the ability of the host immune system to detect and eliminate tumor cells (Burnet, 1970). While the main function of the immune system is protecting the host from foreign cells, the tumor-associated antigens expressed by a tumor cells are the signals that distinguish malignant cells from host cells and trigger the immune system attention to these cells (Smyth, et al., 2001). The concept of cancer immunosurveillance is supported by number of findings in mouse models lacking essential components of the immune response, as well as observations from cancer patients (Dunn, et al., 2004). For instance, mice with non-functional adaptive immune response due to homozygous knockout of the recombinase activating gene RAG-2, which is important in maturation of functional T-lymphocyte and B-lymphocyte receptors (Shinkai, et al., 1992), were more susceptible to develop tumors following treatment with a carcinogen compound (Methylcholnthrene MCA) compared to wild type mice (Shankaran, et al., 2001). Also, the innate immune system has been found to play a role in tumor immunosurveillance. Mice treated with monoclonal antibodies that inhibit NK cells developed more MCA-induced tumors comparing to control mice (Smyth, et al., 2001). The role of the immune system has been observed in human. Epidemiological studies have shown a relative increase in cancer incidence rate

among immunocompromised patients (Birkeland, et al., 1995). Also, histological studies have shown localization of the immune cells into tumor tissues, which indicates an activation and recruitment of these cells into the tumor environment (Naito, et al., 1998). Isolating CD8+ T-cells as well as antibodies against specific tumor associated antigens from cancer patients support the role of adaptive immune system against growing tumors in human (Dunn, et al., 2004).

1.2.1.3- Evasion of the Immune System:

The existence of cancer as a pathological condition in immunocompetent individuals led to suggest a continuous interaction between tumor cells and immune system which has two end points; either the immune system successfully eliminates the tumor cells (immunosurveillance) or the malignant cells evade the immune system (evasion of the immune response) (Dunn, et al., 2004).

Findings from tumor injection studies in mice lacking important immune system components suggest an additional role of the immune system against tumor cells described as immunoediting (Dunn, et al., 2004). Work by Shankaran *et al*. showed that tumors that grew in RAG2 $^{-}/^{-}$ mice without functional adaptive immune system failed to form tumors upon re-injection into wild type mice, but were able to form tumors when they were re-introduced into mice with suppressed immune system (Shankaran, et al., 2001). On the other hand, tumors that grew in mice with a functional immune system were able to form tumors when they re-injected into wild type mice. From these observations,

it has been suggested that the level of immunogenicity of tumor cells is affected by the action of immune response against tumor cells, and the immune system acts as a selective factor that eliminates the highly immunogenic cells (easily detected by immune system) and allows survival of cells that have low immunogenic phenotype.

During tumor progression, malignant cells accumulate genetic and epigenetic alterations that lead to generate a heterogeneous population in the tumor microenvironment. The antitumor effect of the immune system acts as a selective agent against the malignant cells (Vesely, et al., 2011). Cells with immunogenic phenotype are eliminated while cells that develop resistance mechanisms can avoid destruction. Eventually, this set of cells progressively develops and becomes predominant in the tumor site (Birkeland, et al., 1995). Mechanisms that can be developed by tumor cells to avoid the immune system include; reducing tumor immunogenicity, reducing the effect of the CTL and NK cytotoxicity through overexpressing anti-apoptotic molecules or depressing the death signaling pathways; secretion of cytokines that can either inhibit the immune cells activity e.g. interleukin 10 (IL-10), tumor growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) (Khong & Restifo, 2002) or recruit and amplify immunosuppressor cells such as myeloid derived suppressor cells (MDSCs) and regulatory T-cells (Vesely, et al., 2011).

1.2.1.3.1-Reduce Tumor Cell Immunogenicity:

Down regulation of antigens presenting MHC-I molecules is one way which tumor cells can avoid cytotoxic T cells (Garrido, et al., 1997), and has been reported in many tumor types (Algarra, et al., 1997). Down regulation of MHC-I molecules can be through irreversible genetic alterations that affect MHC-I genes or through reversible epigenetic silencing. In addition, reduction in tumor cells immunogenicity can be mediated by silencing the antigens presenting machinery (APM) genes including Psmb8, Psmb9, TAP-1, TAP2 and tapasin (Seliger, 2008). However, as MHC-I molecules are expressed in all nucleated cells, they serve as inhibitory ligands for the inhibitory receptors on NK cells.

1.2.1.3.2-Recruitment and Amplification of MDSCs:

Myeloid derived suppressor cells (MDSCs) are a group of heterogeneous immature myeloid cells. An increase in number of MDSCs has been associated with tumors in mouse models and cancer patients (Gabrilovich & Nagaraj, 2009). In response to different cytokines that are released within tumor microenvironment, which include (granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1 beta (IL-1 β), tumor growth factor beta (TGF-β) and prostaglandins, there is an expansion and activation of the MDSCs population (Naiditch, et al., 2011). In the tumor microenvironment, MDSCs have a negative effect on the immune response through secretion of immunosuppressive factors e.g. arginase and inducible nitric oxide (iNOS) that

cause depletion of important amino acids for T-cell activation such as arginine and cysteine (Gabrilovich & Nagaraj, 2009).

1.2.1.3.3-Regulatory T-cells:

Regulatory T-cells are a group of cells that regulate the host immune response through suppression of CD4 and CD8 cells, and they infiltrate into tumor microenvironment (Piersma, et al., 2008). Depletion of these cells promotes autoimmune response against self-antigens (Yu & Fu, 2006). CD4 regulatory T-cells (CD4+ CD25+ Fox3+ (fork-head box protein3)) are well studied regulatory T-cells, and they mediate their suppresser activity through cell-cell interactions, secretion of inhibitory cytokines such as IL-10 and TGF-β or depleting IL-2 (Schametterer, et al., 2012). Also, studies have shown that CD8 regulatory T-cells infiltrate into tumor tissues and might have similar immunosuppressive function (Wang, 2008).

1.3- Hypothesis:

Along with mutations, epigenetic alterations are associated with tumor development and progression. Previous findings indicated that there is a role of NURF in embryonic development, teratomas formation and regulating important genes in cancer suggest a role of NURF in tumorigenesis. We hypothesized that eliminating NURF function might reduce tumor growth *in vivo*. Toward this end we proposed the following aims;

Aim 1- To investigate tumor growth in mice after injection of Bptf KD 67NR breast cancer cells. Using a syngeneic mouse model, 67NR Bptf knockdown and control breast cancer cells were injected into mammary fat pad of BALB/c mice and NOD SCID gamma (NSG) mice. After 3 weeks, the tumors were surgically removed and tumor weights were measured. Tumors were also processed in aims 2 and 3.

Aim 2- To measure immune cells infiltration into the tumor site following Bptf KD. As findings from the previous experiments suggested a role of the immune system in the observed phenotype, we screened for immune cells infiltration into tumor sites. Using immunofluorescence staining, frozen histological sections were stained with CD8a, CD4, NKp46 and CD11b antibody for CTL, T-helper, NK and MDSC cells, respectively. These experiments attempted to determine if reduction in tumor growth are the result of increased immune cell infiltration

Aim 3- To identify gene expression profile in Bptf KD from tumor tissues. The findings from aim 1 suggested that Bptf KD promote the antitumor immune response against the tumor cells. Tumors from BALB/c mice injected with Bptf KD and control 67NR and 66cl4 cell lines were subjected for microarray analysis.

2- Methods and Materials:

2.1- Mice and Cell Lines:

BALB/c and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ female mice (NOD scid gamma NSG mice) were provided from the Jackson Laboratory (Bar Harbor, ME). Mouse breast cancer cell lines (4T1, 66cl4 and 67NR) were obtained from Dr. Fred R. Miller at Wayne State University (Detroit, MI). Mice were harvested at the barrier facility in the Molecular Medicine Research Building, Virginia Commonwealth University (Richmond, VA). Cells were maintained in 1X high glucose DMEM Dulbecco's Modified Eagle Medium provided by Invitrogen life technology (Grand Island, NY). The media contains 1X Non-essential amino acids, 2 mM L-Glutamine both provided by GIBCO Invitrogen (Grand Island, NY), 10 % fetal bovine serum, 1X penicillin and streptomycin provided by Mediatech Inc. (Manassas, VA). Cells transfected with Bptf short hairpin was maintained in media contains 5 µg/ml of Puromycin, as a selective agent, provided by Invitrogen (Grand Island, NY). Cells were maintained in 6 wells, 12 wells plates or 10 cm dishes at 37 °C and 5 % CO_2 in tissue culture incubator.

2.2- Bptf Stable Knockdown Cell Lines:

In order to generate stable Bptf knockdown (KD) cell lines, the Retro-X™ system (cat. No. 631598) and pSIREN-Retro-Q vector (Cat. No. 631526) were

used, provided by Clontech (Mountain, CA) to generate retrovirus vector with short hairpin specific sequence targeting Bptf gene.

Two short hairpins were used (KD-1 and KD-2) to knockdown Bptf; KD-1 (5'-CGACGATGACTCCGATTATT-TCAAGAG-AATAATCGGAGTCATCGTCG-3'); KD-2(5'-GGCGAAAACCAAGAGTACAT-TCAAGAG-ATGTACTCTTGGTTTCGCC-3'). Non-specific sequence was used as a control (5'-GTGCGTTGCTAGTACCAACTT-TCAAGAG-3'). pSIREN-Retro-Q vector contains shRNA sequence was transfected into PT67 packaging cell line to generate retrovirus vector. PT67 cells were plated in medium without Puromycin for 2-3 days to generate the virus. Then, the medium, which contains the virus, was collected, filtered and added to 67NR breast cancer cells in 6 wells plate for 2-3 days. The medium was then replaced with medium containing Puromycin (5 µg/ ml) for selection of cells that integrated the viral genome. Each well represents a single transduction event.

2.3- Cell Counting:

Cells were plated in 10 cm dish with 10 ml of media for 48 hours. The media was removed and cells were washed with 1 ml of 0.25 % Trypsin and 1 mM EDTA and again incubated in 1 ml of Trypsin + EDTA for 1 to 2 minutes in the incubator to release the cells from the dish. Next, 3 to 5 ml of media was added, and cells were counted using the hemocytometer slide or the Cell meter Auto T4 from Nexcelom Bioscience (Lawrence, MA). 0.2 % Trypan blue provided from Sigma-Aldrich (St. Louis, MO) was used to count cells for injection.

2.4- Population Doubling Time:

In 3 plates of 12 wells plate cells were seeded at $1X10^4$ cells/ ml with 2 ml of media. For each plate 4 wells were used for control and 4 wells for knockdown cells. Cells were counted at 24 hours, 48 hours and 72 hours as one plate for each time point. The population doubling time was measured using doubling time calculator software from Roth V. 2006 http://www.doubling-time.com/compute.php.

2.5- Mice Injection and tumors collection:

6 to 8 weeks of age female mice were anesthetized with isoflurane provided by Clipper distribution company (St. Joseph, MO), and were injected with $1X10^5$ 67NR control shRNA and Bptf shRNA knockdown cells into the mammary fat pad. Cells were diluted into $2X10^6$ cells/ml, and 50 µl was injected into the mice. Three weeks post injection; mice were sacrificed using carbon dioxide. Tumors were surgically removed, and immediately frozen with liquid nitrogen in 15 ml conical tube. Tumor weight was measured by weighing each 15 ml conical tube before and after a tumor is added. The difference between the two weights was used as the tumor weight. Tumor samples were stored at -80°C.

2.6- Western Blotting:

Proteins were extracted from tumor tissues and monolayer cells using TRI Reagent® (as a lysis reagent) provided by Sigma-Aldrich (St. Louis, MO). Tumor tissues were chopped and 0.05 - 0.1 g of tissue was homogenized with 1 ml of TRI reagent using electronic homogenizer. Monolayer cells were washed with 1x PBS, then 1 ml of TRI was added and incubated for 5 minutes. Then, the homogenates were transferred into 1.5 ml tubes, and 200 µl of chloroform was added and the samples were incubated for 10-15 minutes. Then, samples were centrifuged for 15 minutes at 20,000 rcf (relative centrifugal force) at 4°C. Three layers were formed; an aqueous phase contains the RNA, an interphase contains the DNA and an organic phase contains the proteins were in (bottom layer). After removing the aqueous and interphase, 1 ml of isopropanol was added and the tubes were incubated at room temperature for 10 minutes. Then, the samples were centrifuged at 20,000 rcf for 15 minutes at 4°C. The supernatants were discarded and 1 ml of 0.3 M guanidine in 95% ethanol was added for overnight wash at 4°C on shaker. Then, the guanidine was removed by centrifugation and 1 ml 100 % ethanol was added and incubated for overnight at 4°C on shaker. After removing the ethanol, 250 µl of 8 M urea in 1% SDS was added, and samples were incubated at 65°C overnight. Protein concentration was measured using Bio-Rad D_c Protein Assay provided by Bio-Rad Laboratories (Hercules, CA) using BSA standards. The proteins were dissolved in 2 mg/ml concentration. 50 µg of protein was loaded into 4% gel for SDS-PAGE, and run for 1 hour at 200 V and 300 mA. Next, proteins were transferred into

polyvinylidene fluoride PVDF membrane provided by Bio-Rad Laboratories (Hercules, CA) for 17 hours at 20 V and 30 mA. After the transfer the membrans were blocked with 5% non-fat dry milk for 1 hour. Then, Bptf primary antibody was used at 1:5000 dilution and incubated for overnight at 4°C. Following three times washing with PBST (phosphate buffer saline with 0.1% Tween 20) for 5 minutes each, the membrans were incubated with ECL peroxidase labeled anti-rabbit secondary antibody at 1:10,000 dilution for 1 hour. The plots were then washed for 5-10 minutes with PBST for 3 times and developed using supersignal West Femto Substrate from Thermo Scientific (Rockford, IL).

2.7- Immunohistochemistry:

Frozen tumor tissues were embedded in O.C.T compound (Optimal Cutting Temperature) from Sakura Finetek (Torrance, CA). Tissue samples were sectioned at -20 to -25°C with 5 µm thickness using vibratome ultra pro 5000. Cryosections were fixed with acetone for 10 minutes, followed by 10 minutes air dry. Sections were then washed two times with 1 X PBS for 5 minutes each. Then, sections were blocked for 1 hour using 1% BSA (bovine serum albumin) was provided by Sigma-Aldrich (St. Louis, MO). Then, primary antibodies were added at 1:50 dilution for 1 hour. Four rat anti-mouse primary antibodies were used for CD8, CD4, CD11 and NK cells as follow (CD8a cat. No. 550281), (CD4 cat. no.550280), (NKp46 cat no. 560754) and (CD11b cat. no. 557395) provided by BD Biosciences (San Jose, CA). Sections were then washed three times with PBST for 5 minutes and secondary antibody was added. Secondary antibody

was goat anti-rat IgG-FITC antibody Lot# K1711 provided by Santa Cruz Biotechnology (Santa cruz, CA). Then, Slides were washed three times with PBS for 5 minutes each and vectashield® was used as mounting media provided by Vector Laboratories, Inc (Burlingame, CA). Sections were examined using Olympus BX41 Fluorescence microscope under FITC channel.

2.8-RNA Extraction and Quantitative RT-PCR:

Total RNA was extracted using TRI reagent® (as a lysis reagent) provided by Sigma-Aldrich (St. Louis, MO) from the breast cancer cell lines grown in 10 cm plate. The cells were washed with 1X PBS and 1 ml of TRI reagent was added and incubated for 5 minutes. Then, the contents were transferred to 1.5 ml centrifuge tubes and 200 µl chloroform was added. Samples were mixed by vortexing and incubated for 10-15 minutes. Tubes were then centrifuged at 21,000 rcf for 15 min at 4°C. The resulted aqueous phase, which contains the RNA, was transferred into a new 1.5 ml tubes. 100 µl of acidic phenol was added and samples were centrifuged. RNA precipitation was achieved by adding 250 µl of isopropanol and 250 µl of RNA precipitation solution as ¼ the volume of the TRI reagent for each compound. RNA precipitation solution composed of (1.2 M NaCl and 0.3 M disodium citrate). After mixing the contents and incubating the tubes for 10 minutes at room temperature, the tubes were centrifuged at 21,000 rcf for 15 minutes at 4°C. The resulted pellets were washed for two times with 70% ethanol and RNA was dissolved in 50 µl molecular grade water. RNA integrity was investigated by running 1.5% agarose

gel to check for the presence of two un-smeared rRNA bands. The absorbance at 260 and 280 wavelengths was measured by NanoDrop® ND-1000 spectrophotometer provided by Thermo scientific (Wilmington, DE).

RNA was converted to cDNA using Superscript™III kit from Invitrogen life technology (Grand Island, NY). 1 µg of total RNA was used in reaction mixture that include 10 µl of 2X RT reaction mix, 2 µl reverse transcriptase and volume of molecular grad water to make the total volume 20 µl. The thermo cycle was as follow: 25°C for 10 minutes; 50°C for 30 minutes and 85°C for 5 minutes. Then, 1 µl of RNase H was added and tubes incubated for 20 minutes at 37°C.

Quantitative RT-PCR was used to confirm the microarray results. SYBR green ABsolute SYBR Green ROX Mix from Thermo Scientific (Rockford, IL) was used. The reaction mixture was prepared as follow: 5 µl of primers 280 nM (forward and reverse primers), 5 µl of cDNA and 10 µl of SYBR Green ROX Mix. The qRT-PCR condition was as follow: 95ºC for 15min, then 50 cycles of 95ºC for 10 seconds, 60ºC for 30 seconds and 72ºC for 30 seconds. Disassociation curve cycle has been added at the end. Gene expression was calculated using comparative Ct value. β-actin was used as endogenous gene to normalize the gene expression in control and knock-down samples.

-Primer Sequences

Primers were designed using www.ncbi.nlm.nih.gov/tools/primer-blast/ , and provided by eurofins mwg|operon (Huntsville, AL) **(Table 1).**

Table 1)

Gene Symbol		Primer Sequence
H2-Mb2	F	5'-TGTGCCACCCACACCCAACCTT-3'
	R	5'-GTCTCCATTGGGCTGAGCCGT-3'
Cxcl16	F	5'-GACCCTGCCAGGCGATGGCAAC-3'
	R	5'-GGCTTCCCCCACACACGCTTT-3'
Cxcl9	F	5'-TCAGCTCTGCCATGAAGTCCGC-3'
	R	5'-ACTAGGGTTCCTCGAACTCCACAC-3'
H2-Dma	F	5'-TCCCAGTGTCCAGAGGTTTGCCTGT-3'
	R	5'-TGCCTAGCACACCGAGGCCA-3'
β-actin	F	5'-CCCCATTGAACATGGCATTG-3'
	R	5'-ACGACCAGAGGCATACAGG-3'
Lmp7	F	5'-TTGGCCAAGGAGTGCAGGTTGTAT-3'
	R	5'-GTCCCGAGAGCCGAGTCCCAT-3'
Tap2	F	5'-CGCCTTTGCAAGCGCCATCTTT-3'
	R	5'TCGAGTTCAGCTCCCCTGTCTT-3'
Tapbp	F	5'CTGGCTGGTAGCTGCCTACTGGACC-3'
	R	5'-TGAGGGTGGCTTCCACAGACGA-3'
Lmp2	F	5'-CTCTGCTGAGATGCTGCGGGC-3'
	R	5'-CCACTGCTGTTCCCGCTGACAC-3'
H2-D1	F	5'-GAGCCTCCTCCGTCCACTGACTC-3'
	R	5'-CCAGGCAGCTGTCTTCACGCTTTA-3'
Ccnd1a	F	5'-CACAACGCACTTTCTTTCCA-3'
	R	5'-ACCAGCCTCTTCCTCCACTT-3
Ccnd1b	F	5'-GATTTGGCACCTCTCAGCTC-3'
	R	5'-TGGTGAACAAGCTCAAGTGG-3'

2.9- Microarray Analyses:

Microarray experiments and analysis were performed in Dr. Catherine I. Dumur's Laboratory at Molecular Diagnostic Laboratory. RNA extraction, microarray analysis and statistical analysis were performed as described in (Singh, et al., 2011) (Dumur, et al., 2008). Tumor tissues subjected to gross histological

analysis using hematoxylin and eosin stain to determine percentages of tumor cells, necrotic and stromal cells before tissues were isolated for the RNA extraction. Most of the tumors have 100 % to 70 % tumor tissues and necrotic tissues, if present, were selected out by macrodissection. TRIZOL reagent and MagMAX™-96 for microarray total RNA isolation kit provided from Life technology Ambion® (Austin, TX) were used to extract total RNA from frozen tumor tissues in the automated magnatic particle processor MagMAX express from Applied Biosystem. Then, using 2100 Bioanalyzer from Agilent Technologies (Foster City, CA) 1 µl of sampes was applied to RNA ND8000 Lab Chips® to assess the RNA purity and integrity at 260, 270 and 280 nm. Then, 5 µg of total RNA were used for cDNA synthesis and in vitro transcription to generate biotinylated cRNA using the GeneChip® 3' IVT express kit provided by Affymetrix (Santa Clara, CA). Hybridization conditions for the fragmented cRNA on the GeneChip®Mouse Genome 430A 2.0 Array were 16 hours at 60 rpm (round per minutes) at 45°C, and 10 µl of fragmented cRNA were used. Then, using Affymetrix fluidics work station the microarrays were washed and stained with streptavidin phycoerythrin provided from Molecular probes (Eugene, OR). The microarrays were then scanned as previously described using the Affymetrix GeneChip® scanner 3000 and data were saved as .dat and .cel files. The array quality was accepted if the 3'/5' ratio of the housekeeping gene (GAPDH) is less than three and the present gene % is more than 40%.

Microarray Statistical analysis was performed as previously described (Singh, et al., 2011). Log-scale robust multiarray analysis RMA was used for

noise correction, normalization and estimation for probe expression. Relative difference between control and KD samples were analyzed using two-sample-t-test for each pairwise comparison, and to determine differentially expressed probes at univariat level α-level equal 0.01 was used. q-value was used to correct for multiple comparisons in the microarrays experiments, each p-value was corrected for multiple testing using FDR false discovery rate $\leq$ 15%.

Gene Ontology analysis was performed using DAVID the Database for Annotation, Visualization and Integrated Discovery v 6.7 http://david.abcc.ncifcrf.gov/. Functional annotation chart tool was used to determine the highest enrichment terms in the probe set lists that have 2 or more fold changes.

3.10- Statistical Analysis:

Significance difference between control and knockdown samples was determined using two-tail student t-test.

3- Results

3.1- Knockdown of NURF Function Reduces Tumor Growth in a Mouse Model.

NURF is a chromatin-remodeling complex that regulates gene expression by changing nucleosome position. Work by Landry et al., showed that NURF is essential during embryonic development, as mouse lacking Bptf, which is an essential subunit of NURF (Xiao, et al., 2001) is not viable (Landry, et al., 2008). They also showed that ESCs lacking Bptf were unable to form teratomas in NOD/SCID mice. Gene expression profile of Bptf KO ESCs, MEFs and DP thymocyte revealed a role of Bptf in regulating number of genes involved in cancer progression including MHC-I genes, N-cadherin and E-cadherin genes (Landry, et al., 2008) (Landry, et al., 2011). Together these findings led us to hypothesized that eliminating NURF function might reduce tumor growth *in vivo.* To test our hypothesis we chose a BALB/c mouse breast cancer model developed by Fred Miller. This model has many advantages including (i) it is very well characterized mouse model (Aslakson & Miller, 1992), (ii) allows to investigate tumor cells growth *in vivo* with intact immune system (Ottewell, et al., 2006), (iii) it resembles human breast cancer metastasis (Lelekakis,, et al., 1999) and (iv) it is convenient to use as cells can be easily injected into mammary fat pad and form tumors within 3 to 4 weeks. In this model there are five cell lines (4T1, 66cl4, 4T07, 168 FARN and 67NR) that were originally derived from a single spontaneous mammary tumor developed in a BALB/c mouse. These cell

lines differ in their ability to metastasize; 4T1 cells disseminate through blood and metastasize to the lung, liver, brain and bone; 66cl4 cells disseminate through lymph and metastasize to the lung; 168FARN and 4T07 disseminate through lymph and blood, respectively but fail to metastasize and 67NR cells have the ability to form primary tumors, but unable to disseminate from the primary site (Aslakson & Miller, 1992). This work focused on 67NR cell lines. The impact of NURF in the two metastatic cell lines (4T1 and 66cl4) is a subject of another work done by Suehyb Alkhatib in our lab.

First, we wanted to confirm the expression of NURF in the selected cell lines. Work done by S. Alkhatib showed the presence of NURF subunits (Bptf, Snf2L and pRbAp46/48) in these cell lines **(Figure 2).**

Figure 2)

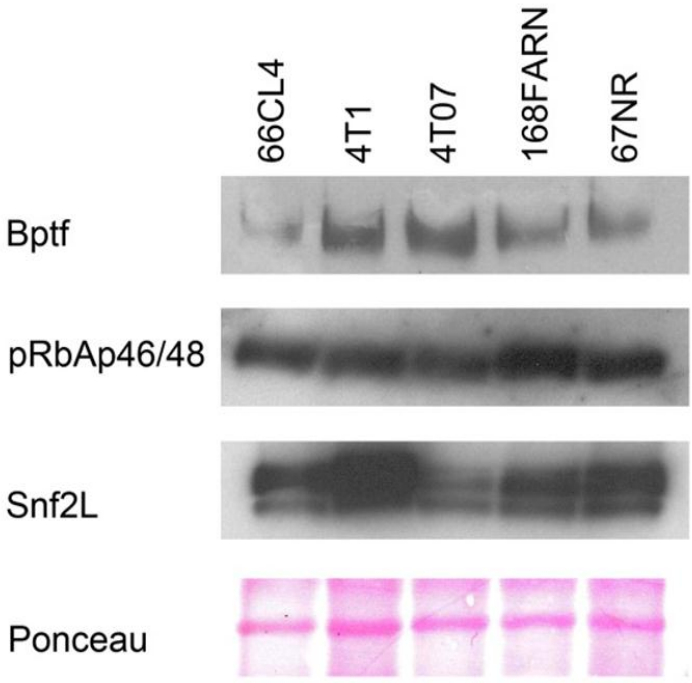


Figure 2: Expression of NURF Subunits in Breast Cancer Cell Lines.

Expression of Bptf, Snf2L and pRbAp46/48 NURF's subunits in 66cl4, 4T1, 4T07, 168FARN and 67NR breast cancer cell lines using Western Blotting. Ponceau stain was used to confirm equal protein loading.

Next, eliminating functional NURF can be achieved by knockdown of its essential and exclusive subunit Bptf (Xiao, et al., 2001) (Landry, et al., 2008). Toward this end we generated stable Bptf KD 67NR cell lines using retrovirus vector to introduce short hairpin targeting Bptf. We used two shRNAs (named as knockdown-1 (KD-1) and knockdown-2 (KD-2)) to create two different Bptf KD cell lines to control for off target effect. A nonspecific RNA sequence was used as a control **(Figure 3).**

Figure 3)

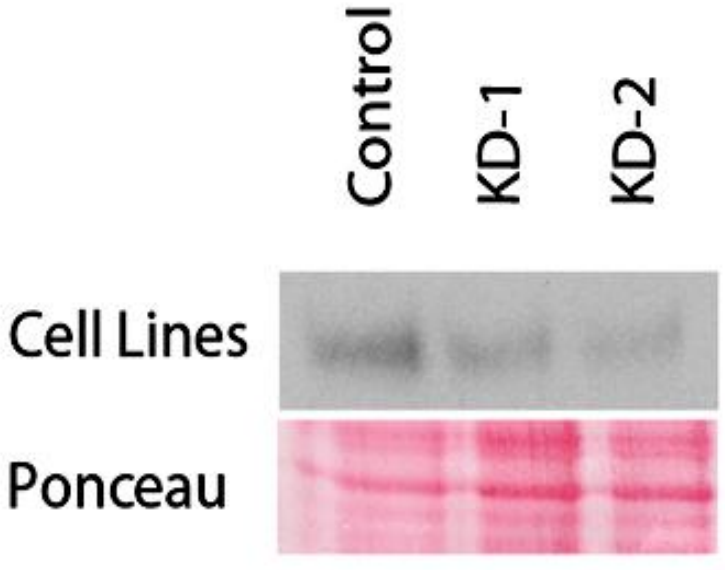


Figure 3: Bptf KD in 67NR Breast Cancer Cell Lines.

Western Blotting is showing Bptf KD using two different shRNAs (KD-1 and KD-2). Non-specific RNA sequence was used as a control. Ponceau stain was used to confirm equal protein loading.

Next, In order to investigate roles of NURF in tumor growth we injected $1X10^5$ 67NR cells into the mammary fat pad of syngeneic BALB/c mice. Mice injected with three group of cells; (i) cells transfected with non-specific shRNA as a control, (ii) cells transfected with KD-1 and (iii) cells transfected with KD-2. The tumors were collected three weeks after injection. We observed significant (p-value < 0.005) reduction in tumors weight and tumors formation in both KDs comparing to the control tumors (**Figure 4).** While all the mice injected with the control cells formed tumors except one, only 3 out of 13 and 6 out of 16 of mice injected with the KD-1 and KD-2 cell lines, respectively, formed tumors. Knockdown of Bptf in the tumor tissues was maintained comparing to the control tumors **(Figure 5).** Similar reduction in tumor growth but not in frequency was observed with the 66cl4 cell lines (by Suehyb Alkhatib, data not shown). These findings suggested that eliminating NURF has a negative effect on tumor growth.

Figure 4)

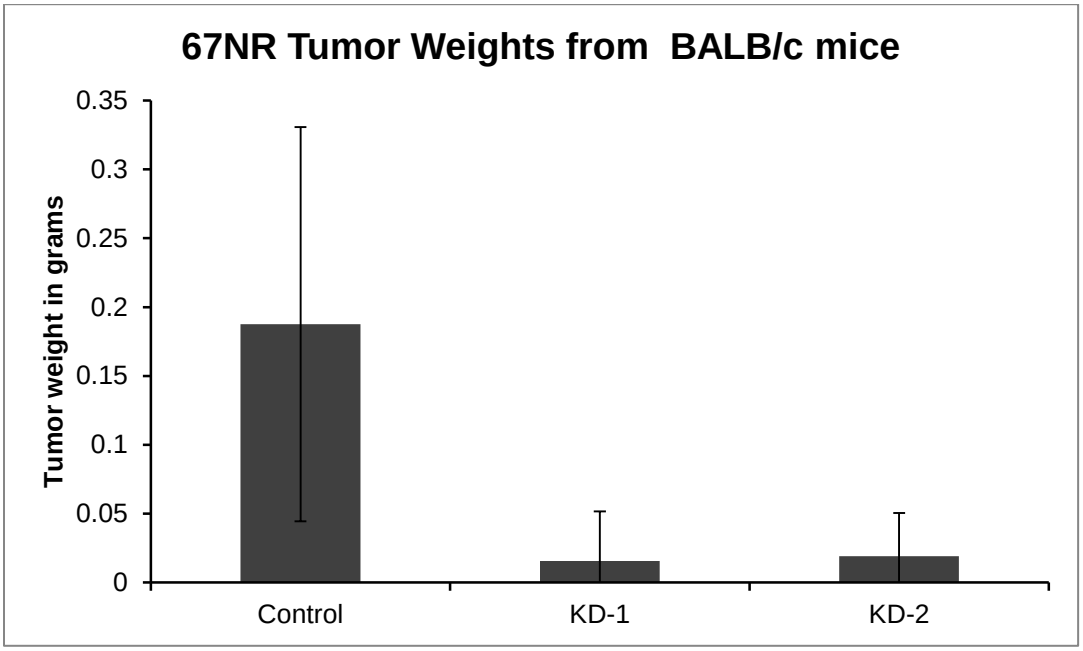


Figure 4: Knockdown of NURF Function Leads to Reduction in Tumor Growth *in vivo.*

Tumors weights form mice injected with control, KD-1 and KD-2 67NR breast cancer cell lines. Significant (p-value < 0.005) reduction was observed in KD-1 and KD-2 tumors relative to the control tumors. Error bar represent standard deviation of 13 tumors for each group.

(Figure 5)

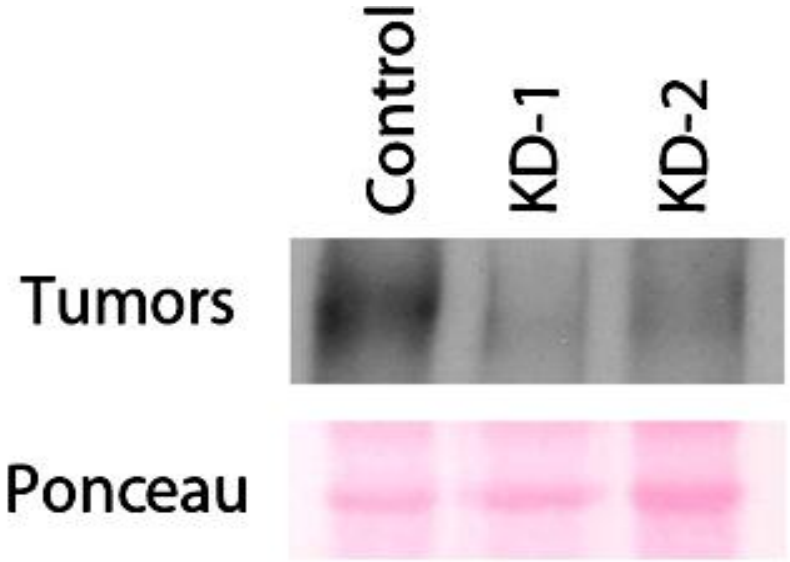


Figure 5: Bptf KD in Tumor Tissues.

Western Blotting is showing Bptf expression level in tumor tissues injected with control, KD-1 and KD-2 67NR breast cancer cell lines. Non-specific control shRNA sequence was used as a control. Ponceau stain was used to confirm equal protein loading.

3.2 – 67NR cells Lacking NURF Proliferate Normally *in vitro.*

The observed reduction *in vivo* can be due to a role of NURF in cellular growth, as a result KD of Bptf can lead to reduce the cell growth efficiency *in vitro*. To exclude the possibility that the observed reduction in tumor growth was due to effect on cellular growth, we measured the population doubling time of the 67NR control and Bptf KDs cell lines *in vitro* (Figure 6). In this experiment, the required time for an entire population of cells to double their number was measured. No significant difference was observed between the control cell lines

and the two KD cell lines. Similar result was observed with 66cl4 breast cancer cell lines (S. Alkhatib, data not shown). This observation suggested that the observed reduction in tumor growth (Figure 4) is due to an effect *in vivo*.

Figure 6)

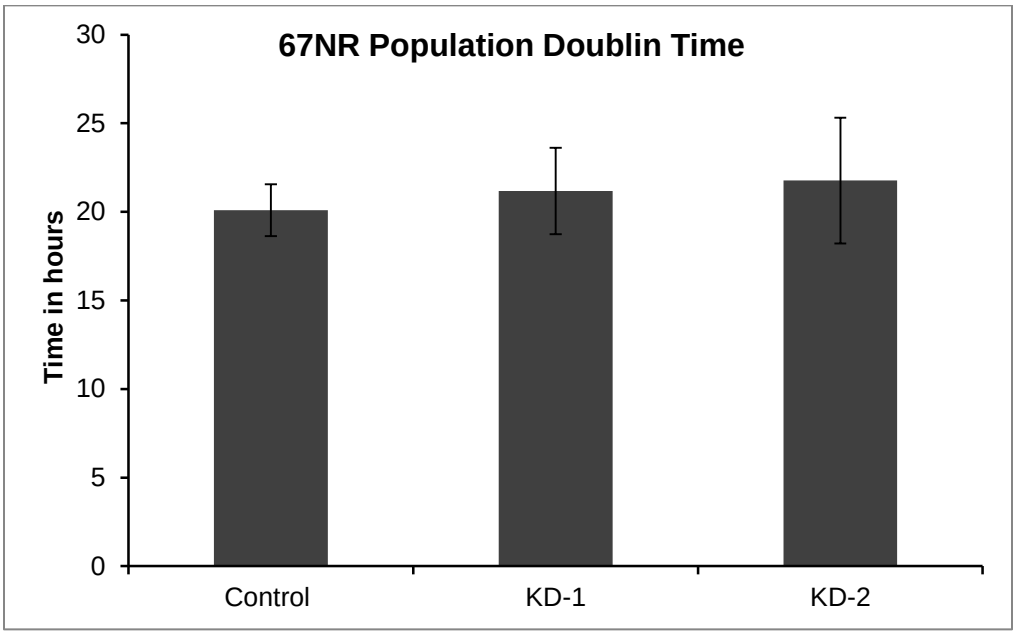


Figure 6: Normal Proliferation Rate for 67NR Cells Lacking NURF *in vitro*.

Population doubling time experiment for control, KD-1 and KD-2 67NR breast cancer cell lines. 1×10^4 cells/ ml were plated in 12 wells plate, and cell count was measured at three time points 24, 48 and 72 hours. The error bars represent standard deviation of three independent biological replicates for each cell line.

3.3 - Reduction in Tumor Growth from Bptf KD 67NR Cells Is Dependent on The Immune System.

Data from the previous experiments suggested a role of NURF in tumor growth *in vivo*. One of the challenges that tumor cells must overcome in order to progressively grow *in vivo* is avoiding the antitumor immune response. The effect of the host immune system on tumor growth have been supported by number of observations, and the ability of the tumor cells to evade the immune system is considered a hallmark of tumor cells (Hanahan & Weinnerg, 2011). The observed deregulation of MHC-I and MHC-II genes in Bptf knockout *in vitro* (Landry, et al., 2008) suggest that the immune system might be responsible for the observed phenotype. We anticipated that the observed reduction in tumor growth might be due to active immune response against the tumor cells. Toward this end, we investigated the effect of Bptf KD in tumorigenesis in immunodeficient mouse model. We chose NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mouse model. This model has two mutations in non-obese diabetic background that lead to a complete absence of the adaptive and innate immune systems. The first mutation is loss of function mutation in Prkdc gene, which is important for functional T-cell and B-cell receptors; as a result it leads to eliminate the adaptive immune cells. The second mutation is knockout for Il2 receptor gamma gene, which encodes important subunit of the IL2 receptor, which plays a role in lymphocytes and other immune cells maturation resulting in elimination of the innate immune cells e.g. NK cells (DiSanto, et al., 1995). Using this model, 1X10^5 67NR cancer cells were injected into the mouse mammary fat pad. Three

weeks after injection, all mice developed tumors. The tumors were collected and no significant difference in tumor weights was observed in both knockdowns comparing to the control tumors **(Figure 7).** This finding suggests that the observed reduction in tumor growth in the BALB/c mice is due to immune system.

Figure 7)

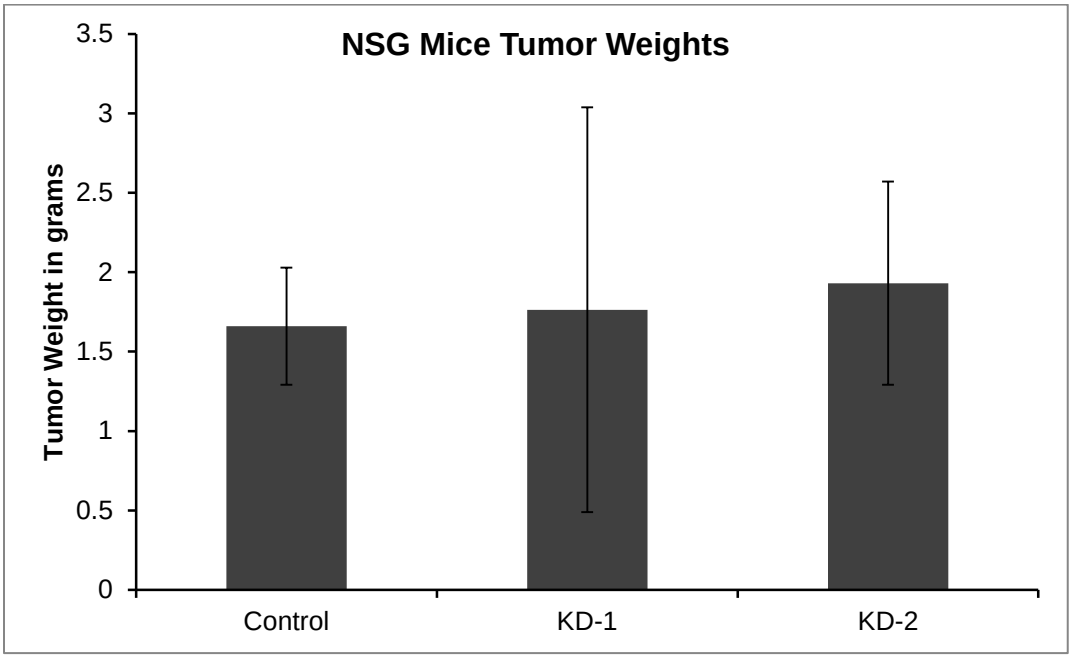


Figure 7: NURF Does Not Affect Tumor Growth in NSG mice:

Tumor weights from mice injected with control, KD-1and KD-2 67NR breast cancer cell lines. The error bars represent standard deviation for 5 mice per each group.

3.4- Normal Immune Cells Infiltration into the Tumor Tissues.

Results from the previous experiments suggested a role of the immune system in the observed reduction in tumor growth. The cellular component of the immune system plays important roles in mediating the antitumor response

against transformed cells. Studies in mice have shown the role of both the innate and adaptive immune cells in mediating cytotoxic activity against malignant cells. Mice lacking CTLs, T-helper or NK cells are more prone to develop induced tumors compared to wild type mice (Dunn, et al., 2004). Another group of cells that infiltrates into the tumor tissues is MDSCs which have an immunosuppressive role against immune cells (Gabrilovich & Nagaraj, 2009). We hypothesized that the reduction in tumor growth might be associated with increase of CTLs, T-helper or NK cells or decrease of the MDSCs infiltration into the tumor tissues.

In order to screen for the immune cells infiltration into the tumor tissue, we used immuofluorescent technique to stain histological sections of tumors tissues. We stained the tumor tissues derived from the BALB/c mice injected with 67NR **(Figure 8)** and 66cl4 **(Figure 9)** cell lines using fluorochrome conjugated antibodies for CD8, CD4, NK and CD11. The preliminary results from the 67NR tumors showed slight increase of the CD8, CD4 and decrease in CD11b cells. However, these results represent one tumor for KD-1 and two tumors for KD-2. More tumors are required to determine the significance of this finding. **(Figure 8 a-d).** For the 66cl4 tumors no significant difference in CD8, CD4, NK and MDSC cells infiltration were observed between the control and knockdown tumors **(Figure 9 a-d).** Although, there is no significant difference in the immune cells infiltration in the 66cl4 tumors, it could be possible that there is increase in the activity and the efficiency of the effector immune cells in targeting the knockdown tumors. (Rosenberg, 2001) (Zitvogel, et al., 2006).

Figure 8)

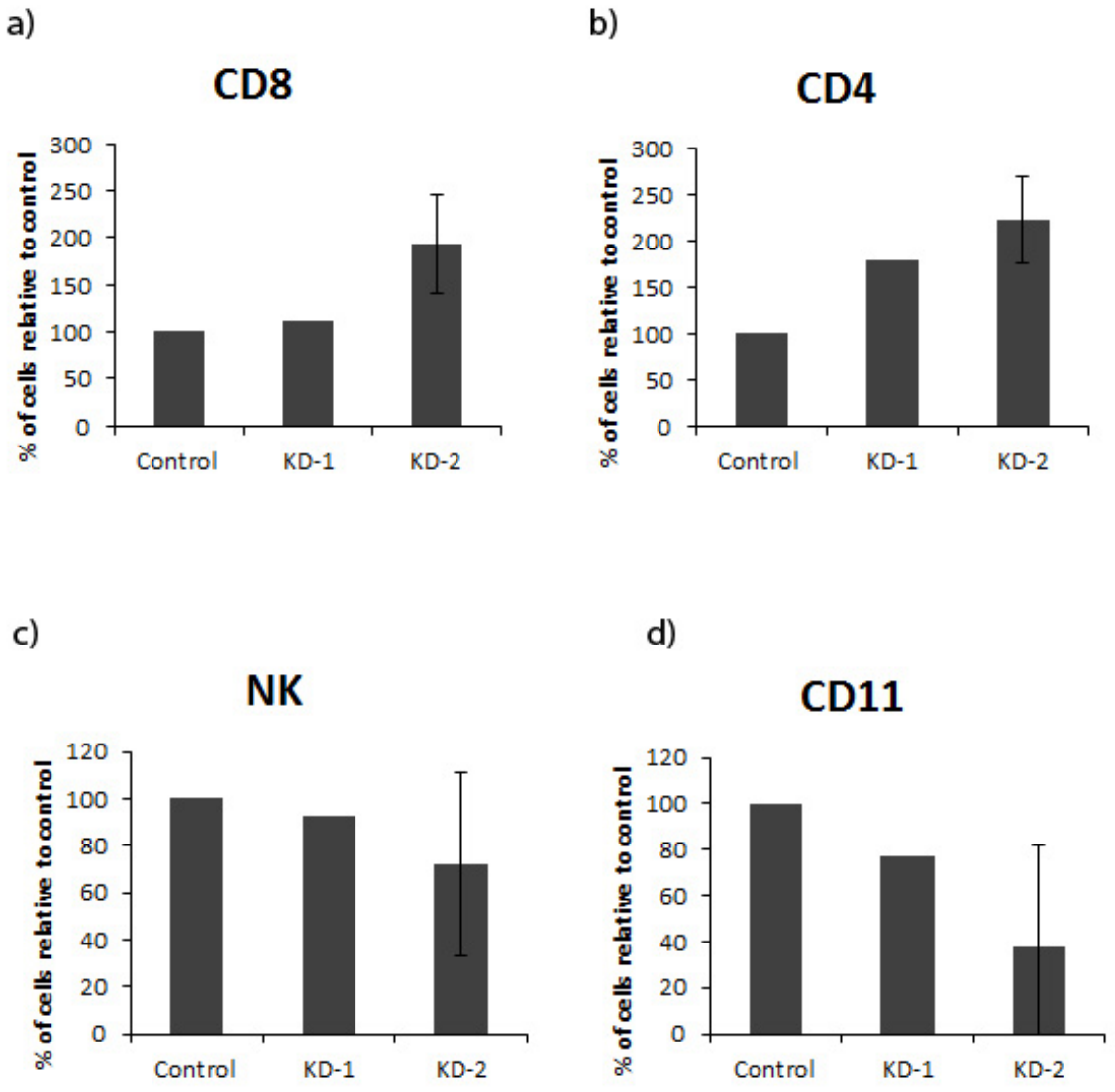


Figure 8: Immune Cells Infiltration into Bptf KD 67NR Tumors:

Number of immune cells infiltrated into the 67NR primary tumors (control, KD-1 and KD-2) developed in the mammary fat pad of BALB/c mice using immunofluorescence staining. Antibodies were used for **a)** CD8a for CTL cells, **b)** CD4 for T-helper cells, **c)** NKp46 for NK cells and **d)** CD11b for MDSCs. Counts were obtained as average cells count from 10 fields relative to the control ((cell counts for KD / Cell count for control) X

100). Only one tumor for KD-1 and 2 tumors for KD-2 are shown. Error bar in the KD-2 represent standard deviation for 2 tumors.

Figure 9)

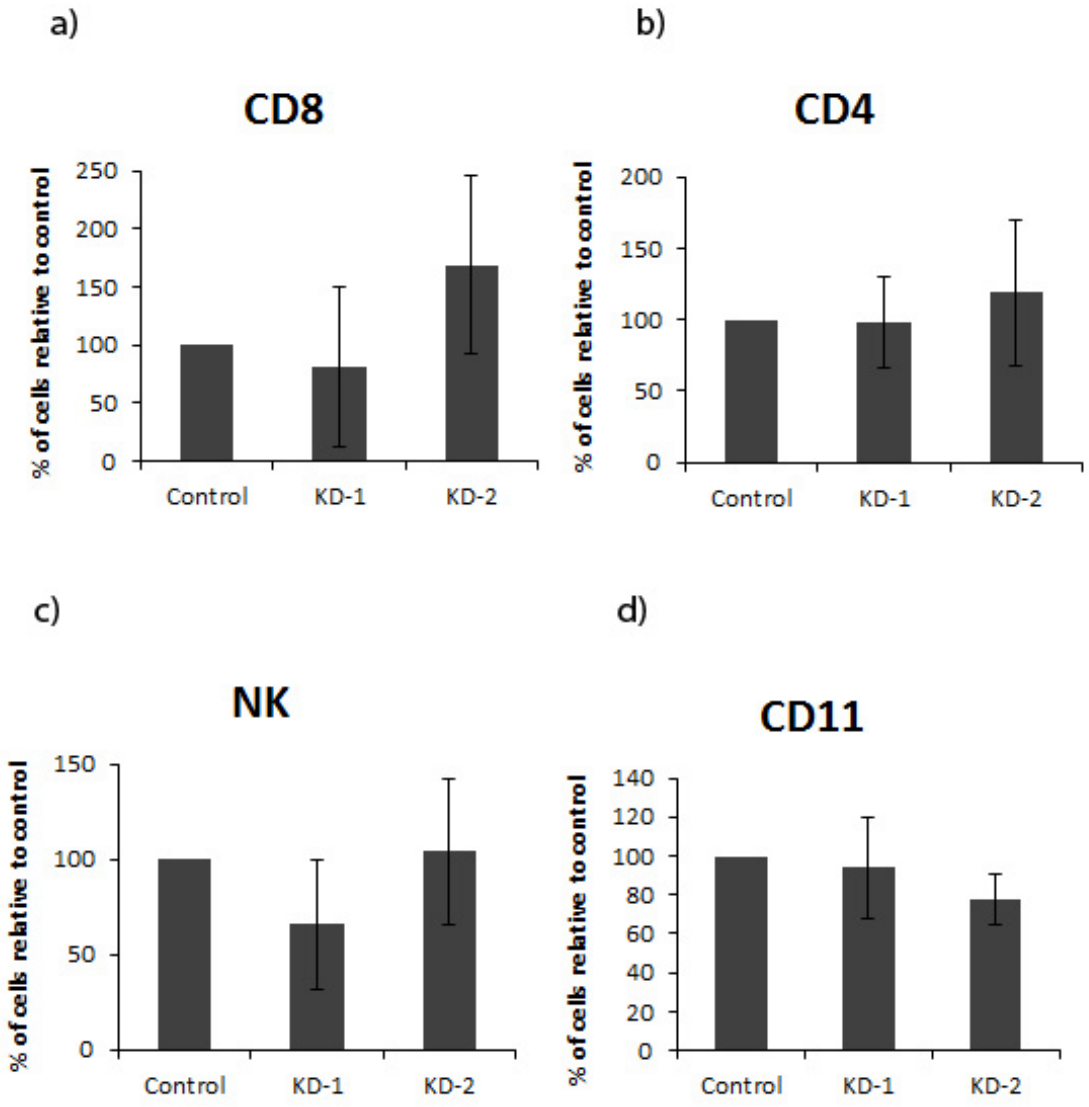


Figure 9: Normal Immune Cells Infiltration into Bptf KD 66cl4 Tumors:

Number of immune cells infiltrated into the 66cl4 primary tumors (control, KD-1 and KD-2) developed in the mammary fat pad of BALB/c mice using immunofluorescence staining. Antibodies were used for **a)** CD8a for CTL cells, **b)** CD4 for T-helper cells, **c)** NKp46 for NK cells and **d)** CD11b for MDSCs. Counts were obtained as average cells count from 10 fields relative to the control ((cell counts for KD / Cell count for control) X 100). Error bars represent standard deviation for 3 biological replicates for each group.

3.5- Microarray Data Show Overexpression of Genes Involved in Immune Response.

The significant reduction in tumors weight from the two different cell lines (67NR and 66cl4) in BALB/c mice after reducing NURF function suggested a role for NURF in regulating genes that has a role in tumor survival *in vivo*. In order to identify NURF dependent genes we measured gene expression profile of tumor tissues obtained from the BALB/c mice injected with the 67NR and 66cl4 breast cancer cell lines **(Figure 10).** Microarray analyses were performed in collaboration with Dr. Catherin Dumur at Virginia Commonwealth University. Tumor tissues were subjected to gross histological examination using hematoxylin and eosin (H&E) stain to determine the percentage of tumor cells. The tumor content of the tissues used for the arrays was 70% to 100% and necrotic tissues, if present, were removed by macrodissection. The following microarray data are preliminary; the 67NR data represent two tumors for the KD-2 and three tumors for the control, and the 66cl4 data represent two tumors for the KD-1, KD-2 and control. More tumors will be used to complete three biological replicates for the

control, KD-1 and KD-2. Unsupervised hierarchical cluster analyses based on 22,960 probe sets showed clustering for KD and control samples **(Figure 10 a, c data obtained from Dr. Dumur).** There were 88 probe sets in the 67NR tumors and 105 probe sets in 66cl4 tumors that showed significant deregulation between the control and KD (p-value $\leq$ 0.01, False Discovery Rate FDR $\leq$ 15%) **(Figure 10 b, d data obtained from Dr. Dumur).** A gene ontology analysis using the database for annotation, visualization and integrated discovery (DAVID) (Huang, et al., 2009) showed that the highest enrichment terms are associated with immune response in both cell lines 67NR **(Table2)** 66cl4 **(Table 3).** Genes from 67NR tumors include genes involved in antigen presentation on MHC-I molecules (TAP2 and Tapbp), MHC-I genes (H2-D1and H2-L) and MHC-II gene (H2-DMa) **(Table 4).** As the tumor tissues that were used for the microarray experiments might be infiltrated with immune cells, identifying Bptf dependent genes will need to be confirmed in cells grown *in vitro.* In the preliminary results of gene expression *in vitro,* we didn't observe overexpression of these genes in the two KD cell lines, which suggests that the observed overexpression of these genes in the array is due to active immune cells infiltrated into the tumor site **(Figure 11 a).** Indeed, enrichment of lymphocyte-associated genes among the overexpressed probes was observed **(Table2).** We detected down regulation of cyclin D1 isoform Ccnd1b gene which is also down regulated in Bptf KO ESCs and MEFs (Dr. Landry unpublished data).

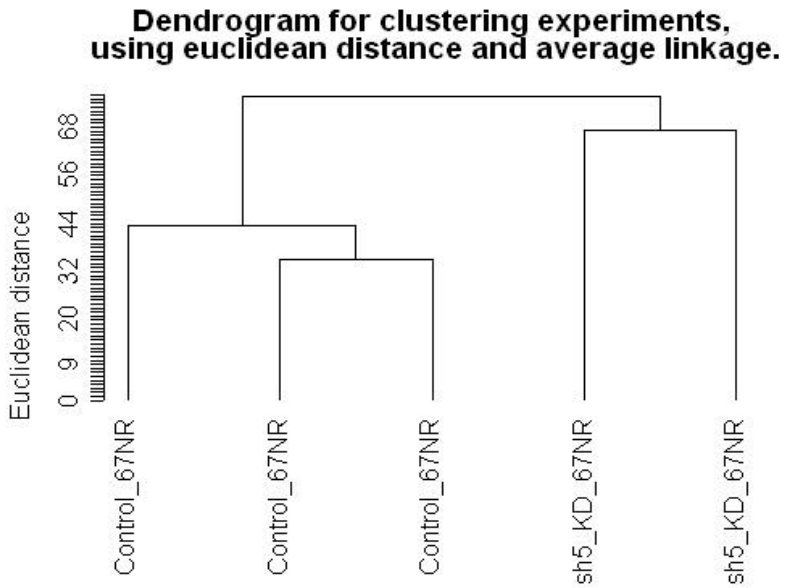
Genes from 66cl4 tumors include genes involved in antigens presentation on MHC-I (Psmb8, Psmb9 and Tapbpl), MHC-I gene (H2-K1), MHC-II gene (H2-DMb2) and chemokine genes (Cxcl16, Cxcl9 and Xcl1) **(Table 5).** We anticipated that the

enrichments of these genes is more likely due to active immune cells infiltrated into the tumor site, as the preliminary results of gene expression *in vitro* did not show overexpression of these genes except Cxcl16 **(Figure 11 b)**. This suggestion is supported by the enrichment of T-lymphocyte genes in the overexpressed genes from 66cl4 tumors **(Table 3).** Cxcl16, which is also overexpressed in the cell culture, is a chemokine that recognized by chemokine receptor Cxcr6, which is expressed on the surface of immune cells e.g. CD8+, CD4+ and NK lymphocytes (Deng, et al., 2010). One of the Cxcl16 roles is inducing chemotactic migration for cells that express Cxcr6.

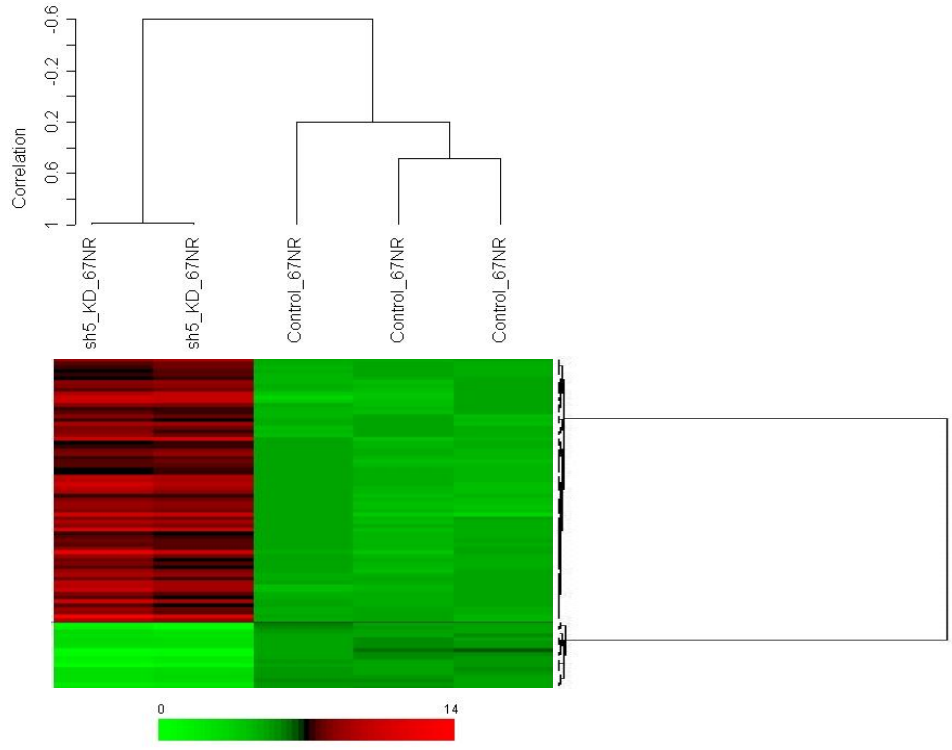
Although more tumors are going to be subjected to the microarray and qRT-PCR, the current data suggested that the immune system is involved in the phenotype observed in BALB/c mice. Complete microarray and *in vitro* gene expression data will be required to identify the potential Bptf-dependent candidate genes.

Figure 10)

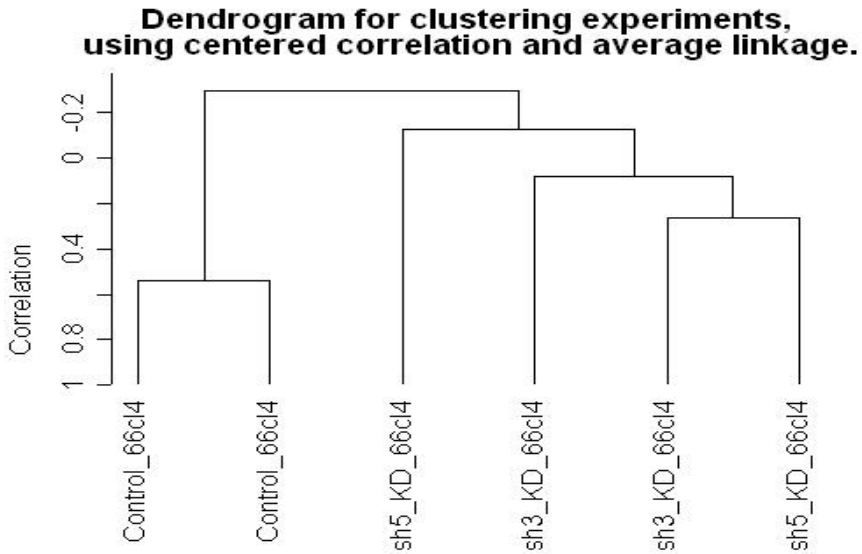
a)


Dendrogram for clustering experiments,
using euclidean distance and average linkage.
Euclidean distance
0 9 20 32 44 56 68
Control_67NR
Control_67NR
Control_67NR
sh5_KD_67NR
sh5_KD_67NR

b)


Correlation
1 0.6 0.2 -0.2 -0.6
sh5_KD_67NR
sh5_KD_67NR
Control_67NR
Control_67NR
Control_67NR
0
14

c)



d)

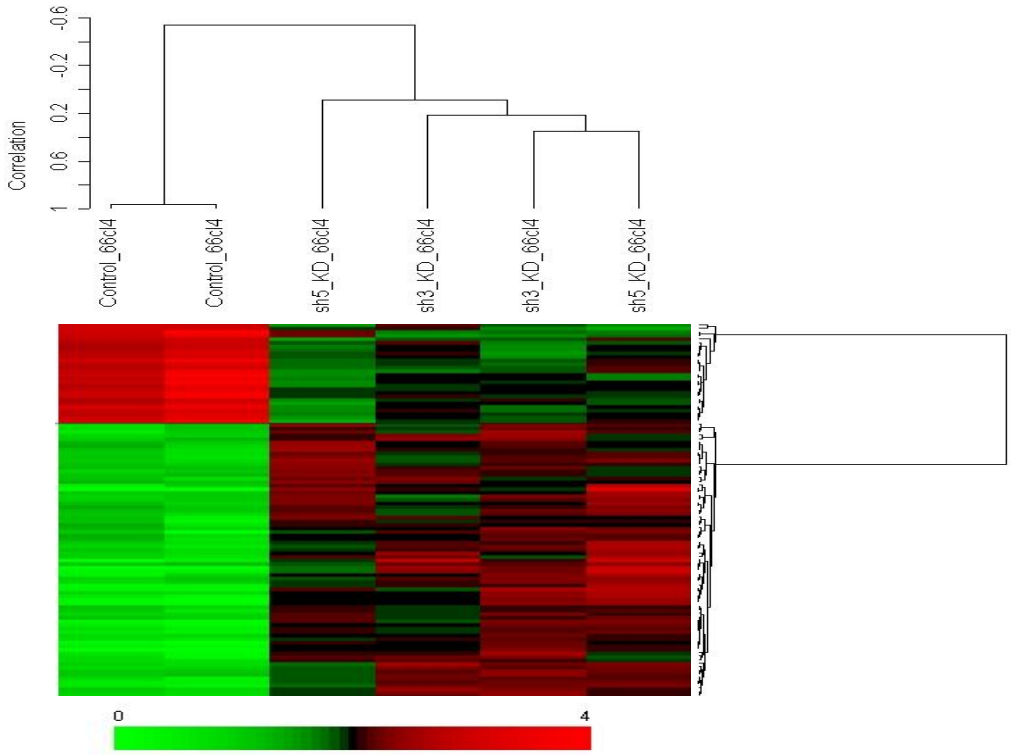


Figure 10: Microarray Analysis for 67NR and 66cl4 Tumors:

a) Unsupervised cluster analysis based on 22,960 probe sets for 67NR tumors using euclidean distance and average linkage. Three control tumors and two KD-2 (short hairpin #5) tumors were used.

b) Supervised cluster analysis for 67NR tumors based on 88 significant (p-value $\leq$ 0.01, False discovery date FDR $\leq$ 12%) probe sets that showed $\geq$ 2 fold change between the control and KD. Three control tumors and two KD-2 (short hairpin#5) tumors were used **C)** Unsupervised cluster analyses based on 22,960 probe sets using centered correlation and average linkage for 66cl4 tumors. Two tumors for the control, KD-1 (short hairpin #3) and KD-2 (short hairpin #5) were used.

d) Supervised cluster analysis for 66cl4 tumors based on 105 significant (p-value $\leq$ 0.01, False discovery date FDR $\leq$ 12%) probe sets that showed $\geq$ 2 fold change between the control and KD. probe sets using centered correlation and average linkage for 66CL4 tumors. Two tumors for the control, KD-1 (short hairpin #3) and KD-2 (short hairpin #5) were used.

Table 2)

	Term	Count	%	P-value	Benjamini
1	Antigen processing and presentation of exogenous antigen	6	9.5	5.60E-08	4.30E-05
2	Antigen processing and presentation of peptide antigen	6	9.5	1.80E-07	7.00E-05
3	Antigen processing and presentation	7	11.1	8.30E-07	2.10E-04
4	Antigen processing and presentation of exogenous peptide antigen	5	7.9	1.50E-06	2.80E-04
5	Immune response	11	17.5	8.10E-06	1.20E-03
6	T cell differentiation	6	9.5	9.20E-06	1.20E-03
7	Thymic T cell selection	4	6.3	2.20E-05	2.40E-03

Table 2: Enrichments of Terms with Immune Response Function in 67NR Tumors:

Gene ontology analysis using DAVID functional annotation chart tools that identify enriched annotation terms in a gene list. Genes list from 67NR tumors for genes that were overexpressed with 2 or more fold changes show the highest significant enrichments terms are associated with immune response.

Table 3)

	Term	Count	%	P-Value	Benjamini
1	Immune response	16	22.9	1.30E-11	6.60E-09
2	Regulation of immune effector process	6	8.6	1.00E-05	2.50E-03
3	Positive regulation of response to stimulus	7	10	3.20E-05	5.20E-03
4	Positive regulation of cytokine production	5	7.1	5.10E-05	6.30E-03
5	Positive regulation of immune system process	7	10	5.60E-05	5.60E-03
6	Regulation of lymphocyte mediated immunity	5	7.1	6.10E-05	5.10E-03
7	Regulation of leukocyte mediated immunity	5	7.1	8.20E-05	5.80E-03

Table 3: Enrichments of Terms with Immune Response Function in 66cl4 Tumors:

Gene ontology analysis using DAVID functional annotation chart tools that identify enriched annotation terms in a gene list. Genes list from 66cl4 tumors for genes that were overexpressed with 2 or more fold changes show the highest significant enrichments terms are associated with immune response.

Table 4)

	Gene Title	Gene Symbol	Fold Change (Geometric)	p-value (α-level: 0.01)	q-value (FDR≤12 %)
1	transporter 2, ATP-binding cassette	Tap2	**4.89**	2.73E-03	1.18E-01
2	histocompatibility 2, class II, locus DMa	H2-DMa	**4.12**	1.60E-03	1.12E-01
3	TAP binding protein	Tapbp	**3.27**	3.07E-04	1.12E-01
4	histocompatibility 2, D region locus 1	H2-D1	**2.09**	1.79E-03	1.12E-01
5	histocompatibility 2, D region	H2-L	**2.07**	6.79E-04	1.12E-01

Table 4: Genes from 67NR tumors:

Candidate genes from 67NR tumors that show significant change compared to control tumors (p-value α-level0.001 FDR $\leq$ 12%).

Table 5)

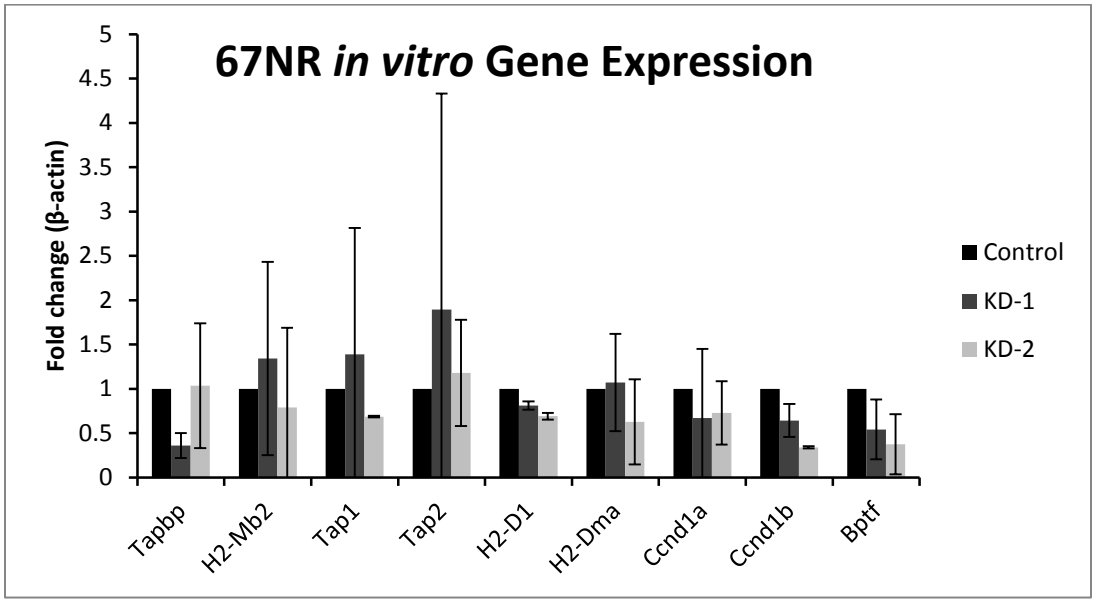
	Gene Title	Gene Symbol	Fold Change (Geometric)	p-value (α-level: 0.01)	q-value (FDR≤15 %)
1	proteasome (prosome, macropain) subunit, beta type 9	Psmb9	**2.4**	1.21E-04	1.21E-01
2	TAP binding protein-like	Tapbpl	**1.8**	7.32E-03	1.47E-01
3	histocompatibility 2, class II, locus Mb2	H2-DMb2	**1.6**	4.74E-03	1.47E-01
4	chemokine (C-X-C motif) ligand 16	Cxcl16	**2.4**	3.38E-03	1.47E-01
5	chemokine (C-X-C motif) ligand 9	Cxcl9	**5.1**	6.75E-03	1.47E-01
7	proteasome (prosome, macropain) subunit, beta type 8	Psmb8	**2.4**	2.38E-03	1.47E-01
8	histocompatibility 2, K1, K region	H2-K1	**1.7**	6.84E-03	1.47E-01
9	chemokine (C motif) ligand 1	Xcl1	**2.4**	1.95E-03	1.47E-01

Table 5: Genes from 66cl4 tumors:

Candidate genes from 66cl4 tumors that show significant change comparing to control tumors (p-value α-level0.001 FDR $\leq$ 15%)

Figure 11)

a)



b)

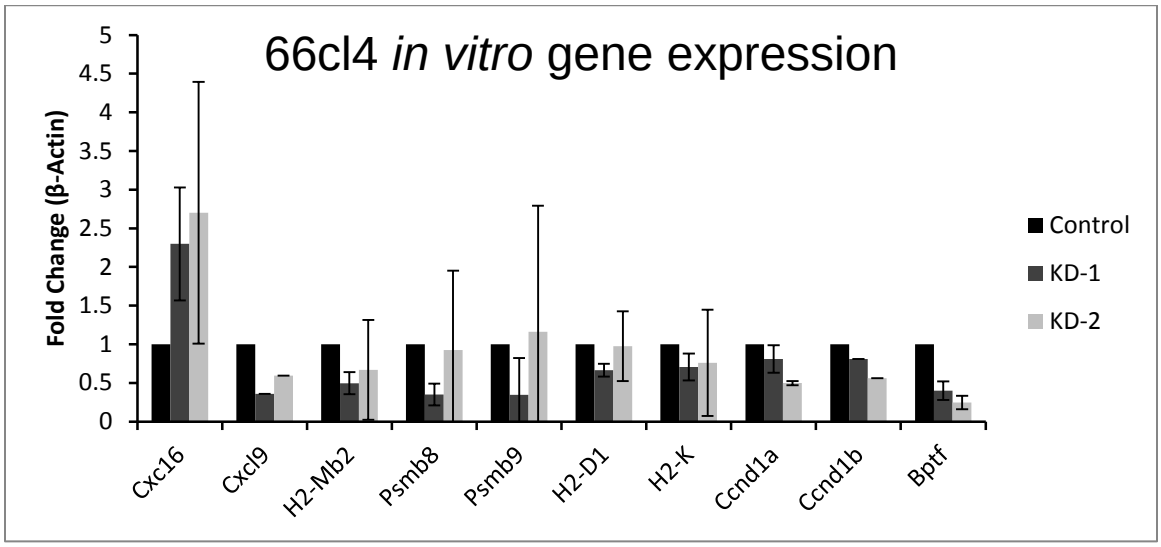


Figure 11: Q RT-PCR for 67NR and 66cl4 Breast Cancer Cell Lines:

a) Differential gene expression of Tapbp, H2-Mb2, Tap1, Tap2, H2-D1, H2-Dma, Ccnd1a, Ccnd1b and Bptf were analyzed in the 67NR breast cancer cell lines using qRT-PCR. β-actin was used as a housekeeping gene for normalization. Error bars represent standard deviation of two biological replicates.

b) Differential gene expression of Cxcl16, H2-Mb2, Psmb8, Psmb9, H2-D1, H2-K1, Cxcl9, Ccnd1a, Ccnd1b and Bptf were analyzed in the 666cl4 breast cancer cell lines using qRT-PCR. β-actin was used as a housekeeping gene for normalization. Error bars represent two biological replicates for Cxcl16, Psmb8, Psmb9, H2-D1, H2-K1, Ccnd1a and Bptf.

4- Discussion and Future Directions

4.1- Discussion.

Epigenetic alterations are important factors in tumorigenesis (Jones & Baylin, 2002). The reversible nature of epigenetic changes makes these types of aberrant alterations potential targets for cancer therapy. Extensive research in understanding DNA methylation and histone modifications provide broad understanding of these mechanisms, which led develop drug therapies targeting these mechanisms (Sharma, et al., 2010). Chromatin remodeling complexes are epigenetic regulators that affect gene expression by changing the chromatin structure (Clapier & Cairns, 2009). NURF is an ATP-chromatin remodeling complex that is essential for normal embryonic development through regulating important developmental pathways (Landry, et al., 2008). ESCs lacking NURF through KO of its unique subunit Bptf were unable to form teratomas in NOD/SCID mice (Landry, et al., 2008). Moreover, Bptf KO in ESCs, MEFs and DP thymocytes showed deregulation of genes involved in tumor progression e.g. MHC-I molecules and E- and N- cadherin genes (Landry, et al., 2008) (Landry, et al., 2011). These findings suggested that NURF might have an impact on tumor growth. Prior to this work, the role of mammalian NURF in tumorigenesis was unknown.

The subject of the current work is to study the role of NURF in tumorigenesis *in vivo.* We hypothesized that eliminating NURF function might reduce tumor growth *in vivo.* Our findings suggest that abolishing NURF function in tumor cells reduces the tumor growth in the presence of intact immune system.

We used a syngeneic breast cancer mouse model to study the impact of eliminating NURF on tumor growth. Using shRNA technology we generated two stable KD breast cancer cell lines with two different shRNA targeting NURF essential subunit Bptf. In order to investigate the impact of eliminating NURF in the tumor growth *in vivo*, we injected the Bptf KD breast cancer cell lines into the mammary fat pad of BALB/c mice. After three weeks of injection of the 67NR cells, we found significant reduction in tumor growth in mice injected with KD cell lines comparing to mice injected with control cells. While 12 out of 13 mice injected with the control cells formed tumors, only 3 out of 13 and 6 out 16 mice injected with KD-1 and KD-2 cells, respectively, developed tumors. A similar reduction in tumor size but not frequency was observed with the 66cl4 breast cancer cells (work done by S. Alkhatib data not shown). Reduction in primary tumor growth in BALB/c derived 4T1 breast cancer mouse model have been observed in number of study that target genes involved in tumor survival (Nasrazadani & Lynn Van Den Berg, 2011) (Hong, et al., 2009) . One study showed significant reduction in mammary tumor growth in mice injected with 4T1breast cancer cells stably express shRNA targeting IL-17 receptor (Nam, et al., 2008). These tumor cells were less response to IL-17 that is secreted by immune cells such as CD8+ T-cells which acts as survival signal for tumor cells.

Next, it was important to determine if the observed reduction in tumor growth is due to a change in growth capacity *in vitro* following Bptf KD. By measuring the population doubling time for the breast cancer cell lines *in vitro*, we found that KD of Bptf does not affect the growth capacity of the tumor cells. The obtained population

doubling time in our experiment is similar to previously published results (Eckhardt, et al., 2005). This suggests that the observed reduction in tumor growth is due to an effect that the tumor cells encounter *in vivo*.

One important step during tumor growth is the ability of the transformed cells to avoid the antitumor response mediated by the host immune system (Dunn, et al., 2004) (Hanahan & Weinnerg, 2011). As previously observed *in vitro* that Bptf deregulates MHC-I and MHC-II genes (Landry, et al., 2008), which are important for proper immune response. Deregulation of MHC-I and MHC-II genes are observed in number of tumors such as breast cancer, prostate cancer and melanoma (Campoli & Ferrone, 2008). We hypothesized that the immune system might preferentially target the Bptf KD tumors cells. To test this, we used NSG mouse model which has loss of functional innate and adaptive immune system. Bptf KD-1, KD-2 and control 67NR breast cancer cell lines were injected into the mammary fat pad of these mice with the same number of cells that were injected in the BALB/c mice. After three weeks of injection, all the mice injected with the KD-1, KD-2 and control cells developed tumors. The obtained tumor weights showed no significance difference between the KDs and control tumors. Similar finding was obtained using the 66cl4 cells (work done by S. Alkhatib data not shown).

Our finding that showed reduction in tumor growth in BALB/c mice but not in immunodeficient mice in agreement with previously published results that showed a significant reduction in primary tumor growth in BALB/c mice but not in immunodeficient mice after injection with 4T1 breast cancer cells lacking indolamine 2,3-dioxgenase that promote immune escape capability of the transformed cells. In this study, KD of

indolamine 2,3-dioxgenase (IDO1) which is an enzyme responsible for tryptophan catabolism in 4T1 cells showed reduction in tumor growth after injection of these cells into the syngeneic BALB/c mice but not in mice lacking functional immune system (Levina, et al., 2012). Over expression of IDO1 in tumor microenvironment is known to promote tumor cells to escape the antitumor immune response likely by inhibiting T-cells activity (Prendergast, 2008). This suggests a similar role of NURF in transformed cells, in which Bptf KD in tumor cells; promote active antitumor immune response against these cells.

The antitumor response of the immune system is mediated mainly through the cellular components of the innate and the adaptive immune system like NK, CTLs and T-helper cells. While the antitumor role and the favorable outcome are associated with lymphocytes infiltration into the tumor microenvironment (Naito, et al., 1998), MDSCs are known to oppose the immune response and act as immunosuppressive cells (Gabrilovich & Nagaraj, 2009). We screened for CTLs, T-helper, NK and MDSCs cells infiltration into the tumor tissues isolated form the BALB/c mice injected with 67NR and 66cl4 breast cancer cell lines using immunofluorescence staining with antibodies for CD8a (CTLs), CD4 (T-helper cells), NKp46 (NK cells) and CD11b (MDSCs). As not all the mice injected with the 67NR KD cells form tumors, we only subjected one tumor for KD-1 and two tumors for KD-2 for the staining. We observed a relative increase in CD8a and CD4 and decrease in CD11b from KD-2 tumors. However, more tumors need to be used in order to confirm the significance of any observed findings. From mice injected with the 66cl4 cells, we did not observe a significant difference between the control and KD tumors in the CD8a, CD4, NKp46 and CD11b cells. Although no

significant increase in the immune cells infiltration into KD tumor microenvironments, there might be an increase in the cells efficiency or activity in or decrease in regulatory cells that have immunosuppressive role, which can't be distinguish by using single antibody for each cell type (Rosenberg, 2001) (Zitvogel, et al., 2006). In order to differentiate between the effector T-cells and regulatory T-cell populations infiltrated into the tumor sites, fluorescence activated cell sorting (FACS) analysis needs to be performed using specific markers for each cell type such as Fox3+ that distinguish regulatory T-cells from effector T-cells (Bui, et al., 2006).

To identify NURF dependent genes in tumor cells, we subjected the tumor tissues isolated from BALB/c mice to microarray analysis. From our preliminary microarray data we observed enrichment of genes involved in immune response pathways. Among the overexpressed genes from the 66cl4 KD tumors, there is enrichment of lymphocyte-associated genes, which indicates infiltration of active immune cells into the KD tumors. The observed overexpression of MHC-I, MHC-II and APM (Psmb7and Psmb8) genes in the array is more likely due to immune cells infiltration since the qRT-PCR experiment showed down regulation of these genes in the tumor cells *in vitro*. Another group of genes that is overexpressed in 66cl4 tumors is a set of chemokines (Cxcl16, Xcl1 and Cxcl9) which induce the migration of the immune cells. Cxcl16 is of particular interest since it is also overexpressed in 66cl4 cell lines *in vitro,* and it has a role in immune cells infiltration (Hojo, et al., 2007). A colorectal cancer study showed an association between Cxcl16 expression in tumor cells and CD4+ and CD8+ T-lymphocyte infiltration (Hojo, et al., 2007). In this study it has been shown that overexpression of Cxcl16 also associated with favorable

prognosis. Xcl1 is another chemokine that induce infiltration of CD8+ dendritic cells in mouse and CD141+dendritic cells in human which are specialized in antigen presentation to CTLs and augment CTL cytotoxic activity (Lei & Takahama, 2012). A study in human breast cancer cell lines showed overexpression of XCL1 following treatment with DNA methylation and histone acetylation inhibitors (Keen, et al., 2004). In order to confirm whether Xcl1 expression is Bptf dependent, the expression will be tested using 66cl4 cell lines grown *in vitro*. Cxcl9 is a chemokine that induce attraction for immune cells, and it has antitumor immune response (Walser, et al., 2007). The preliminary result from *in vitro* gene expression for one replicate showed down regulation of Cxcl9. More samples need to be tested to confirm whether Cxcl9 is Bptf-dependent or not.

Data from the 67NR only represent 2 tumors for the KD-2 and 3 tumors for the control. We also, observed enrichment of genes involved in the immune response in the KD tumors. The observed overexpression of MHC-I (H2-D1), MHC-II (H2-Dma) and APM (Tapbp and TAP2) in the microarray but not in cells growing *in vitro* more likely was due to immune cells infiltration. We detected down regulation of cyclin D1 b isoform *in vitro*. This in agreement with a previous finding that showed cyclin D1 down regulation in Bptf KO ESCs, MEFs (Dr. Landry unpublished data). Along with its classical role as a cell cycle regulator, cyclin D1 plays a role as a regulator of gene transcription through its interaction with transcription factors as well as HATs and HDACs coactivators (Velasco-Velazquez, et al., 2011). We did not observe a reduction in cell growth *in vitro* and tumor growth in NSG mice, which indicates that reduction of cyclin D1 does not affect the cellular proliferation in Bptf KD cells. Knockout of cyclinD1

promote MEF differentiation to adipocyte, which indicates that cyclinD1 involved regulation of genes control this differentiation (Fu, et al., 2005). Down regulation of cyclin D1 in the 67NR might altered expression of genes that enhance the tumor cell immunogenicity e.g. overexpression of genes that might serve as tumor associated antigens and enhance the tumor cell recognition by the immune cells. Currently we don't have complete data from KD-1 and KD-2 67NR tumors. We expect that more tumors will help in identifying set of genes, such as chemokines, that might account for the observed phenotype.

The microarray experiment is currently ongoing and we anticipate that complete microarray and qRT-PCR data will provide us with a better set of potential NURF-dependent candidate genes that account for the observed phenotype in tumor growth.

In conclusion, our findings that KD Bptf reduces tumor growth *in vivo* with an intact immune system, but not *in vitro,* and that reduction is retained in immunedepleted mice support our hypothesis that eliminating NURF function in tumor cells reduces the tumor growth *in vivo* likely through an increased active antitumor immune response. At present time we don't have complete data from the microarray and qRTpPCR to identify the Bptf dependent genes that account for the observed phenotype. However, enrichment of genes associated with immune response supports the role of the immune system in the observed phenotype.

4.2- Future Directions.

The ultimate goal for studying roles of NURF in tumorigenesis is to provide complete understanding of how NURF might be involved in tumor growth. Four

questions need to be addressed in order to achieve this goal. **First**, what component of the immune system is active against tumor cells lacking Bptf? **Second**, what are the NURF dependent genes that underlie the observed phenotype? **Third**, is the observed phenotype specific for the breast cancer models or similar finding can be obtained in other solid tumor models? **Fourth**, does the observed phenotype also occur in humans?.

As the data suggests that eliminating NURF in tumor cells promotes the antitumor immune response, it is important to determine what component of the immune system is involved. The two important main cells that mediate the tumor cytotoxicity are NK and CTL cells (Russell & Ley, 2002). To answer this question investigation of the NK and CTL activity against Bptf KD tumor cells *in vitro* will be performed. Next, the *in vitro* study will be followed by *in vivo* study using animal model lacking the immune effector cell population either by genetic modification or antibody treatment.

Complete data from the microarray will provide a set of candidate genes that are NURF dependent. Molecular analysis will be performed to identify the role of NURF in regulating these genes. DNase I-hypersensitivity analysis and chromatin immuneprecipitation (ChIP) assay will help to determine whether NURF directly regulates these genes.

It is important to determine whether the role of NURF in tumor growth is not limited to the breast cancer model that is used in this work. Towered this end, studying the effect of NURF in other solid tumor models e.g. melanoma, will be essential.

Finally, many promising gene targets using mouse models don't translate to human therapies. It is important to know if NURF dependent pathways and genes identify in the mouse model also apply to human.

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