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Role of NF-*k*B Signaling in Multiple Myeloma

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Role of NF-κB Signaling in Multiple Myeloma

by

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Certificate of Originality

To Whomever it may concern

This is to certify that the thesis entitled "**Role of NF-κB Signaling In Multiple Myeloma**" submitted by Ms. Rachel Pallapati embodies original work done by her under the guidance and supervision of Dr.SivaKumar Vallabhapurapu, Department of Cancer and Cell Biology, University of Cincinnati, Ohio.

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DEDICATION

To my Mom and Dad, who were and are my first teachers; my amazing sister; and my encouraging brother.

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ABSTRACT

Genomic DNA was purified from Multiple Myeloma Cell Line JJN3, and BMF promoter region was PCR amplified and cloned into pCR 2.1 TOPO vector using One Shot® Chemically Competent E. coli. After confirmation of the clone through polymerase chain reaction (PCR) and restriction analysis, BMF promoter (2.5 Kb) was released from TOPO vector and subcloned into pGL3 promoter vector. The resulting pGL3-BMF-promoter vector was further amplified for MaxiPrep preparation. This plasmid can be used to study the regulation of BMF promoter by various transcription factors including NF-κB family members.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter I - Introduction

What is Cancer?

The living body is made up of millions of cells which, when normal, grow, divide, and die. In a young person, the cells normally divide faster, allowing growth to take place, whereas in an adult, the cells divide only to replace dying cells or to repair injuries. When these normal cells do not divide in a controlled fashion, cancer develops with an uncontrolled growth of cells. Unlike the normal cells, cancerous cells grow and give rise to new and abnormal cells. Cancer cells also have the potential to grow and metastasize to new tissues. These abnormalities are characteristics features of cancer cells. The fundamentally important material of the living cells is DNA, which controls the functionality of cells. Mutations, deletions, or damage in DNA can cause malignant transformation of cells, causing cancer. These cancer cells form a mass, which is called a tumor. While many cancers such as lung, liver, breast, gastric, kidney and brain cancers form solid tumors, cancers relating to blood, such as leukemia, do not often form solid tumors (American Cancer Society, 2011).

What is Multiple myeloma?

Multiple myeloma is a cancer that develops due to malignant transformation of antibody-producing plasma cells. Multiple myeloma is also known as plasma cell myeloma, plasmacytic myeloma, and myelomatosis and Kahler disease. Multiple myeloma is a neoplastic disorder distinguished by the multiplication of a clone of plasma cells that are derived from the B (B lymphocytes) cells (Kyle, 2003). Plasma cells form a malignant neoplasm, which accumulates in bone marrow, causing the destruction of the bone and bone marrow failure, leading to multiple myeloma, also known as bone marrow

cancer (Alsina & Bensinger, 2011). It is a post-germinal center tumor formed by extensive somatic hyper mutation and antigen selection (Demchenko & Kuehl, May 2010). Afro-Americans and males have a higher incidence of the disease, whose causes are unknown (Kyle, 2003; Angtuaco, 2004). It accounts for over 10% of the hematological and about 1% of the other types of malignancies. In 1873, J von Rustizky introduced the term *multiple myeloma* (Kyle, 2003).

The B and T cells play an important role in fighting diseases and infections. When the B cells counter antigens causing infection, they mature into plasma cells within the germinal center (GC). These plasma cells then start producing antibodies or immunoglobulins to fight the infection. The long lived plasma cells migrate to the bone marrow, and within the bone marrow microenvironment plasma cells receive the survival signals to live for several days and keep producing antibodies. Under normal circumstances the plasma cells eventually die due to programmed apoptosis. However, due to a range of different mutations, plasma cells undergo malignant transformation and become resistant to apoptosis, leading to the development of multiple myeloma. The condition of myeloma begins with the plasma cells being abnormal; they then divide and make many copies, producing more and more abnormal cells. These abnormal cells are termed *myeloma* cells. The myeloma cells make the antibodies called M-proteins. Upon growing uncontrollably, the myeloma cells start to leave the bone marrow and form the tumor elsewhere by the process of metastasis. Moreover, by producing factors such as Receptor Activated Nuclear Factor of Kappa-B ligand (RANK-L), myeloma cells lead to bone lesions by promoting extensive osteoclastogenesis. At the late stage of the disease,

myeloma cells may even cause lesions in the skull, leading to the death of the patient (National Cancer Institute of the NIH, 2008).

In Multiple myeloma, the abnormal growth of the plasma cells in the bone marrow leads to low count in blood cells causing anemia (loss of red blood cells), leading to fatigue and weakness. It also causes the lowering of the number of platelets, causing thrombocytopenia, which leads to increased bleeding. Leukopenia is also caused, which is a result of the lowering of white blood cells, which fight infections. Myeloma cells also obstruct the activity of the bone-forming and bone-destroying cells called osteoblasts and osteoclasts, respectively, and send signals by producing a substance to the osteoclasts to speed up the process of dissolving the bone. The osteoblasts do not receive a signal regarding formation of a new bone, which further leads to breaking of the older bone before a new one is substituted. In this diseased condition, the myeloma cells outnumber the normal plasma cells, so there will be no more antibodies made to fight infections (American Cancer Society, 2011).

Currently multiple myeloma is considered an incurable disease. Although drugs such as Velcade® (Bortezomib) are currently in the clinic for the treatment of multiple myeloma, resistance to Velcade has been often reported, presumably due to the inefficiency of Velcade against the multiple myeloma progenitor cells. For all tumors it is well known that the tumor progenitor cells are the key cell types that form the tumors, and to completely eradicate the tumors, it is essential to target these progenitor cells and induce apoptosis in these cells. Therefore, it is essential to identify novel molecular targets which are absolutely essential for the survival and tumor formation by the Multiple myeloma tumor progenitor cells (Field-Smith, 2006).

What is MGUS?

The antibodies made by the myeloma cells are less efficient in fighting infections as they are copies produced by one plasma cell. The condition is called *monoclonal gammopathy*. The condition of monoclonal gammopathy of undetermined significance (MGUS) may develop into multiple myeloma or some other related diseases. The production of excess amounts of a particular type of gamma globulins monoclonal leads to monoclonal gammopathy of undetermined significance (MGUS). It does not affect a person's health but may eventually lead to amyloidosis and multiple myeloma. Some of the recent studies on genes of plasma cells of patients with MGUS revealed that these plasma cells have a genetic make-up that is much more similar to the myeloma plasma cells than the normal plasma cells, which suggests that these cells are malignant and divide rapidly. Compared to multiple myeloma, MGUS represents 10% or less of the mononuclear cells in the bone marrow, where the tumor cells continue to be stable but for a Progression to malignant multiple myeloma (American Cancer Society, 2011).

Risk Factors for Multiple myeloma

Changes in a person that increase the chances of acquiring a disease are termed *risk factors*. The risk of multiple myeloma increases with age and is more likely to develop in men than in women. It is more common among African Americans. Exposure to any amounts of radiations and workplace related exposures may also increase the chances of the disease. Plasma cell diseases like MGUS and Solitary Plasmacytoma will eventually develop multiple myeloma (American Cancer Society, 2012).

How is Multiple myeloma developed?

The causes of most cases of multiple myeloma are still unknown. In spite of this, scientists have come to a point where they are able to recognize the specific changes in DNA that can cause the normal plasma cells to become abnormal cancer cells. Genes present in DNA that enhance cell division are potential oncogenes, and genes that reduce the process of cell division are called tumor suppressor genes. The disease can be caused by mutations in DNA that signal the oncogenes (like c-myc, N- ras, and K-ras) and the tumor suppressor genes (like p53) to turn on and off, respectively. In a normal human, 46 chromosomes that control cell growth and other metabolic functions are present. However, in myeloma cells, some parts of chromosome 13 are missing; these deletions enable myeloma to be more antagonistic and resistant to any kind of treatment. Most myeloma patients have irregularly trans-located chromosomes, chromosomes with parts from other chromosomes (About Us: Multiple Myeloma Research Foundation, 2011).

Recent studies revealed that the plasma cell tumors develop more when there is an excess production of the cytokine Interleukin-6 (IL-6) by certain cells in the bone marrow called dendritic cells, which stimulate the growth of normal plasma cells (American Cancer Society, 2011; About Us: Multiple Myeloma Research Foundation, 2011).

Genetic abnormalities of multiple Myeloma

Genetic abnormalities have shown to considerably impact most of the hematological malignancies. Recent research studies showed that several genetic factors have an influence in the development of multiple myeloma. Chromosomal aberrations like difference in structure and number constitute the genetic factors. Also, with the advancement of technology in mapping the human genome, scientists have discovered

various genes that are associated with early relapse of the disease (National Cancer Institute of the NIH, 2008).

Chromosomal abnormalities of Multiple myeloma

Chromosomal abnormalities have shown to play an important role in most of the hematologic malignancies. Recent studies by Debes-Marun et al. on the abnormal karyotypes of myeloma revealed that regular patterns of chromosomal changes were identified which classify the cytogenetics of the disease. It was also denoted that the most frequent chromosomal abnormalities are del(13) and 14q32, which are interconnected. The stage of the disease increases with the increase in the translocations involving the immunoglobulin heavy chain, IgH, which is normally 60-65% in intramedullary multiple myeloma and 70-80% in extramedullary multiple myeloma.

The four major chromosomal partners involved in this translocation are: 11q13 (cyclin D1), 6p21 (cyclin D3), 4p13 (FGFR3 and MMSET) (Malgeri, 2000) and 16q23 (c-maf) (Bergsagel, 2001). A novel translocation was recently identified which is the t(4;14), (p16.3;q32), which causes the deregulation of FGFR3 and MMSET genes (Malgeri, 2000).

What is NF-κB?

Nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB, was first discovered by Ranjan Sen and David Baltimore (Gilmore, 1986; Singh, 1986; Sen, R 1986), with the vital roles in the regulation of apoptosis, cell growth, and regulation of innate immunity and inflammatory responses (Zhuo, 2008). NF-κB also participates in the development and the activity of the central nervous system (Memet, 2006).

The NF-κB Family

All of the NF-κB family proteins have a Rel Homology Domain (RHD) in their N- terminus. The NF-κB super family of transcription factors in mammals includes the following five transcription factors: p50 (NF-κB1/p105), p52 (NF-κB2/9100), RelA (p65), RelB and c-Rel. The RHD region is required for DNA-binding, dimerization, and interactions with the inhibitory κB proteins (IκBs) (TD Gilmore, 2006; Hoffmann A., 2006; Moynagh, 2005) and also contains the nuclear localization sequence. NF-κB is a dimer of members of the Rel family of proteins (Grilli M, 1993; Kopp E, 1995). The first discovered NF-κB molecule was a heterodimer with the sub-units, p50 and p65 (Kopp, E., 1995; Verma, I.M., 1995). C-terminal transcriptional activation domains (TADs; Figure 1) exist in RelA, c-Rel, and RelB, enabling the activation of targeted gene expression (Karin, M., 2009). On the contrary, p50 and p52 lack the C-terminal TADs that enable those homodimers to repress transcription unless they bind to a protein containing a TAD, such as RelA, c-Rel, or RelB or Bcl-3. The p50 and p52 family members are derived from large precursors, namely p105 and p100, respectively. The transcriptional activity of NF-κB is silenced by the interactions with inhibitory IκB proteins present in the cytoplasm (Hooper, 2010).

A variety of homo- and heterodimers are formed by the sub-units p50, p52, c-Rel, RelA (p65), and RelB, in the mammalian NF-κB transcription complexes. The complexes bind to the κB-sites, which are the 9-10 base pair DNA regulatory sites to activate the specific target gene expression (Gilmore, 2006). The target gene specificity of these dimers is believed to have arisen from a number of factors: the specific NF-κB complexes in various cell types, the different protein-protein interactions, and posttranslational

modifications that NF-κB complexes undergo in different circumstances (Hoffmann, 2006; Perkins, 2006).

Inhibitory NF-κB proteins

To date, there are seven known IκB (Inhibitors of NF-κB) molecules identified: IκBα, IκBβ, Bcl-3, IκBε, IκBϒ, and the precursor proteins, p100 and p105. The two precursor proteins are distinguished by the presence of ankyrin repeats, which contain multiple copies of a 30-33 amino acid sequence. (Hooper, 2010) These ankyrin repeats interact with a region in the RHD of the NF-κB proteins. The IκB molecules have different affinities for individual NF-κB dimers. All the IκBs are similar in structure (Figure 2), but each has its own binding preferences and depends on the transcriptional regulation by NF-κB family members (Karin, 2009). The inhibitory IκB proteins regulate the activity of the NF-κB by interacting with them. Each of the IκBs is regulated differently by phosphorylation and proteolysis, and they show differences in their tissue-specific expression patterns. From the structural views (Ghosh, 1999), it is also known that IκBα makes multiple contacts with NF-κB (Gilmore, 2006).

Phosphorylation of IκBs results in their ubiquitination and subsequent degradation by the multicatalytic ATP-dependent 26S proteasome complex (Maniatis, 1999). Regulation of the NF-κB pathway is brought about by multiple post-translational modifications that control the activity of the core components of NF-κB signaling: the IκB kinase (IKK) complex. (Perkins, 2006)

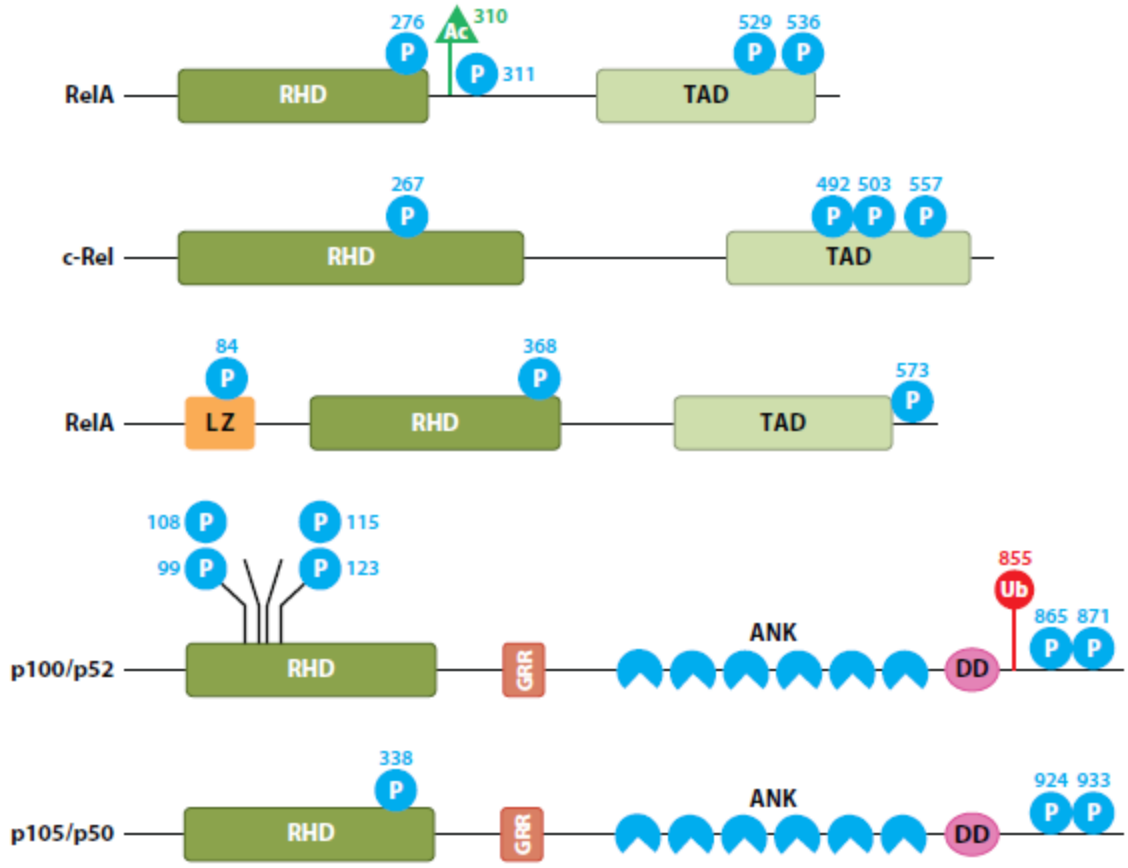

Figure 1. The NF-κB family - Diagram for different NF-κB members contains domains including ankyrin repeats (ANK), glycine-rich region (GRR), leucine zipper motif (LZ), Rel homology domain (RHD) and transcription activation domain (TAD). Phosphorylation (P), acetylation (Ac), and ubiquitination (Ub) are the Post-translational modifications. (Karin, 2009)

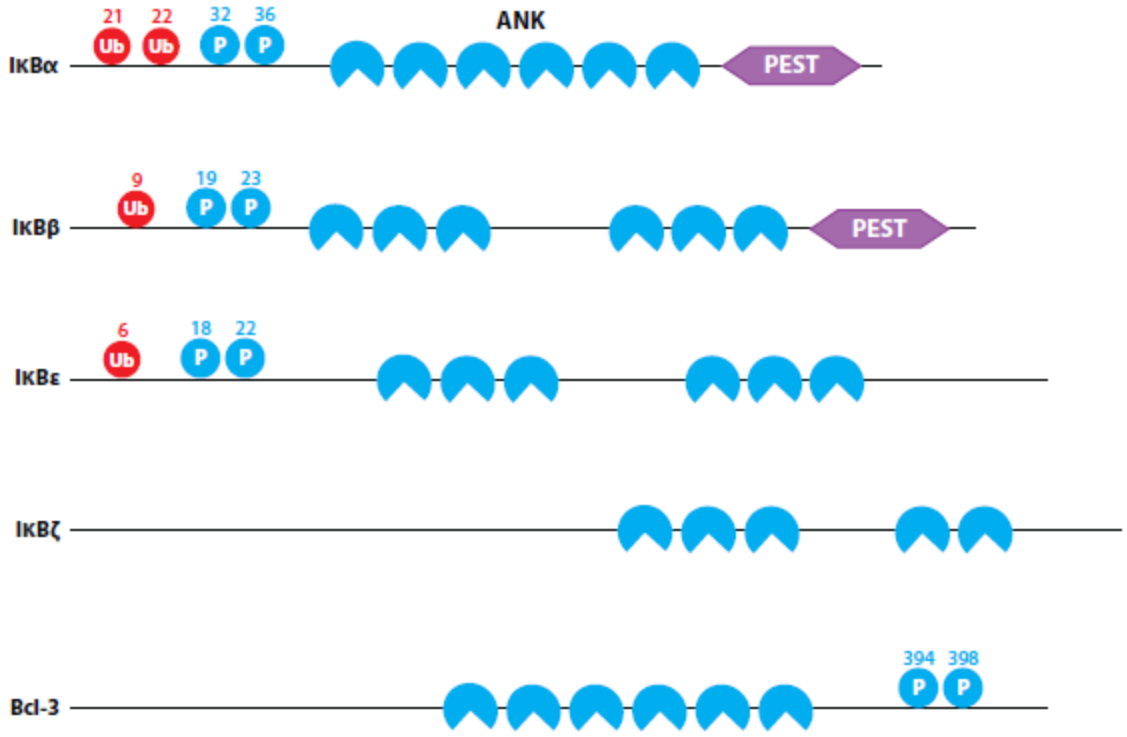

Figure 2. The IκB family diagram showing the IκB proteins with several ankyrin repeats (ANK) and Phosphorylation and ubiquitination at specific amino acid residues as indicated. The PEST domains are indicated: Proline (P), glutamic acid (E), serine (S), and threonine (T). (Karin, 2009)

In addition to the classical IκB family of proteins, the novel IκB-like proteins, BCL3 (B cell CLL/ lymphoma 3), IκBζ, and IκBNS, have a different type of regulation and serve different functions. BCL3 has been associated with cancer development, as it has the ability to regulate NF-κB activity, where it functions as a critical regulator of cyclin D1 transcription. IκBζ was recently identified with a weak homology to other family members. It associates with the p50 homodimers and may function in a similar manner to BCL3. IκBNS is also a unique IκB-like protein that is rapidly produced upon ligation of the T cell receptor (TCR) in thymocytes and plays an important role in negative selection of the T-cells (Rabek JP, 1998).

The IκB Kinase Complex

The Inhibitors of NF-κB Kinase (IKK) complex consist of the sub-units IKKα, IKKβ, and IKKϒ, which is also called *NEMO, NF-κB essential modulator*. The IKK activation occurs upon activation of different types of signaling receptors, including the receptors of the TNFR family of receptors. The activated IKK complex phosphorylates the IκBα leading to polyubiquitination. The ubiqutinated IκBα is degraded by the 26S proteasome while exposing a nuclear localization signal on RelA and induces the nuclear translocation of the dimmers, RelA: p50. Among the three IKK subunits, IKKβ and IKKα are the catalytic subunits whereas IKKϒ is the regulatory subunit. Among IKKα and IKKβ, the latter is the major kinase for phosphorylating IκB-alpha, while in its absence, IKKα provides residual kinase activity towards IκB-alpha. Also, deletion of IKKα in the cells that express IKKβ has no effect on the classical IKK activity. An important and unique function of the IKKα is the activation of the alternative NF-κB pathway (Karin, 2009).

The NF-κB Signaling Pathways

In the cytoplasm, NF-κB is present as a latent, inactive, IκB-bound complex. Two distinct pathways are known to activate the NF-κB. These two main pathways are called the canonical and non-canonical pathways. They are also termed the classical (p50-RelA) and alternative pathways (p52-RelB), respectively. While the classical pathway relies on the IKKα-dependent phosphorylation and degradation of IκB-alpha, the alternative pathway depends on NF-κB inducing kinase (NIK) and IKKα dependent phosphorylation and processing of p100 to p52 and subsequent nuclear translocation of p52-RelB heterodimers. Once in the nucleus, classical p50-RelA heterodimers or the

alternative RelB-p52 dimers regulate the expression of respective target genes (Karin, 2009).

Classical/Canonical Pathway

The Classical Pathway is the well-studied NF-κB activation pathway and is activated by most stimuli, which mainly depends on the RelA: p50 heterodimers. The basis of this pathway is the activation of the trimeric IκB kinase (IKK) complex, which consists of IKKα and IKKβ and IKKγ/NEMO (Karin, 2000) and phosphorylation of IkBα leading to its degradation and nuclear translocation of p50-RelA heterodimers. Most inflammatory receptor signaling pathways such as the Tumor Necrosis Factor Receptor (TNFR) activation results in the recruitment of the IKK complex to the TNF Receptor (TNF-R) with the binding of the NEMO to a K63-ubiquitinated RIP1 (an adapter protein kinase essential for NF-κB activation by TNF-α) adaptor molecule on the TNF-R. Further, the activity of the IKKβ kinase is increased by two phosphorylations in its activation loop, resulting in the downstream events of NF-κB signaling (Herscovitch, 2006).

Upon being activated, IKKβ causes phosphorylation of two serine residues, Ser-32 and Ser-36 on IκB-alpha leading to the complete degradation of the latter and nuclear translocation of p50-RelA heterodimers. Similar to TNFR signaling, in many other cases, ligand binds to a cell surface receptor (e.g., Toll-like receptors) and recruits adaptors (e.g. TRAFs and RIP) to the cytoplasmic domain of the receptor leading to subsequent recruitment of the IKK complex to the receptor proximal site where activation of the IKKβ occurs, which then phosphorylates IκB at two serine residues, leading to its K48

ubiquitination and thereby degradation by the proteasome. Now, NF- κB enters the nucleus (Figure 4) to turn on target genes (Gilmore, 2006).

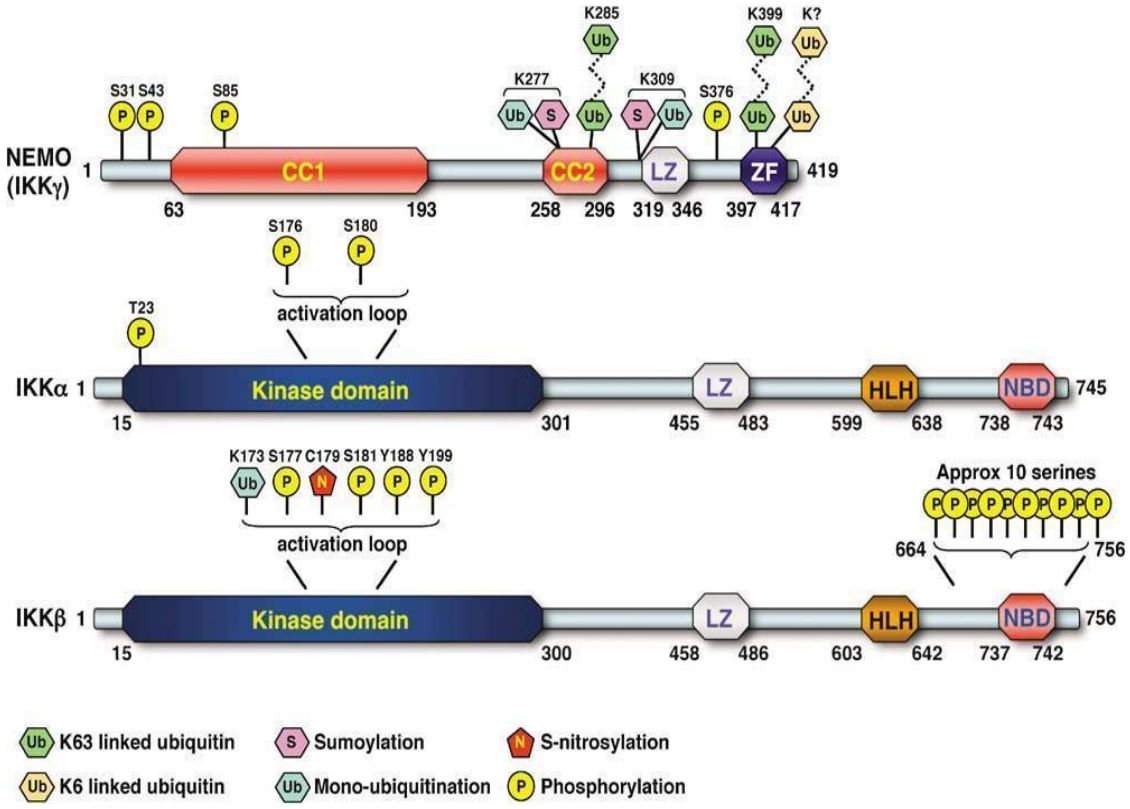

Figure 3. Post-translational modifications and structure of the IKK subunits (Perkins, N.D., 2006)

Alternative/Non-Canonical Pathway

The "non-canonical" NF-κB pathway depends on the activation of NIK and IKKα by a subset of the TNFR family of receptors such as CD40, BAFFF-R, lymphotoxin beta receptor, and so on. The action of NIK and IKKα results in the processing of the p100 subunit to p52 (Bonizzi, 2004). In this pathway, p100/RelB is the inactive cytoplasmic NF-κB complex. The IKKα homodimer complex becomes activated by certain receptor signals, and phosphorylates the p100 at two C-terminal serine residues. Then, ubiquitination of p100 and its proteasomal processing to p52 results in the nuclear

translocation of a p52/RelB complex. While NIK and IKKα are absolutely essential for the activation of the alternative NF-κB pathway, IKKβ and IKKϒ are completely dispensible for this pathway. Moreover, unlike the classical pathway, the functions of the alternative pathway are not well understood. However, recent reports indicated that the alternative NF-κB pathway plays an important role in the organogenesis of secondary lymphoid organs (Karin, 2009; Figure 4).

Interestingly, activation of the phosphatidylinositol-3-OH kinase (PI(3)K) pathway, in some cells, has been shown to lead to the phosphorylation of IKKα at Thr-23 and subsequent activation of the non-canonical pathway (Gustin, J.A., 2006). It can be inactivated by somatic mutations or deletions, and in some A20-deficient cells, re-expression of A20 leads to suppression of cell growth and NF-κB activity (Demchenko, 2010).

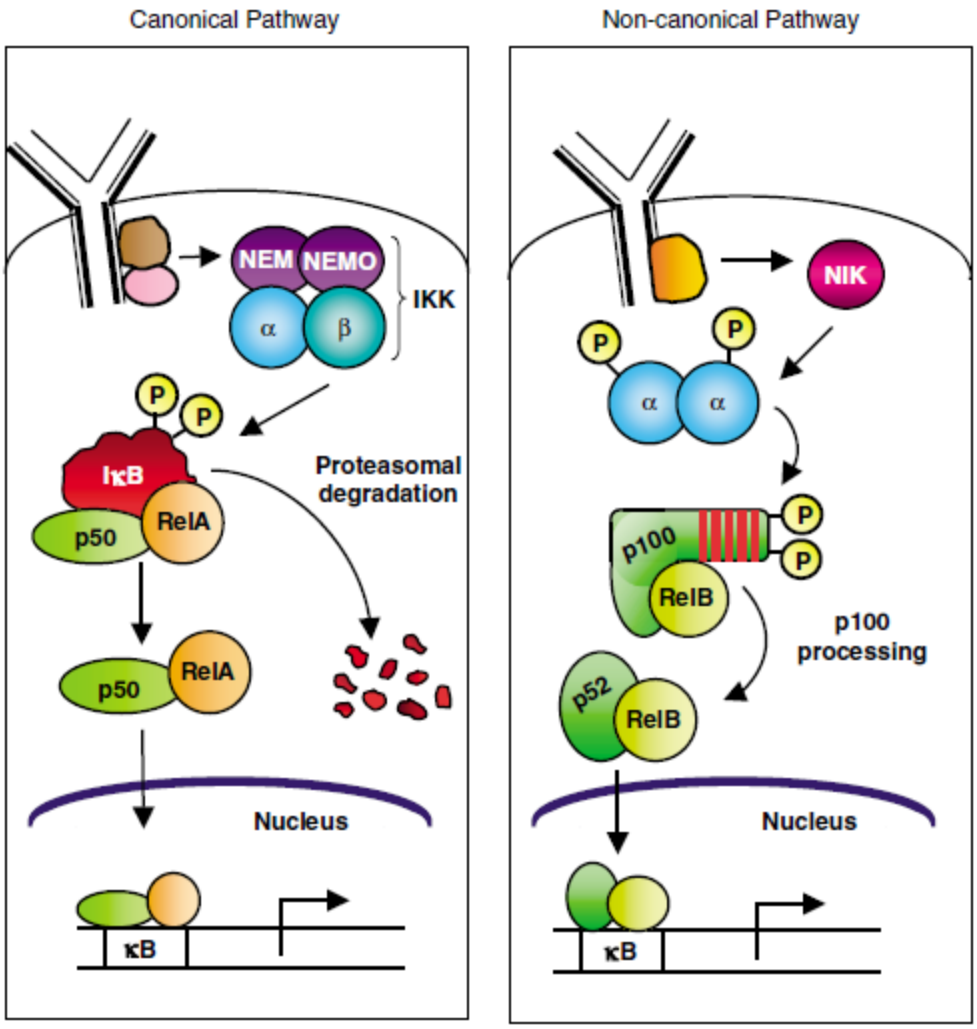

Figure 4. NF-κB signal transduction pathways (Canonical or Classical and Non-Canonical or Alternative Pathways). (Gilmore, 2006)

NF-κB and Multiple myeloma

Abnormal activation of NF-κB contributes to inherent drug resistance in numerous cancers, including multiple myeloma. The target genes of the NF-κB pathway vary depending on the type of inducer and the cells (Feinman, 2004). TRAF3 (TNF receptor-associated factor 3), which has been recognized as a regulator of the alternative NF-κB pathway, has never been involved in a malignant process. But a study (Keats,

2007) suggested that TRAF3 is frequently inactivated by mutations or deletions of this gene, and the loss of TRAF3 has been associated with constitutive activation of both classical and the alternative NF-κB pathways leading to drug resistance and growth advantage of multiple myeloma tumors. In this line, it can be suspected that TRAF3 abnormalities may induce other malignancies as well. Another abnormality frequently found in most of the B-cell neoplasms is the loss of the function of the A20 protein, which is a key negative regulator of the classical pathway.

NF-κB and Lymphoid Cancers

The NF-κB family of transcription factors plays an important role in regulating gene expression essential for the generation of both innate and adaptive immune responses. While NF-κB promotes the proliferation, differentiation, and survival of lymphocytes, abnormal activity of NF-κB significantly adds to the pathogenesis of many lymphoid malignancies. (Nishikori, 2005) In some of the normal cells like B cells, T cells, Sertoli cells, and neurons, NF-κB is mainly located in the cytoplasm under resting conditions. However, in many cancer cells (including breast cancer, lymphoid cancers, and probably many others), NF-κB is active and is present in the nucleus due to mutations in the negative regulators of NF-κB or activating mutations in some of the upstream kinases. (Vallabhapurapu et al., 2009) Some of the human lymphoid cancer cells have mutations in the genes coding the Rel/NF-κB transcription factors. Most of the multiple myelomas have mutations in genes encoding NF-κB signaling regulatory proteins that lead to constitutive activation of NF-κB. It has been known that continuous nuclear Rel/NF-κB activity protects cancer cells from apoptosis and in some cases

enhances their growth, which makes it the reason why many of the current anti-tumor therapies prevent NF-κB activity to reduce tumor growth (Gilmore, 2006).

The first relation between NF-κB and lymphoid malignancies was obtained from studies on the viral oncogene v-Rel, a homolog of the cellular c-Rel that causes leukemia and lymphoma (Gilmore, T.D., 1999). Subsequently, other studies revealed various genetic alterations involved in the NF-κB activity in B and T cell malignancies. In Hodgkin's lymphoma, characterized by Hodgkin and Reed-Sternberg (HRS) cells, transcriptionally active forms of p50 homodimers complexed with BCL3 were detected (Mathas et al., 2005). Some studies suggest that elevated NF-κB in HRS cells is due to the activation of receptors such as CD30, CD40 and RANK (Thomas, 2004). Surprisingly, in large B cell lymphoma, CD40 forms complexes with c-Rel in the nucleus and enhances the expression of NF-κB target genes, including CD154, Blys/BAFF, and Bfl-1/A1, which indicates that the association between nuclear CD40 and c-Rel is important in the regulation of lymphoma cells (Zhou, 2007).

While many studies involving NF-κB and tumorigenesis have been focused on the classical NF-κB pathway, the role of alternative NF-κB tumor cell survival and growth has been elusive. Interestingly, a recent report showed that RelB is required in radiation-resistant stromal cells to promote T cell leukemia (dos Santos, 2008). Thus, in addition to its role in cancer cells, the alternative NF-κB signaling also plays a critical pro-oncogenic role in non-hematopoietic stromal cells by regulating their role with the leukemic cells (Karin, 2009).

BCl2 Modifying Factor (BMF)

BMF, known as Bcl2-modifying factor, is a pro-apoptotic BH3-only member. It belongs to the Bcl2 family of apoptosis-related proteins. It contains only one BCL2 homology domain 3 (BH3) and has been shown to function as an apoptosis activator. Also, it is associated with dynein light chain 2 and has been known to be an apoptotic "trigger" protein, which initiates programmed cell death in some epithelial cells when their attachment to the basal lamina is lost (Puthalakath, 2001).

Bim (Bcl-2 interacting mediator of cell death) has an important biological role to play, compared to the other members of the BH3-only proteins, whereas little is known about Bmf, which is its closest relative. Bmf has a limited role to play while supporting Bim in some apoptotic processes. Bmf was originally discovered when performing the Y2H screen of a mouse embryonic cDNA library using Mcl1, although Bmf has lesser affinity to Mcl1 (Chen L, 2005), which recommends that amino acid residues outside the core of the BH3 domain are likely to add to identifying the binding specificities of Bmf in vivo. The presence of the functional BH3 domain was adequate for cell killing and binding and neutralization of Bcl-2-like molecules. Nonetheless, Bmf showed some degree of proapoptotic ability compared to other Bcl2-like molecules like Bim (Bcl-2 interacting mediator of cell death; Pinon, 2009).

Multiple isoforms of Bmf are found in the hematopoietic tissues, mostly in immature B and T cells (Labi, V, 2008). Two splice variants of BMF, Bmf II and Bmf III, were discovered in the healthy and malignant human B cells of patients with B cell lymphocytic leukemia. Remarkably, these variants lack the functional BH3 domain, and a different carboxyterminus was seen in Bmf III. Increased colony-forming potential was

seen in HeLa cells that express Bmf II and Bmf III, but Bmf I, the original form, was pro-apoptotic. (Morales AA, 2004) In mouse, Bmf is located on chromosome 2 and in humans, on a syntenic region of chromosome 15 (Rodin SN, 2005). Bim and Bmf were hypothesized to be released from the cytoskeleton in response to the loss of adhesion and/or integrin signals preceding a distinct form of cell death, called anoikis, which is observed in fibroblasts, epithelial, or endothelial cells and prevents detached cells from colonizing elsewhere (Puthalakath, H., 1999).

The Bmf gene expression is regulated by methylation-sensitive CpGs that are present in its promoter region, at the epigenetic level. Also, recent advances in research reveal that the protein expression in Bmf can be induced in melanoma and adenocarcinoma and in other cell lines of oral and esophageal cancers by the addition of new histone deacetylase inhibitors (Zhang, Y., 2006a). The Y2H screens also showed that Bmf is capable of binding to dyenin light chain 2, which is similar to the binding of Bim to the light chain 1. A highly conserved motif enables the binding of Bmf and Bim to the actin cytoskeleton and the microtubules, respectively, at their N-termini. It is believed that these proteins were circulated from the cytoskeleton in response to anoikis (Puthalakath, 1999).

The presently known physiological role of Bmf is mainly from the gene-targeting experiments performed in mice. Importantly, studies from BMF-deficient mice indicated that BMF can function as a tumor suppressor owing to its role as a pro-apoptotic molecule. Moreover, lack of BMF impaired B-cell homeostasis and resulted in the B-cell restricted lymphadenopathy and gamma irradiation-induced thymic lymphomas (Labi, V., 2008a). It has also been suggested that apoptosis due to the loss of cytokines in the

bone marrow progenitor B cells and the regulation of the developmental splenic B-cells are controlled by Bmf and also Bim (Pinon, 2009). From earlier studies, it has been derived that Bmf has a function in the anoikis of epithelial cells (Puthalakath, 2002).

BMF and Malignancies

From studies (Pinon, 2009) conducted earlier in Myc-transgenic mice, it was clear that Bim acts as a tumor suppressor. In line with its role as a pro-apoptotic and tumor suppressor, in human Burkitt's lymphoma, Bim was found to be silenced (Mestre-Escorihuela C, 2007). Similar to the role of Bim in oncogenesis, the role of Bmf as a tumor suppressor has been drawing attention. It is important to note in this context that the loss of BMF enhances the gamma-irradiation-induced thymic lymphoma (Labi, V, 2008a).

Bmf and anti-tumor therapies

Novel anti-tumor therapies aim to induce apoptosis in cancer/tumor cells. Bim, which is an effective initiator of apoptosis, was shown to be involved in some therapies as an important pro-apoptotic initiator. Induction of pro-apoptotic BH3-only proteins is therefore employed as a tool to induce apoptosis of tumor cells. Irrespective of the type of pro-apoptotic BH3-only proteins operating, they are important in the initiation of apoptosis owing to the fact that they can bind to the anti-apoptotic Bcl2 family members and neutralize them. This idea led to the development of imitative BH3 molecules that neutralize the anti-apoptotic Bcl2 members that are overexpressed in various cancers and initiate the release of proteins like Bmf (Labi.V, 2008b). These BH3 mimetics show an association with therapeutic compounds like tyrosine kinase inhibitors (TKIs), which include imatinib and erlotinib, though they lack efficacy for a single-compound. These

compounds have shown to be effective as specific compounds in the treatment of Chronic Myelogenous Leukemia (CML) and Non-Small Cell Lung Cancer (NSCLC) (Pinon, 2009).

In the novel clinical therapies, induction of apoptosis by Bmf has not been clearly defined. Although Bmf was upregulated in response to TKIs, its role in tumor apoptosis and its use in clinical therapies has been less studied. Bmf, along with Bim, was shown to be involved in the induction of apoptosis with respect to treatment of Acute Lymphoid Leukemia (ALL) with glucocorticoids (Ploner C, 2008) and arsenic oxide in the treatment of multiple myeloma (Morales, 2008). In most of the cancers, after treating the cells with HDACi, Bmf appeared to be upregulated (Zhang, 2006a). These HDACi's belong to a class of capable anti-cancer agents, whose molecular mechanisms, in future, would lead to the invention of valuable therapeutics in combination with others, for treatment of the various types of cancers. Given the vast amount of information, Bmf, like Bim, will give the researchers a plethora of opportunities to develop the particular apparatus needed to treat malignancies (Pinon, 2009).

Given the importance of BMF in the induction of tumor cell apoptosis, it is necessary to understand how BMF is regulated transcriptionally. In this study, I focused on how NF-κB might regulate BMF in multiple myeloma.

The Present Study and its Objective

Current Objective:

To clone a 2.5kb promoter region of BMF gene into luciferase reporter construct (pGL3-Promoter vector).

The specific role of classical vs. alternative NF-κB in the survival and growth of multiple myeloma has been unclear. In particular, how the classical vs. alternative NF-κB pathways regulate pro-apoptotic genes such as BMF has not been clear. In order to study how these two pathways regulate the BMF expression, it is necessary to clone the BMF gene promoter into a reporter plasmid.

Therefore, I aimed to clone a 2.5Kb promoter region of BMF gene into pGL3-promoter vector upstream of the luciferase gene. Prior to cloning the BMF promoter into the luciferase reporter construct, I first planned to clone it into the TOPO-T vector and eventually planned to subclone the BMF promoter into the pGL3-Promoter vector for performing luciferase reporter assays.

Chapter II – Materials and Methods

Cell Line

Multiple myeloma cell line JJN3 was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and was cultured in a humidified atmosphere containing 5% CO2 at 37 °C. The cells were sub-cultured and were maintained as needed.

Enzymes

Restriction endonucleases and enzymes used in cloning DNA were obtained from New England BioLabs.

Isolation of genomic DNA and PCR amplification

Genomic DNA from the JJN-3 myeloma cell line was isolated by conventional phenol-chloroform method. Isolated DNA was seen on 2% agarose gel. Bmf promoter region (2.5 Kb) has been amplified by polymerase chain reaction (PCR) using the forward and reverse primers. The 40 µl reaction mixture contained 20 µl of mixture containing the Elongase enzyme, PCR buffer, 10 mM dNTP mixture and magnesium chloride, 2 µl each of the forward and reverse 2.5 Bmf primers, 1 µl autoclaved water, and 5 µl of 1:10 diluted JJN3 DNA. The reaction mixture was incubated in a thermal cycler for initialization at 94°C for 5 min, 35 amplification cycles at 94°C for 30 seconds, 1 min at 70°C Annealing temperature, followed by a final hold time of 5 min at 68°C. The PCR product was analyzed on 2% agarose gel, extracted, and purified by using QIAquick PCR Purification Kit (Qiagen).

Cloning of Bmf promoter into pGL3 promoter vector

The cloning vector, pGL3 promoter vector is CIPped (Calf Intestinal Phosphatase, CIP- treated DNA fragments lack 5´ phosphoryl termini required by ligases and hence

cannot self-ligate). The purified 2.5 Kb Bmf promoter was ligated into pGL3 promoter vector (5010 bp), which contains the promoter-luc+ transcriptional unit, by using DNA ligase enzyme. The reaction mixture (CIP, pGL3 vector DNA, Bmf DNA, Ligase) was incubated at 37°C for about 2 hours.

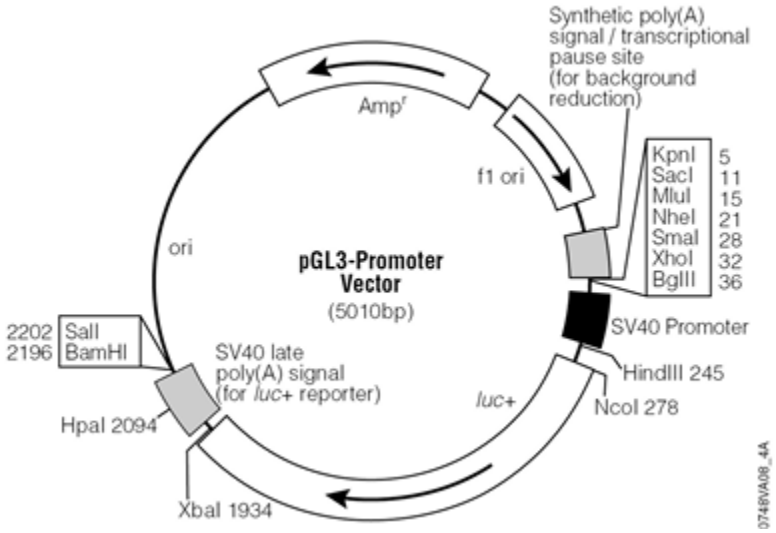

Figure 5. pGL3 promoter vector with the restriction enzyme sites of BamHI, XbaI and SacI (Promega)

The ligated mixture was transformed into E.coli DH5α competent cells (Promega, Madison, WI). Transformed (Heat shock) cells were selected from the LB (Luria-Bertani) agar plates (medium containing ampicillin, 100 µg/ml, and LB broth). The agar plates were incubated at 37°C overnight. Positive clones were identified by blue and white colony screening. The plasmid DNA was electrophoresed on 2% Agarose gel.

Cloning of Bmf gene (2.5 Kb) into TOPO TA (3.9 Kb) Vector

The recombinant plasmid pGL3 containing the Bmf promoter was digested with BamHI and XbaI restriction enzyme pair and double digested with KpnI and NheI. The pCR®2.1-TOPO® vector was also digested with the same enzymes. The restricted fragment as well as the double digested TOPO vector were run on 2% agarose gel, purified, and then ligated to the TOPO TA vector using DNA ligase. The ligated product was then transformed into One Shot® competent cells (TOPO TA cloning® of Invitrogen™). Positive clones were confirmed.

Expression of Bmf promoter in One Shot® competent cells

A single transformed colony from LB agar plate (containing ampicillin) was picked up and inoculated into 50 ml of LB medium containing ampicillin (100 mg/ml). The medium was incubated overnight at 37°C in shaking incubator. 1 ml of overnight grown culture was then inoculated to 100 ml of the above mentioned LB medium and incubated at 37°C in shaking incubator. The culture was centrifuged at 6000 g for 10 min. The supernatant was discarded, the pellet was saved, and the plasmid DNA was eluted and purified by Qiagen Maxiprep Purification. The 6.4 Kb recombinant plasmid DNA was confirmed on 0.8% Agarose gel followed by staining with ethidium bromide and visualizing under the UV light in the Gel Doc System.

Preparation of plasmids

pGL3 vector and pRL-TK Renilla vector encoding Firefly Luciferase and Renilla Luciferase, respectively were transformed into E.coli, isolated and purified using Maxiprep Plasmid Purification (Qiagen). Also, the DNA from the genes of interest, NF-κB p52, p100 and RelB, was purified and further amplified for the purpose of transfection.

Cell line and culture medium

Human embryonic kidney cell line (293T) was cultured in Dulbecco's modified eagle medium (DMEM). The medium was complemented with 10% fetal calf serum (FCS), streptomycin at 100 µg/ml and penicillin at 100 U/ml. Cells were harvested at 37°C in a 95% air and 5% CO2. Cells were split using trypsin/ethylenediaminetetraacetic acid (EDTA) medium as were maintained as needed.

Transfection Protocol

Vector pGL3 alone, pGL3 with the Bmf insert (both contain the luciferase activity site) were taken in separate tubes with combinations of the amplified p52, p100, and RelB. The combinations taken for two wells each are pGL3 vector alone, pGL3-Bmf alone, pGL3 with RelB, pGL3-Bmf with RelB, pGL3 with RelB, and p52, and pGL3-Bmf with RelB and p52.

For transfection, plain DMEM medium (without serum and antibiotics) was taken. 200 µl of Lipofectamine® (Invitrogen™, Life Technologies, California) reagent was added to the above plasmid combinations taken in the respective tubes, and the tubes were incubated for 30 minutes at room temperature. The cells were sub-cultured and maintained as necessary.

Luciferase Reporter assay

HEK-293T cells were transfected with the following plasmids in different combinations that express p52 (600 ng), and RelB (650 ng) as well as pGL3-Luc plasmid (200 ng), pGL3- BMG-luc plasmid (200 ng) and renilla luciferase plasmid (50ng) in the 24-well plates. After incubation for about 24-36 hours, the cells were washed with phosphate-buffered saline (PBS) and then lysed in the passive lysis buffer supplied with the luciferase assay kit. Relative levels of luciferase were measured by measuring the

light units using a luciferase assay kit (Promega) with a luminometer as described in the manufacturer's protocol. The data were expressed as relative light units (RLU) per well.

Chapter III - Results & Discussions

Cloning of Bmf promoter into pGL3 vector

Although human BMF promoter may previously have been cloned into reporter plasmids by other investigators, for our convenience we intended to clone a 2.5 Kb proximal region of human BMF gene promoter from Myeloma cell lines into pGL3-promoter vector. To this end, JJN-3 genomic DNA was isolated by Phenol-Chloroform method. Amplification of the Bmf gene promoter was performed by polymerase chain reaction using primers that would amplify a 2.5 Kb region of BMF promoter. The PCR cycles were approximately 36 at an annealing temperature of 70 ºC. The 2.5 Kb band of Bmf promoter obtained from the PCR was cut from the gel and purified by using QiaQuick gel extraction kit (Qiagen).

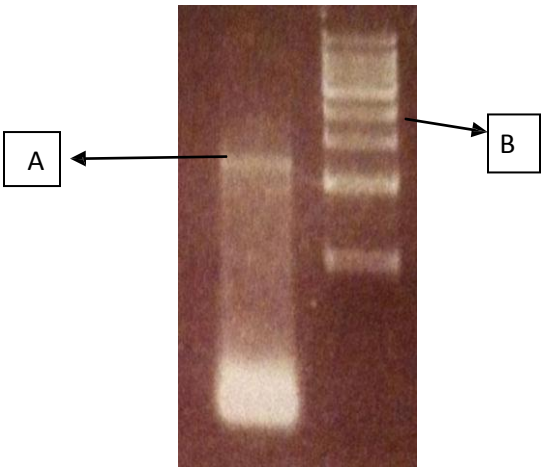

Figure 6. "A" shows the 2.5 Kb band separated from the JJN3 genomic DNA by electrophoresis on a 2% agarose gel with "B", the 10 Kb ladder.

The initial PCR reaction gave a smear in addition to the 2.5 Kb band for the BMF promoter. This could be due to a high concentration of genomic DNA. Therefore, I diluted the DNA 1:10 and 1:20, and the PCR has been performed again. As can be seen from Figure 7, 1:10 diluted genomic DNA gave a crisp band of 2.5 Kb size, which was then eluted from the agarose gel by QiaQuick gel extraction kit. The PCR primers used to amplify the 2.5Kb promoter region for BMF gene contained KpnI and NheI restriction sites to clone into the KpnI and NheI sites of the pGL3-Promoter Luciferase reporter vector.

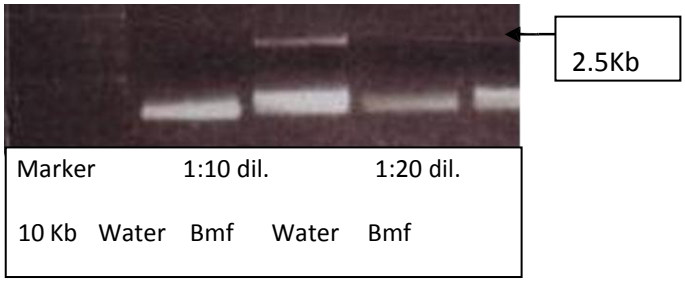

Figure 7. PCR Amplified 2.5 Kb Bmf promoter on a 2 % agarose gel.

Prior to cloning into the pGL3-Promoter vector, the BMF gene promoter was cloned in pCR TOPO 2.1 vector (3.9 Kb) by quick ligation reaction at room temperature for 5 minutes. The ligated product was transformed in the One Shot® competent cells (Invitrogen, Life Technologies, California). Several colonies were screened for the presence of BMF promoter, and one of the positive clones was sequenced to confirm that the BMF promoter that we obtained has the correct sequence.

The 2.5 Kb BMF promoter fragment was released from the TOPO2.1 vector by KpnI and NheI digestion and ligated with pGL3 promoter vector that has also been

digested with KpnI and NheI. Prior to ligation, the pGL3 vector was treated with CIP to prevent vector self-ligation. Upon ligation, the pGL3 recombinant plasmid containing the 2.5Kb BMF promoter fragment (pGL3-Bmf) was transformed into competent cells of E. coli DH5α.

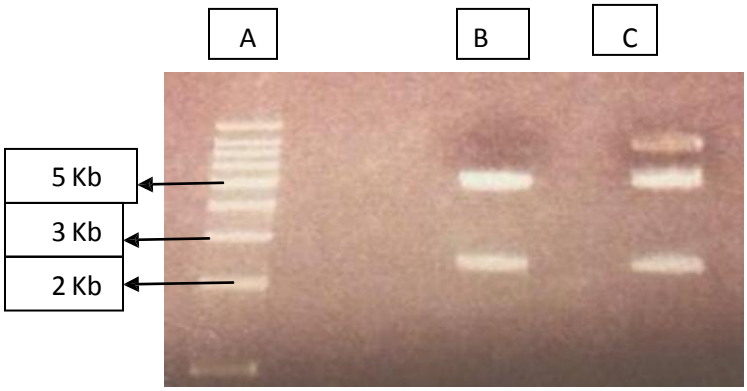

Figure 8. Release of 2.5Kb BMF promoter fragment from pGL3-BMF vector upon digesting with KpnI and NheI enzymes. "A" is the 10 Kb Ladder, "B" and "C" show the digestion fragments for clones 2 and 5, respectively.

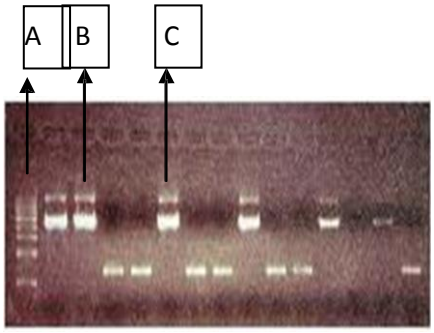

Figure 9. MiniPrep plasmid purification. A and B are Clones 2 & 5 respectively. C is the DNA ladder of 10 Kb length.

Positive clones were identified using blue and white screening. Several clones were screened by restriction digestion for the presence of the 2.5 Kb BMF promoter

fragment (Figure 8). Two different clones (Clones 2 and 5) were selected eventually for Maxiprep Plamid Purification (Qiagen) and confirmation by sequencing.

From the two clones obtained (clones #2 and #5), clone #5 was used to investigate how RelB and p52 may regulate the BMF promoter. However, initial studies involving luciferase assays did not produce convincing results about whether RelB and p52 regulate the BMF promoter. Further studies are required to conclude about the role of RelB and p52 in the regulation of BMF promoter.

Future Perspectives

How tumors become resistant to apoptosis is an important question to address. While hyper-expression of anti-apoptotic genes has been extensively studied in various tumors, regulation of pro-apoptotic genes has not been clear. Especially, tumor-specific regulatory networks that achieve a finely balanced expression of pro- and anti-apoptotic genes seem to play an important role in tumor cell survival. To this end, regulation of BMF, a pro-apoptotic gene, is of great significance in the survival of not only multiple myeloma but also other types of tumors. Since NF-κB signaling is involved in various survival strategies that are operated in different tumors including multiple myeloma, it could be that BMF expression is under the regulation of NF-κB. Therefore, reporter studies such as luciferase assays to investigate BMF promoter regulation by NF-κB are an ideal experiment to perform. To this end, the pGL3-BMF-promoter-Luciferase reporter construct made in this study is of significant use for future studies to delineate how NF-κB and/or other transcription factors regulate the expression this important regulator of apoptosis in tumor cells.

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