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Investigating the Roles of Zinc-Binding Protein (ZBP-89) and Rela (NFkB-p65) in the Regulation of Matrix Metalloproteinase 1 (MMP1) Gene Expression

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Thesis for the Master of Science degree in Biomedical Sciences
By Nelly Khaselev

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Abstract

ROLE OF ZBP-89 AND NFKB (P65) IN MMP-1 GENE REGULATION
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Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases with the unique ability to breakdown virtually the entire extracellular matrix (ECM). Through ECM remodeling, MMPs play an important role in normal development, tissue repair, angiogenesis, and apoptosis. Studies have shown that unregulated MMP expression plays a role in many cancers and chronic inflammatory diseases. Previous research has used gene sequence analysis of the MMP-1 promoter to identify a putative ZBP-89 binding site at -1969 bp. Chromatin immuneprecipitation experiments showed that both ZBP-89 and RelA (p65) could bind to this binding site. In this thesis research, transfection experiments were used to explore the role of ZBP-89 and p65 in regulating MMP-1 gene expression. Two versions of a MMP-1 plasmid were used: a "Long MMP-1 plasmid" with a longer MMP-1 promoter (2.2 kb) that contains the distal putative binding site and a "Short MMP-1 plasmid with a shorter promoter (1.1 kb) that does not. Results showed that ZBP-89 alone can increase long MMP-1 plasmid expression in COS-1 cells and ZBP-89 and p65 synergistically increase long MMP-1 plasmid expression in a dose-dependent manner. This suggests that ZBP-89 may cooperate with NFkB-p65 in MMP-1 gene regulation. ZBP-89 and p65 did not increase short MMP-1 plasmid expression in COS-1 cells or A549 cells. These results support our hypothesis that ZBP-89 and p65 work directly through the putative binding site at -1969 bp. This research further expands our understanding of MMP-1 gene regulation and can aid the development of MMP targeted therapy.

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List of abbreviations

AP-1 - Activator Protein-1

ATL - Adult T-cell leukemia

ChIP - Chromatin immunoprecipitation

CoMTb - Conditioned media from Mtb-infected monocytes

EC - Endothelial cells

ECM – Extracellular Matrix

ERK - extracellular signal-regulated kinases

FBS - Fetal bovine serum

FGF-1 - Angiogenic fibroblast growth factor-1

HCC - Hepatocellular carcinoma

HPX - Hemopexin-like domain

HTVL-1 - Human T-cell lymphotrophic virus type-I

IKK - IkB kinases

IL-1 - Interleukin-1

JNK - c-Jun N-terminal kinases

KD – (gene) knock-down

LPS - Lipopolysaccharides

MAPK - Mitogen-activated protein kinase

MMP – Matrix Metalloproteinase

NFkB – Nuclear factor kappa B

NC - negative control

NSCLC - Nonsmall cell lung carcinoma

shRNA - Small hairpin RNA

TB - Tuberculoses

TIMP1 - Endogenous tissue inhibitor

TNF – Tumor necrosis factors

ZBP – Zinc- Binding Protein

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1. Introduction

1.1 MMP Background

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases involved in the breakdown of extracellular material. The first member of the MMP family was discovered in 1962 by Dr. Jerome Gross and Dr. Charles Lapiere, having collagenolytic activity in amphibian tissue, since then at least 24 other MMPs have been discovered (Gross & Lapiere, 1962; Yan *et al.,* 2007). This family of proteins garnered further attention as the complexity of its role in both physiological and pathological processes became increasingly evident. MMPs are known to be involved in wound healing, tissue remodeling, angiogenesis, apoptosis and various other physiological processes. Interestingly, MMPs have been shown to have both a positive and a negative role in pathologies such as cancer and chronic inflammatory disorders (reviewed in Malmud, 2006). It is therefore important to further our research of MMP regulation to better understand MMPs' role in physiological and pathological processes.

1.2 The Structure-Function Relationship

The MMP family has the capacity to break down most, if not all, of the macromolecules of the extracellular matrix (ECM), a process essential for normal organ development and growth. This family of proteins includes collagenases, matrilysin, metalloelastase, gelatinases, enamelysin, stromelysins, and others. These endopeptidases share a number of similar structural features, yet they have distinct but overlapping

substrate specificities. Moreover, many may serve other functions beyond matrix breakdown (Verma *et al.*, 2007).

MMPs can be either secreted or membrane-bound as cell surface enzymes. They mainly consist of five distinct domains: N-terminal pre-domain, pro-domain, catalytic domain, a hinge region, and C-terminal hemopexin-like domain (HPX). Membrane-type MMPs also contain a transmembrane domain, and a short cytoplasmic signaling tail (Sternlicht & Werb, 2015). A schematic of the basic MMP family protein structure is shown below (Figure 1), (Kandasamy, *et al.*, 2009).

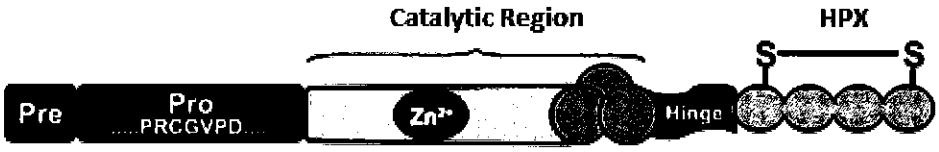

Figure 1: Common MMP Protein Structure, (Modified from Kandasamy, *et al.*, 2009)

The pro-domain contains a conserved Cys residue that forms a complex with the zinc ion in the active site to inhibit catalysis. All MMPs are initially synthesized as an inactive zymogen form. Disrupting or removing the Cys-Zinc molecular complex activates the zymogen MMP, and catalysis can proceed (Van Wart *et al.,* 1990).

Within the catalytic domain, three histamine residues encoded by the conserved sequencing motif HExxHxxGxxH bind the Zinc ion that facilitates catalytic activity (Verma *et al.*, 2007). Interestingly, Cha *et al.* have shown that replacing the Zinc ion of MMP-3 with other +2 cations maintained the catalytic activity, but subsequent geometric

changes affected the substrate specificity (1998), thus characterizing the role of the Zinc ion in MMPs in both substrate specificity and proteolysis.

Most MMPs contain a C-terminal hemopexin domain (HPX) that supports protein-protein interaction, substrate specificity, and protein activation or inhibition. For example, Dr. Murphy and colleagues showed that removal of the collagenase HPX altered MMP-1's interaction with endogenous tissue inhibitor (TIMP1) thus indirectly increasing MMP-1 protease activity (1991). Interestingly, Dr. Remacle and colleagues, identified inhibitors that target the HPX domain and found they reduced tumor growth, providing preclinical evidence of a new therapeutic target – the HPX domain (2012).

Understanding the structural similarities and differences of MMPs can provide further insight into the complex and sometimes contradictory roles MMPs play in both physiological and pathological processes.

1.3 MMP breakdown of extracellular matrix and other physiological functions.

The extracellular matrix (ECM) provides structural support for organs and tissues as well as creates the microenvironment that influences many important biological processes. The ECM provides anchorage to cells, it can aid or hinder cell migration, and it sequesters various cytokines and growth factors that, when released, modulate cell behavior and growth. Lastly, physical properties of the ECM such as stiffness and biomechanical force can influence cell behaviors such as cell differentiation (Lu *et al.*, 2011).

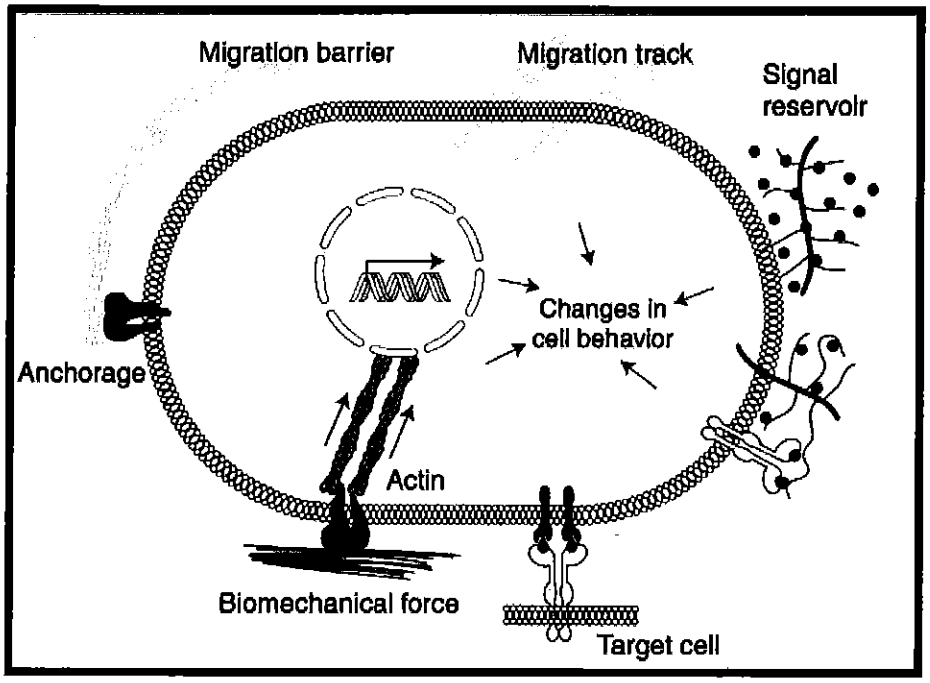

Figure 2: Extracellular Matrix function leads to changes in cell behavior (Modified from Lu *et al.*, 2011)

MMPs are by far most commonly known as important extracellular matrix regulators. MMPs through ECM remodeling help regulate cell differentiation, angiogenesis, bone remodeling, and wound repair. Furthermore, the breakdown of ECM-specific substrates can produce bioactive cleavage fragments, providing further cell communication. In contrast, abnormal ECM regulation leads to uncontrolled cell proliferation, differentiation, and failure of apoptosis, leading to various pathologies (Lu *et al.*, 2011).

A study that investigated the role of collagenase (MMP-1) in wound healing used in situ hybridization to localize the expression of MMP-1 in samples of pyogenic granuloma, a common skin growth. The study found an increased expression of MMP-1 in migrating keratinocytes near the edge of all lesions, but not in tissue samples without

ulcerations (Saarialho-Kere *et al.,* 1992). MMP-1 specifically degrades collagens types I and III, which aids keratinocyte migration at sites of focal adhesion attachment to the dermal substratum (Martin, 1997).

In a more recent study looking at wound healing in diabetic patients with foot ulcers, results indicated that high levels of MMP-1 are vital for proper wound healing. An excess of MMP-8 and -9, however, are harmful and can delay wound healing, showing the complexity of MMPs role and importance of protein balance. Wound fluid from neuropathic diabetic foot ulcer patients was analyzed for MMP-1, -8, -9 and TIMP-1 (an endogenous tissue inhibitor of MMPs), during a 12-week period. Good healers, defined by a decrease of wound surface by at least 82 percent by four weeks, showed a decreased level of MMP-8 and -9 by inflammatory cells and a significant increase in MMP-1 by week two. Poor healers, defined by a decrease of wound surface less than 82 percent by four weeks, showed higher levels of MMP-8 and -9 and an initial level of MMP-1 similar to that of good healers but no significant increase as time progressed. Better understanding of the regulation of MMPs can help direct treatment. Further research for topical treatments aimed at MMPs can have significant clinical implications in diabetic patients (Muller *et al.,* 2007).

MMP-1 is also important in angiogenesis, as shown in a study of the migration of microvascular endothelial cells (EC) – an early stage of angiogenesis. In this study, investigators overexpressed angiogenic fibroblast growth factor-1 (FGF-1) and MMP-1 in cultured postcapillary venular endothelial cells and found that chimeric FGF-1 transfected cells migrated two times faster in a pure collagen I matrix as compared to vector control transfected cells. A selective MMP-1 inhibitor abolished this increase in

migration. These results showed the important function of MMP-1 and how crucial proper regulatory factors are in mediating MMP-1 activity (Patridge *et al.*, 2000).

Although most MMPs are secreted and function primarily as external proteins, these same proteins can have an intracellular function. One study found that intracellular MMP-1 levels were highest during mitosis in glial Müller cells. Using immunohistochemical staining, investigators found a clear association of MMP-1 with mitochondria and cell nuclei in both glial and nonglial cells upon induction of apoptosis. RNA interference and an MMP inhibitor showed that inhibition of MMP-1 speeds up the degradation of lamin A, activates caspases, and increases DNA fragmentation as compared to uninhibited cells. This suggests that MMP-1 has an important role in cell cycle regulation and can help cells resist apoptosis (Limb *et al.,* 2005). Likewise, this provides one explanation to the common association of MMP-1 with cancer cell survival and metastasis.

For decades now, MMPs have been investigated for their important role as extracellular matrix regulators. Their important physiological role in cell-cell and cell-ECM communication is evident in normal development and maintenance of human tissue and organs. Interstitial collagenase, MMP-1, has been implicated in cell migration, wound healing, angiogenesis, and cell survival as described above.

1.4 MMP's role in cancer biology.

MMPs play a complex and often contradictory role in cancer. MMPs can be both positive and negative prognosis predictors depending on the cancer and cell type. MMPs

role in cancer can be cancer-stage specific, tissue specific, or cell specific.Through both extracellular and intracellular functions, the MMP family can regulate cell survival, differentiation, proliferation, and migration, affecting virtually all aspects of cancer progression. MMP-1 overexpression has been studied in breast cancer (Xuan *et al.*, 2014), colon cancer (Lu ZH *et al.*, 2011), and lung cancer (Schutz *et al.*, 2015), as well as several others. Further understanding of MMP expression can provide novel insight to its role in tumorigenesis.

The simplest explanation for MMP-1's involvement in cancer is the role it plays in digesting ECM macromolecules and thus paving the way for tumor cell migration. Indeed, the cause of death in many cancers is due to metastasis of tumor cells that invade the peripheral tissue, blood, and lymphatic vessels, thus entering circulation (Kessenbrock *et al.*, 2015). In one study, MMP-1 expression was detected via immunohistochemical staining of four different tissues: 1) non-specific invasive ductal carcinoma (IDC) of the breast, 2) cancer-adjacent normal breast tissue, 3) lymph node metastases of non-specific IDC of the breast, and 4) normal lymph node tissue. Analysis of the results showed that positive MMP-1 expression in non-specific IDC (54.5%) was significantly higher than in normal tissue adjacent to cancer (20.6%). Likewise, the positive MMP-1 expression was significantly higher in IDC metastatic lymph node tissue (66.7%) than in normal lymph node tissue (0%) (Xuan *et al.*, 2014). This is one example of many that show an association between MMP-1 and tumor metastases.

MMP-1 seems to have a stage-specific significance in colon carcinoma. The expression pattern of MMP-1 was studied using immunohistochemistry on the tissue of 620 colon carcinoma patients. Data analysis revealed that positive MMP-1 expression

was significantly higher in cancer tissue than in normal colon tissue. Dividing patients by cancer stage revealed that positive MMP expression is also significantly higher in patients with Stage I and II colon cancer when compared to patients with Stage III and IV colon cancer. In Stage I and II, high MMP-1 expression was associated with poor prognosis; while in stage III and IV, high MMP-1 expression was associated with improved prognosis. This data presents MMP-1 as an independent prognostic factor in colon carcinoma with differing prognostic implications depending on the stage of the cancer (Lu ZH *et al.*, 2011).

MMP-1 and MMP-9 levels have been implicated in lung cancer prognosis as well. Gouyer et al. examined nonsmall cell lung carcinoma (NSCLC) in lung cancer patients that underwent complete resection. Their study found that high MMP-1 expression significantly correlated with tumor-lymph node metastasis and lower survival rates (Gouyer *et al.,* 2005; Schutz et al., 2015). MMP-1 levels increased with tumor stage progression, with a significant difference between Stage IA and Stage IIB disease. Interestingly, where MMP-1 seems to be more associated with metastasis progression, MMP-9 seems to be involved in tumor cell growth. MMP-9 expression correlated with an increase in T classification, which measures tumor size (Gouyer *et al.,* 2005). Both MMPs were overexpressed in tumor cells but their impact on cancerous tumors differs.

The human proteolytic system that breaks down tissue is remarkable, involving over 500 genes encoding for proteases or protease-like proteins. Nevertheless, among all the enzymes potentially associated with tumor growth and tumor metastases, the MMP family draws extra attention as promising targets of cancer therapeutics on the basis of being commonly overexpressed in malignant cells, strongly associated with poor

prognosis and uniquely able to degrade virtually the entire extracellular matrix (Folgueras *et al.,* 2004). Preclinical studies in various tumor models showed great efficacy of MMP inhibitors, and clinical trials began quickly. The results of these trials were disappointing, largely due to drug-related side effects, toxicity and lack of specificity. Although MMPs physiological role in tissue remodeling and digestion of ECM is a compelling argument for their role in cancer, we now know that MMPs play a much more complex role than previously expected. Researchers began to re-investigate MMP biology – its regulation and function in pathology.

1.5 The role of MMPs in Chronic Inflammatory Diseases

Inflammation is the body's natural response against harmful stimuli such as toxins, infections, and injury. Upon stimulation, cells release inflammatory cytokines that can activate a protein-kinase signaling pathway. This results in increased expression of various proteins that help eliminate the cause of injury, and begin the process of tissue repair. Inflammatory cytokines, such as IL-1 and TNFα have been shown to increase MMP gene expression (reviewed in Malmud, 2006). Prolonged inflammation causes cartilage, bone, and tissue damage - the hallmark symptoms of many chronic inflammatory diseases such as periodontitis and rheumatoid arthritis. Increased levels of MMPs contribute greatly to these chronic inflammatory diseases.

One of the first connections between pathology and MMPs was made in Rheumatoid arthritis (RA) (reviewed in Brinckerhoff, 2002). One of the main components of articular cartilage is type II collagen, while type I collagen is the major

type found in tendon and bone. Elevated levels of inflammatory cytokines, IL-1β and TNFα in synovial fluid stimulate the expression of collagenases, MMP-1 and MMP-13. These collagenases degrade collagen type I and II causing much of the damage in RA (Brinckerhoff, 2002). With the discovery of MMP's important role in synovial joint damage, researchers quickly began to investigate MMP inhibitors as a therapeutic approach for RA. Adalimumab, a TNFα monoclonal antibody, binds to TNFα thus preventing it from stimulating inflammatory signaling pathways. In a clinical trial, Adalimumab was found to reduce MMP-1 expression in patients with RA receiving concomitant methotrexate. Adalimumab successfully reduces many symptoms of RA (Malmud, 2006).

One of the better understood inflammatory diseases, as it pertains to MMPs, is periodontitis. This chronic inflammatory disease affects the gums and teeth, and results from opportunistic bacterial infection. Bacterial antigens, such as lipopolysaccharides (LPS), stimulate cells such as fibroblasts, macrophages, neutrophils, and even pulp tissue itself (odontoblasts and odonotclasts) to secrete cytokines and subsequently increase MMP expression (Jain, 2015). Several studies have shown elevated levels of MMP-1, as well as other MMPs, in periodontitis patients, which results in substantial tissue destruction and even tooth loss (Popat *et al.,* 2014; Shindo *et al.*, 2014). Treatment with antibiotics, tetracycline or doxycycline, which inhibits MMP expression and activity, can prevent and treat periodontitis. Periostat is a tetracycline that chelates the zinc ion, thus effectively inhibiting MMP activity. It is the only MMPI currently approved by the FDA for medical use and prescription (Brinckerhoff, 2002; www.accessdata.fda.gov).

1.6 MMP-1 Gene regulation.

MMP-1 is ubiquitously expressed in low levels in most healthy tissues and is involved in a variety of physiological processes, such as tissue remodeling. Gene expression is complex and tightly controlled to maintain appropriate physiological levels. However, when the regulation pathway is hijacked in neoplastic cells or altered by immune system dysfunction, gene expression is drastically increased, promoting progression of the pathology. In order to fully understand pathologies such as cancer and chronic inflammatory diseases, and how we can therapeutically intervene, it is critical to understand which transcriptional factors and which signaling pathways lead to increased gene expression.

MMP-1 expression can be induced by oxidative stress, cytokines, growth factors and other environmental signals, and multiple signaling pathways lead to transcription factors binding the MMP-1 promoter. Figure 3 below represents the MMP-1 promoter with important transcription factor binding sites.

Figure 3: Schematic diagram of the MMP-1 gene promoter. Transcriptions factor binding sites for AP-1, ETS, NF-kB and ZBP-89 are shown. Note that the distal NF-kB binding site is not within the MMP plasmid constructs used in these experiments. (Figure based on information from Okeane et al 2010, Spinale et al. 2007, Cartharius et al. 2005, McCreechy et al 2005.)

Activator Protein-1 (AP-1), a heterodimer of Fos and Jun family proteins, has a common binding site at -70 kb in many MMP genes. AP-1 can be activated through the mitogen-activated protein kinase (MAPK) pathway (Figure 4). Cytokines, osmotic stress, apoptotic signals, and growth factors begin a signaling cascade that leads to the

stress, apoptotic signals, and growth factors begin a signaling cascade that leads to the phosphorylation and activation of c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs), two members of the MAPK family. As a result, JNKs and ERKs translocate into the nucleus where they can phosphorylate and activate c-Jun proteins, which can dimerize with c-Fos to form an active AP-1 molecule capable of stimulating MMP-1 gene transcription (Benbow et. al., 1997; Kida et. al., 2005, Hong et. al., 2015).

MMP-1 has a pivotal role in cancer progression and metastasis, and is commonly found to be overexpressed in many cancers (as discussed previously). It is not surprising that important MMP-1 regulators, such as AP-1 are also overexpressed in cancers. For example, Dr. Belguise and colleagues found that breast cancer patients with more invasive prognosis have higher levels of Fra-1 expression. Fra-1 is a transcription factor in the FOS family. Belguise overexpressed as well as silenced the Fra-1 gene in MCF7 ER+ cells, a breast adenocarcinoma estrogen receptor positive cell line. Belguise found that overexpression of Fra-1 increased expression of wildtype MMP-1 but not MMP-1 with a mutated AP-1 site. Also, silencing of Fra-1 inhibited cell proliferation and drastically decreased DNA content (4.5 fold) as compared to control cell line (Belguise et. al., 2004).

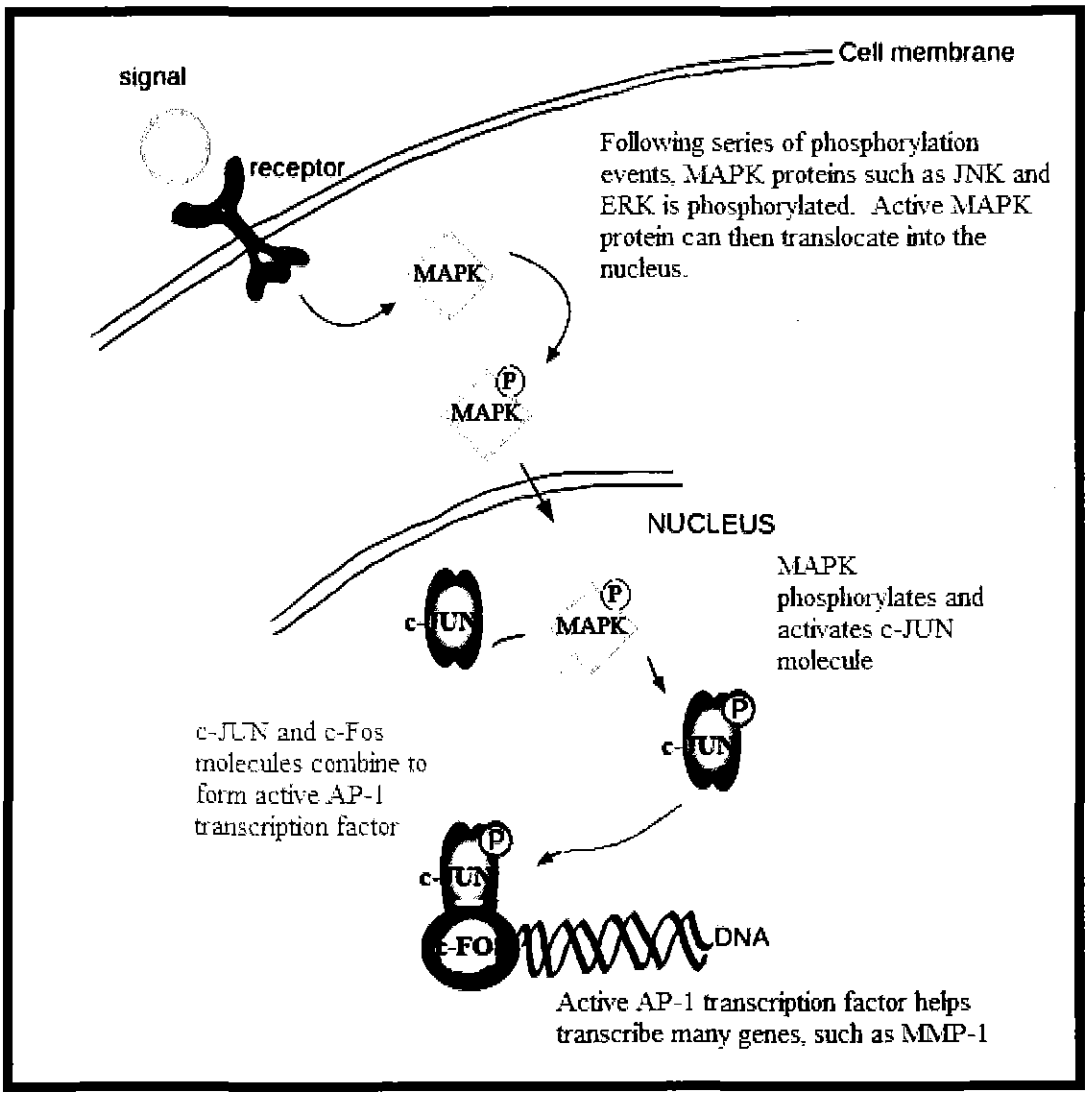

Figure 4: Schematic of AP-1 Activation Pathway (image modified from Buddhini Samarasinghe 2014 based on information from Benbow et. al., 1997; Kida et. al., 2005, Hong et. al., 2015).

Interestingly, the MMP-1 promoter has a common polymorphism that creates a functional transcription factor binding site. This polymorphism at -1607bp, is commonly found in many cancer patients, as well as patients with chronic inflammatory conditions. Due to the insertion of a guanine nucleotide, an Ets binding site is created. Studies looking at hundreds of patients with ovarian, breast, melanoma, endometrial carcinoma, and glioblastomas all found similar results – the cancer patient population had an increased prevalence of the 2G/2G genotype compared with healthy controls. Patients with the 2G polymorphism had tumors with significantly increased MMP-1 expression, which correlated with more aggressive cancer and metastasis (McCready *et al.,* 2005; Nishioka *et al.,* 2000; Kanamori *et al.,* 1999). Remarkably, this same polymorphism has been shown to increase periodontitis and arthritis susceptibility (Li, Feng *et al.,* 2015; Lepetsos *et al.,* 2014; Li, Peng *et al.,* 2015).

1.7 Transcription Factors of Interest: NF-kB and ZBP-89

This research project focuses on two transcription factors: Nuclear factor – kappaB (NFkB) and Zinc-Binding Protein-89 (ZBP-89). NF-kB is a family of proteins that function as important transcription factors. The dimeric transcription factor binds to DNA and regulates the expression of many genes involved in immunity, inflammation and cell cycle regulation. The NF-kB family of proteins is composed of two classes: class I - the 'NF-kB' proteins and class II - the 'Rel' proteins.The Rel proteins include c-Rel, RelB, and RelA (p65); while the NF-kB proteins include p105 and p100.Class I proteins, p105 and p100, cannot activate transcription on their own, unless bound with a class II-

Rel protein. Moreover, p105 and p100 must become shorter active proteins either by limited proteolysis or arrested translation; p105 and p100 become p50 and p52, respectively. This allows for a diverse combination of hetero and homodimers, which increases specificity, and range of gene targets (Reviewed in Gilmore, 2006).

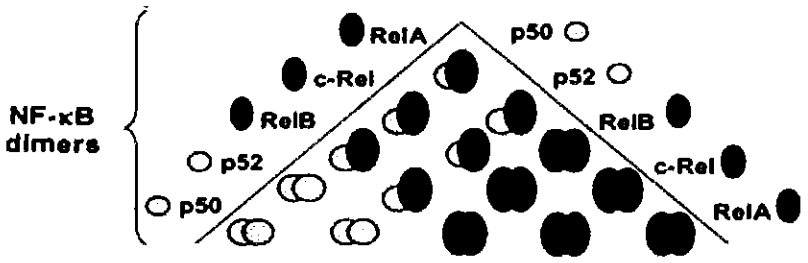

Figure 5: NF-kB possible dimers provide diverse gene targets (modified from Hoffmann, 2006)

Various signals such as oxidative stress, cytokines, viral and bacterial components can stimulate a cascade of signaling events leading to the activation of the NF-kB family of proteins. The predominant transcription factor dimer in most cells consists of RelA (p65) and p50 and is activated by the classical NF-kB signaling pathway, as depicted in Figure 6. In normal physiological states, IkB proteins in the cytoplasm bind to and inhibit NFkB, keeping it inactive until the cell is stimulated. Once the cell is stimulated by proinflammatory cytokines, LPS, or growth factors, IKK proteins (IkB kinases) are activated. As a result, active IKK phosphorylates the IkB molecule attached to NF-kB. Phosphorylated IkB molecule is ubiquitinated and targeted for proteasomal degradation, thus releasing the NF-kB transcription factor. Active NF-kB is translocated into the nucleus where it can bind to DNA and increase or decrease gene transcription (Vincenti et. al. 2002; Kida et. al., 2005).

Whether NF-kB acts as an activator or repressor of gene expression depends on various factors: the dimer composition, the signaling pathway, the cofactors, the target gene and the cell type. For example, NF-kB helps regulate fibrosis and extracellular matrix remodeling by decreasing collagen expression and increasing MMP gene expression. Rippe *et al.* conducted transfection experiments with NF-kB p50, RelA (p65), and c-Rel plasmids in collagen producing cell lines. Dose-response curves showed that p65 inhibited alpha1 collagen gene expression the most. Nuclear run-on assay showed that p65 also decreased endogenous alpha1 collagen gene expression (1999).

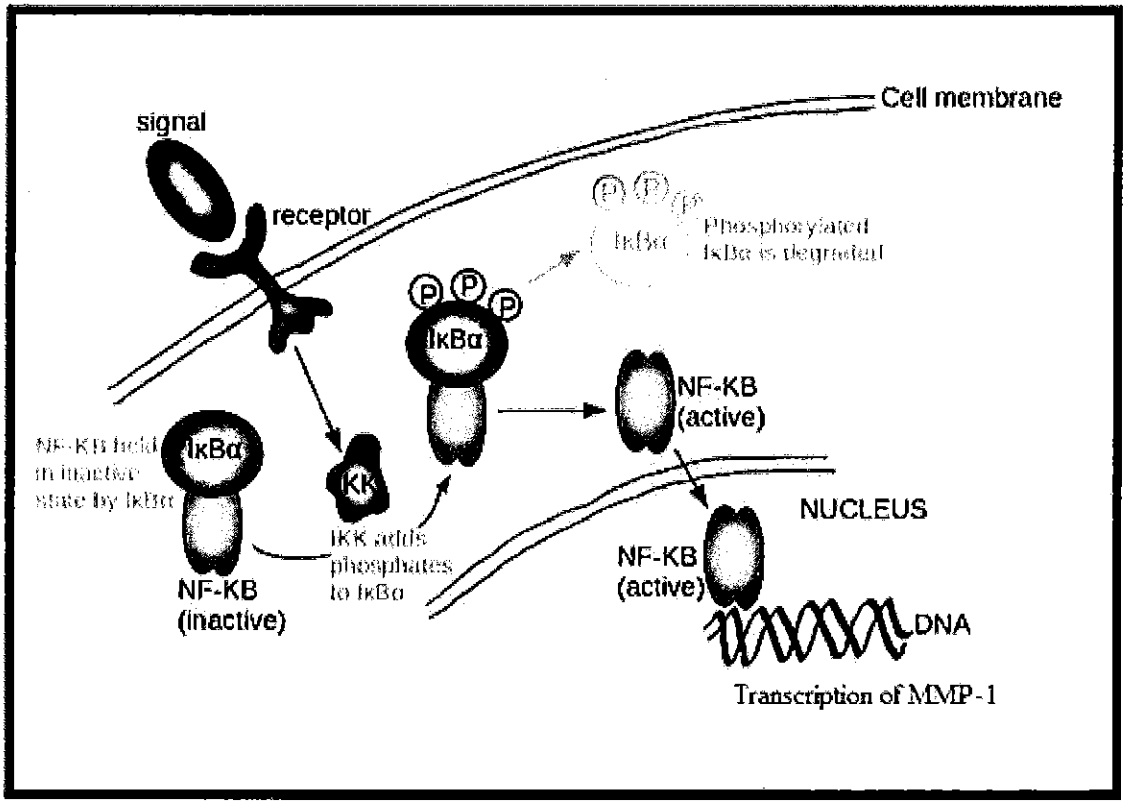

Figure 6: NF-kB Canonical/Classical Activation pathway (modified from Buddhini Samarasinghe 2014). In a stimulated cell, phosphorylation of IkB by IKK renders NF-kB free to translocate into the nucleus and bind to DNA.

Protein-protein interactions are an important factor that affects NF-kB transcriptional activity. For example, NF-kB has been found to have both negative and positive effects on activity of p53, a tumor suppressor protein. Wu and Lozano demonstrate cytokine induced NF-kB binding to the p53 promoter thus increasing gene expression and active p53 protein (1994). Jeong *et al.* studied the regulation of p53 in adult T-cell leukemia (ATL), and HTLV-1 transformed cells. This study found a new interaction between NF-kB and p53 that inhibited NFkB expression and activity. The human T-cell lymphotrophic virus type-I (HTVL-1), expresses a transactivator of viral protein, Tax. This study demonstrates that Tax induced NF-kB activation leads to p65-p53 protein-protein interaction and p53 inhibition. In addition, posttranslational modification of p53, such as phosphorylation, affects its activity and this study demonstrated that phosphorylation of p53 is essential for the p65-p53 complex formation in tumor cells (2004).

One of the first fundamental studies looking at cytokine induced MMP-1 expression found NF-kB to be an important gene regulator. This study looked at IL-1 induced MMP-1 expression. IL-1 is an important pro-inflammatory cytokine that triggers various catabolic reactions – such as the breakdown of extra cellular matrix. Vincenti *et al.*, found that NF-kB activates gene expression in rabbit synovial fibroblasts through a distal binding site on the MMP-1 promoter. This study helped our understanding of MMP-1 gene regulation and its implication in inflammatory disease, such as rheumatoid arthritis (Vincenti *et al.*, 1998).

A more recent study investigated the role of NF-kB induced expression of MMP-1 in tuberculosis (TB). TB is an infectious disease caused by mycobacteria and is

commonly characterized by scarring and lung tissue damage as a result of increased MMP-1 expression. In this study, human lung fibroblast cells were stimulated with conditioned media from Mtb-infected monocytes (CoMTb). Expression experiments and immunohistochemistry showed that CoMTb increased MMP-1 protein synthesis. Deleting the kB site (NF-kB binding site at -2,878kb) from the MMP-1 promoter abolished this induced response from CoMTb (O'kane *et al.,* 2010).

Zinc-Binding Protein-89 (ZBP-89) is a zinc finger transcription factor, which binds to GC-rich DNA, and often works in conjunction with other transcription factors to either increase or decrease gene expression. ZBP-89 is known to regulate many genes involved in cell growth, differentiation, and apoptosis and has a complex role in pathologies such as cancer (Yan *et al.*, 2014; Cai *et al.,* 2012). ZBP-89 can also have an epigenetic function, as it contributes to the regulation of histone and DNA modification - acetylation and methylation (Ye *et al.,* 2013; 2015).

Studies have shown that ZBP-89 can act either as a repressor or activator of gene expression, although the mechanism for this bi-functional activity is unknown. ZBP-89 is a gene activator of p21, T cell α- and β- receptor, tyrosine kinase (lck), and type 1 collagen but a gene repressor of gastrin, p16, SOX18, and vimentin as well as others (reviewed in Zhang *et al.* 2010). It is thought that ZBP-89's bi-functional activity is influenced by the gene sequence itself and protein-protein interactions (Zhang *et al,* 2010). On genes that it suppresses, ZBP-89 often competes for binding with other transcription factors, especially members of the Sp1 family (Law, *et al.* 1998; Keates, *et*

al. 2001). The competition between transcription factors Sp1 and ZBP-89 allows for balanced gene expression.

Although not much is known about the regulation of ZBP-89, studies have found that post-translation modification can influence ZBP-89's activity. For example, Bai et. al, has shown that ATM kinase phosphorylates ZBP-89 which weakens its ability to increase p21 expression (2007). In addition, Borghaei *et al.* have shown that inflammatory cytokine IL-1β inhibits ZBP-89 gene expression, suggesting that ZBP-89 is regulated during inflammation (2009).

As a transcription factor, ZBP-89 has been shown to act directly and indirectly. For example, ZBP-89 has been shown to bind directly to the Bak promoter and increase expression in hepatocellular carcinoma cells and significantly increase tumor apoptosis (To *et al.*, 2011). Interestingly, Ye el al. found that ZBP-89 can also enhance Bak expression by suppressing epigenetic enzymes, HDAC3 and DNMT1. Ye *et al.* used xenograft mouse tumor model to show that ZBP-89 repression of HDAC3 maintained histone acetylation of the Bak gene in HCC cells and increased Bak expression (Ye, 2013). Although studies have shown that ZBP-89 can enhance gene expression through histone modification, other studies have shown that ZBP-89 can repress gene expression through histone modification as well. For instance, Feng *et al.* have demonstrated that ZBP-89 recruits HDAC3 and inhibits expression of p16, an inhibitor of cyclin dependent kinases such as CDK4 and CDK6 (2009).

ZBP-89 interactions with other transcription factors, including p300, HDAC, Sp1, YY1, STAT3, p53 and NF-kB can determine its effects on gene expression (Bai et 2000, Zhang, *et al* 2003; Boopathi, *et al* 2004, Wu et al., 2004; Wu *et al.* 2009, Woo *et al.*

2011, Bai, *et al* 2001; Borghaei, *et al* 2016). For example, ZBP-89 has been shown to cooperate with STAT3 to increase vimentin gene expression (Wu *et al.* 2004). However, a few years later it was reported that vimentin gene expression is inhibited instead of enhanced when ZBP-89 cooperated with HDAC (Wu *et al.* 2009).

The most well studied protein interaction is between ZBP-89 and tumor suppressor protein, p53. Bai et al. showed ZBP-89 can induce apoptosis in human gastrointestinal cell lines through a p53 mediated pathway. This study found that ZBP-89 physically bound to p53, stabilizing it and preventing it from leaving the cell nucleus, which allowed for increased p53 activity (Bai *et al.,* 2001). Later studies further demonstrated the ZBP-89 –p53 interaction in hepatocellular carcinoma cells. Chen *et al.* found that ZBP-89 co-localized with p53 in the nucleus in 67% of all HCC patients positive for p53. This study also found that patients who had ZBP-89/p53 co-localized in the nucleus where more responsive to treatment (Chen *et al,* 2006). This is consistent with Dr. Zang's study that found patients with increased expression of ZBP-89 in cancerous HCC cells as compared to adjacent non-cancerous liver cells showed better survival (2012).

Recent studies have found an interesting relationship between ZBP-89 and NF-kB. Both transcription factors can act as activators or repressors and have been shown to interact with many of the same proteins, (i.e. p53). Both have also been implicated in MMP gene regulation. For example, ZBP-89 binds to a polymorphic site in the MMP-3 promoter with NF-kB (Borghaei *et al.,* 2009). Recent evidence is more consistent with cooperation rather than competition of this binding site. This study also showed that ZBP-89 can physically interact with p50 and p65 (Borghaei *et al.* 2016). Ye *et al.,*

suggests that ZBP-89 may have a role in regulating NF-kB activity. This study indicates that ZBP-89 can increase levels of phosphorylated IkB, thus increasing active NF-kB levels (2015). These recent developments prompt further curiosity of the relationship between NF-kB and ZBP-89.

1.8 Preliminary data:

After sequence analysis of the human MMP-1 promoter found two possible ZBP-89 binding sites, this lab began to investigate ZBP-89's role in MMP-1 gene expression. Preliminary experiments looked at ZBP-89's effect on basal and cytokine induced expression of MMP-1 in MG-63 osteosarcoma cell lines. To do this, a ZBP-89 knockdown cell line was created using RNA interference. Using RT-PCR, ZBP-89 gene knock-down was shown to decrease cytokine induced MMP-1 gene expression (Figure 7). Furthermore, a chromatin immunoprecipitation (ChIP) assay showed increased ZBP-89 and NF-kB binding to the endogenous MMP-1 promoter in the negative control line treated with TNFα, as compared to the untreated cells (Figure 8).

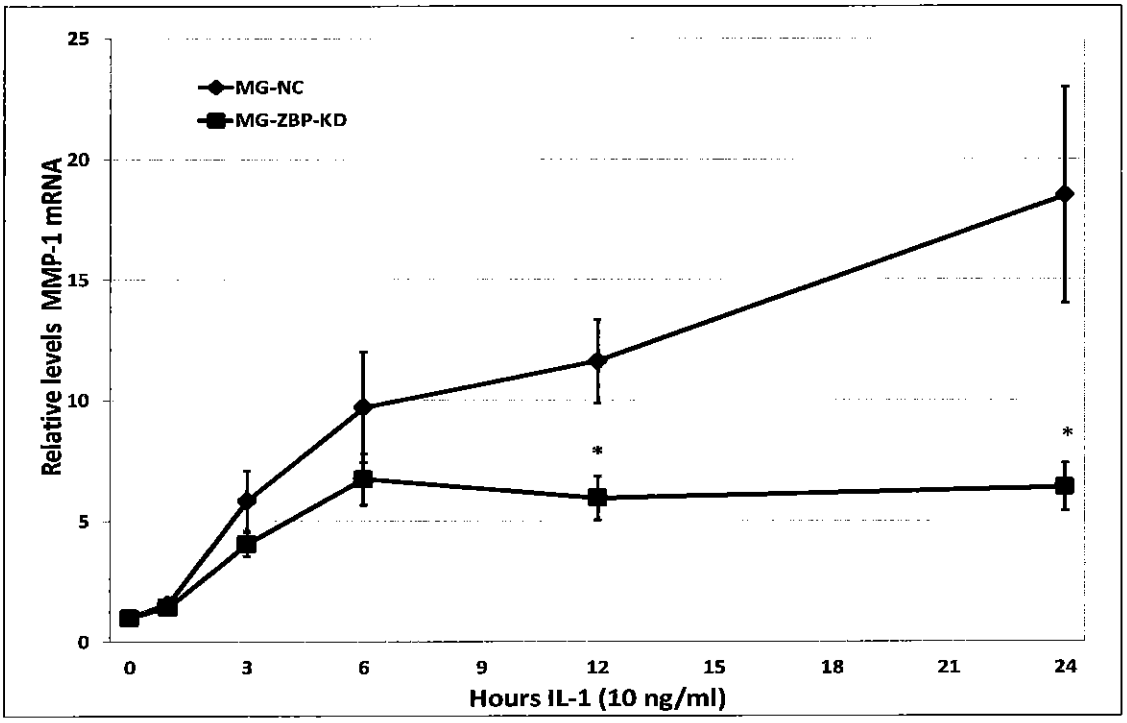

Figure 7: Effect of ZBP-89 knockdown on cytokine-induced expression of MMP-1 mRNA in MG-NC and MG-ZBP-KD cell line. Total RNA was harvested from ZBP-89 knock-down (MG-ZBP-KD) and negative control cells (MG-NC) left untreated or treated with IL-1b for the indicated times. MMP-1 mRNA levels were quantified by real-time PCR, normalized to levels of GAPDH mRNA, and expressed relative to levels in the untreated cells. Similar results were found when treated with TNFa (from Borghaei *et al* 2016).

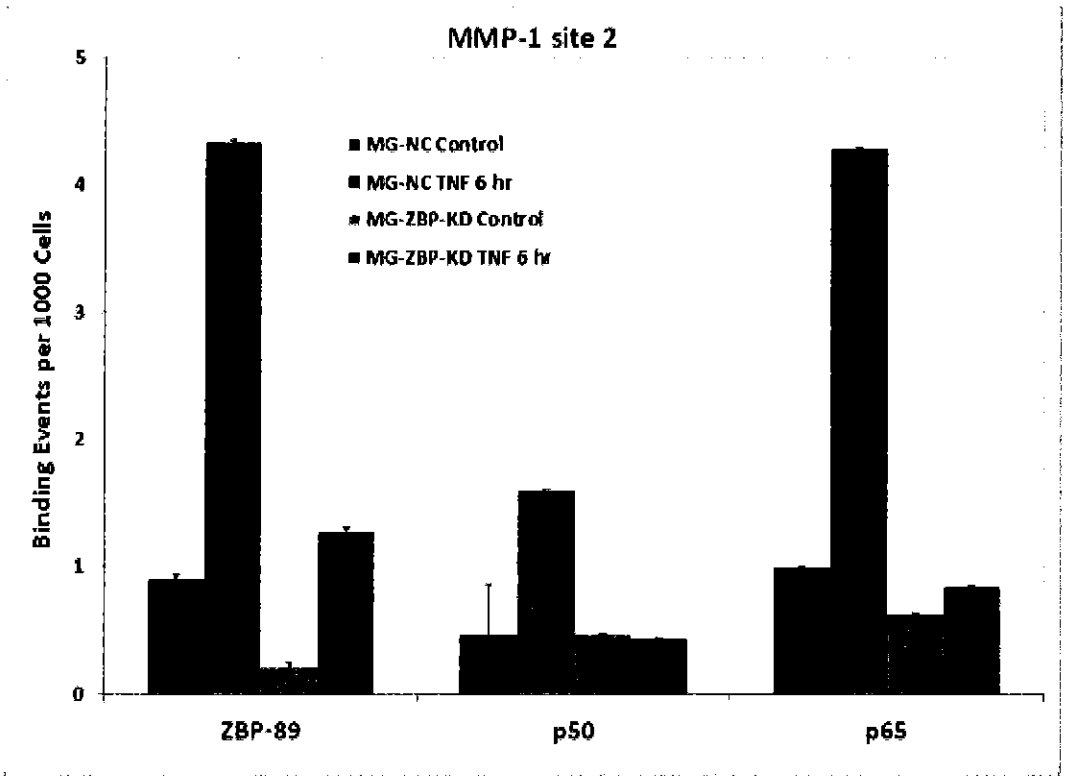

Figure 8: MG-NC and MG-ZBP-89 cells were left untreated (control) or treated with TNFα for 6 hours. ChIP assays were completed using the ChIP-IT High Sensitivity kit (Active Motif) and analyzed using the ChIP-IT qPCR Analysis Kit (Active Motif) with primers to amplify -1970 to -1790 of the MMP-1 promoter. Binding units per 1000 cells were normalized to levels of binding of RNA polymerase II to the GAPDH promoter (from Borghaei *et al* 2016).

The same Figure shows binding of NF-kB on the same MMP-1 promoter site in the negative control cell line treated with TNFα. Even more interesting, binding of both ZBP-89 and NF-kB decrease in the ZBP-89 knockdown cell line. It is this experiment that initiated our interest in the interaction between ZBP-89 and NF-kB on MMP-1 promoter. It is important to note that MG63 cells lack a functioning p53 gene due to a mutation between the first and second exon (Masuda *et. al.,* 1987; Roepke *et. al.* 2007). Tumor suppressor, p53 is known to interact with both ZBP-89 and NF-kB, and can play

an important role in gene regulation (refer to section 1.7).

1.9 Research goal and hypothesis

MMPs are a promising target for therapeutics in many pathologies. Clinical trials of synthetic MMP inhibitors and small-biological molecule inhibitors have failed much due to lack of specificity and side-effects. Furthering our knowledge of how MMP-1 gene expression is regulated can provide novel insight into a different therapeutic approach. This research aims to investigate the roles of ZBP-89 and NF-kB in basal and cytokine induced MMP-1 gene expression. We hypothesize that ZBP-89 has a direct role in MMP-1 gene expression by binding to the site at -1969 bp of the promoter, in cooperation with NF-kB, and together these transcription factors increase cytokine-induced transcription.

2. Methods and Materials

2.1 Cell cultures and treatment

The monkey (*Cercopithecus aethiops*) fibroblast-like kidney cell line, COS1, was obtained from the American Type Culture Collection (ATCC CRL-1650). All cells were maintained in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic/Antimicotic (Gibco). Cells were incubated at 37°C in 5% CO_2. The passage number for the COS1 cells was 10-12.

Human lung carcinoma cell line, A549 was obtained from ATCC (CCL-185). A549-ZBP-KD3 cells were derived by stable transfection of small hairpin RNA (shRNA) ZBP-89/ZNF148; and A549-NC cells were derived by stable transfections of a scrambled shRNA negative control (SABioscinces). The two cell lines were maintained in parallel in ATCC-formulated F-12K Medium, which is supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic/Antimicotic (Gibco). The cells were incubated at 37°C in 5% CO2.

2.2 Plasmids

pGL3-MMP1 (referred to as "longMMP-1") contains 2.2kb of the human MMP1 promoter within the pGL3 luciferase reporter plasmid. This MMP1 promoter includes the putative ZBP-89 binding site at -1969, but lacks the NF-kB site at -2878. The reporter plasmid includes the *luc*+ gene, encoding firefly luciferase; and Amp^r, the gene providing ampicillin resistance in *E. coli*. The pGL3-MMP1 was obtained from Dr.

Maryam Rohani, Cedars Sinai Medical Center (J. Investigative Dermatology 2014, 134 (5) 1230 – 1237).

pLightswitch-MMP1 (referred to as "shortMMP-1) contains 1.12kb of the human MMP1 promoter within the lightswitch reporter plasmid. The shorter MMP1 promoter lacks the putative ZBP-89 binding site but maintains proximal AP-1 and Ets sites. The reporter plasmid includes *RenSP*, an optimized Renilla luciferase gene and the *Amp*^{r} gene providing ampicillin resistance in *E. coli*. The pLightswitch-MMP1 was purchased from Switchgear Genomics.

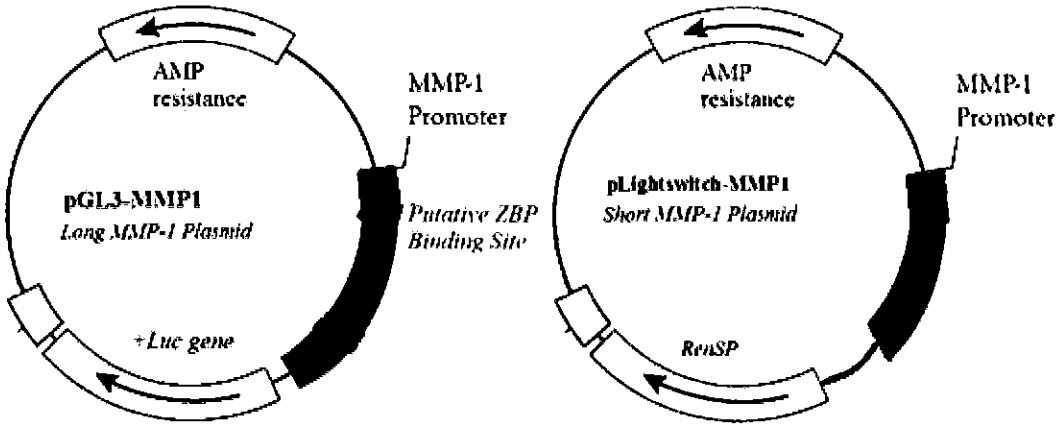

Figure 9: Schematic of Long and Short MMP-1 Plasmids

The ZBP-89 expression plasmid contains the ZBP-89 coding sequence with an N-terminal Myc-DDK tag under the control of the cytomegalovirus promoter (pCMV6-XL4 vector), as well as *Kan*^{r}, a gene providing Kanamycin resistance in *E. coli*. This plasmid was purchased from OriGene.

pCMV-βgal contains a cytomegalovirus promoter driving expression of the *GLB1* gene, coding for β-galactosidase, and the *Amp*^{r} gene providing ampicillin resistance in *E.*

coli. The pCMV- βgal was purchased from Clontech. The pCMV- βgal is co-transfected with pGL3-MMP1 as a normalizing control for transfection efficiency.

pLightswitch GAPDH contains the glyceraldehyde 3-phosphate dehydrogenase promoter within the lightswitch reporter plasmid. The reporter plasmid includes *RenSP*, an optimized Renilla luciferase gene, and the *Amp*^{r} gene providing ampicillin resistance in *E. coli*. The pLightswitch GAPDH is transfected in parallel with pLightswitch-MMP1 as a positive control. The pLightswitch GAPDH was purchased from Switchgear Genomics.

pBlueScript II is an empty cloning vector containing only Amp^r. It is used instead of the ZBP-89 and/or p65 expression plasmids to maintain consistency of DNA quantities in experiments comparing MMP1 expression with and without ZBP-89. The pBlueScript plasmid was purchased from Agilent Technologies.

pCMV-NFkB-p65 contains a cytomegalovirus promoter driving expression of *RELA* gene, coding for nuclear factor-kappa-B p65 subunit, and the *nptII* gene providing kanamycin resistance. The pCMV-NFkB-p65 was purchased from OriGene.

2.3 Transformation and Plasmid Isolation

The transformation was conducted within chemically competent D5H-α *E. coli* cells (Invitrogen) which are stored in -80°C. Cells and plasmid DNA are thawed on ice prior to starting transformation. 50 µl of D5H-α *E. coli* cells are incubated with 5-20 µg of desired plasmid DNA (roughly 2 µl) on ice for 30 minutes, followed by heat shock in a 42°C water bath for 45 seconds. The transformed cells are incubated in 500 µl Lysogeny

broth (LB medium – 10g Bactotryptone, 5g bacto-yeast extract, 10g NaCl per liter of media) with the appropriate antibiotic for the plasmid being transformed (100ug/ml of antibiotic) in a 37°C shaker for 1hour. 75µl of the transformed cells are plated on agar plates. Agar plates are prepared with antibiotic containing media, (10g bactotryptone, 5g bacto-yeast extract,10g NaCl, and 15g agar per 1L). The plates are incubated overnight in an incubator at 37°C.

A single colony was selected to inoculate an overnight culture, from which the plasmid is isolated using the ZymoPURE Plasmid Maxiprep Kit (ZymoReaserch) with slight modification of the manufacturer's suggested protocol. 50ml of cultured cells is centrifuged for ten minutes at 3,400xg. The supernatant is discarded and the pellet is resuspended in 10ml of Buffer P1. 10ml of Buffer P2 is added to the solution and incubated for 2-3minutes at room temperature. 10ml of Buffer P3 is added to the solution and mixed by pipetting up and down. This solution is poured into a capped syringe and incubated at room temperature for 10minutes. The solution is pushed through the filter into a new 50ml conical tube. 10ml of binding buffer is added and the solution is mixed by inverting 10 times. Using the provided V-P column, the entire solution is vacuumed through the filter. 5ml of wash 1 is added and vacuumed. 5ml of wash2 is added and vacuumed. This step is repeated once more. The final spin column is centrifuged for 1 minute to let any excess buffers pass through. 60µl of Elution buffer is added directly to the column matrix and incubated for 5minutes at room temperature. The collecting tube is centrifuged at 10,000 rpm for 1 minute to collect the plasmid DNA in solution. The integrity of the isolated plasmid DNA is confirmed by agarose gel electrophoresis and concentration is determined by measurement in a spectrophotometer (NanoDrop 2000).

The ratio of absorbance at 260nm/280nm is used as an indication of purity, and plasmids with ratios near 2.0 are suitable for transfection.

2.4 Transfection

Approximately, 5 x 10^4 COS cells/well are plated in a 6 well plate and transfected 24 hours later, when the cells have reached 70-90% confluence. The ZBP-89 or pBlueScript is transfected along with pCMV-βgal and pGL3-MMP1 plasmids, using Lipofectamine 2000 DNA as the transcfection reagent (Invitrogen). 10μl of Lipofectamine reagent is diluted in 500μl of Opti-MEM Medium (Invitrogen). 4μg of total plasmid DNA (1.3 μg of each plasmid) is diluted in 500μl of Opti-MEM Medium. The diluted DNA is added to the diluted Lipofectamine (1:1 ratio), and this mixture is left at room temperature for 20 minutes. The Opti-MEM-Lipofectamine-DNA mixture is added to each well of cells and incubated at 37°C for 5 hours, then 2ml of DMEM growth media is added to each well. The 6 well plate is then incubated at 37°C for 48 hours. The transfected cells are analyzed using the Luciferase Assay with reporter lysis buffer (Promega) and the β galactosidase Assay (Clonetech) according to the manufacturers' suggested protocols. The results are read using a TD-20/20 Luminometer.

For transfection of pLightswitch GAPDH and pLightswitch-MMP1 plasmids, approximately 2 x10^4 cells (either COS-1 or A549 cells) are plated per well in a 96 well plate. Twenty-four hours later, when the cells have reached 70-90% confluence, plasmids are transfected using the FuGene HD Transfection reagent (Promega). 2μg of total plasmid DNA (1μg of each plasmid) is added to 30μl of DMEM. 4μl of FuGene is

added to this solution and incubated at room temperature for 30 minutes. 5µl of the solution is added to each well of a 96-well plate, which is then incubated at 37°C for 48hours. The transfected cells are analyzed using the LightSwitch Luciferase Assay Kit (Active Motif) according to the manufacturer's suggested protocol. Results are read using a BioTek synergy2 microplate luminometer and Gen5 software.

Transfection of pGL3-MMP1 plasmids in A549 cells is accomplished with a slightly modified protocol using FuGene reagent in 6 well- plates. 4 µg of total plasmid DNA is added to 150µl of A549 specific – F 12K media. 10µl of FuGene is added to this solution and incubated at room temperature for 30 minutes. 150µl of the solution is added to each well, incubated at 37°C for 48hours. After 24 hours 10ng/ml of cytokine IL-1 is added in specified experiment wells. The transfected cells are analyzed using the Luciferase Assay with reporter lysis buffer (Promega) and the β galactosidase Assay (Clonetech) according to the manufacturers' suggested protocols. The results are read using a TD-20/20 Luminometer.

2.5 Statistical Analysis:

Data is expressed as the mean of three or more experiments $\pm$ standard error of the mean (SEM). Statistical analysis was determined using t-test for paired data, and one way ANOVA with post-hoc Bonferroni for analysis of multiple groups. A probability of less than 0.05 was considered statistically significant.

3. Results

3. 1 Effect of ZBP-89 transcription factor on short and long MMP-1 expression in COS-1 cells

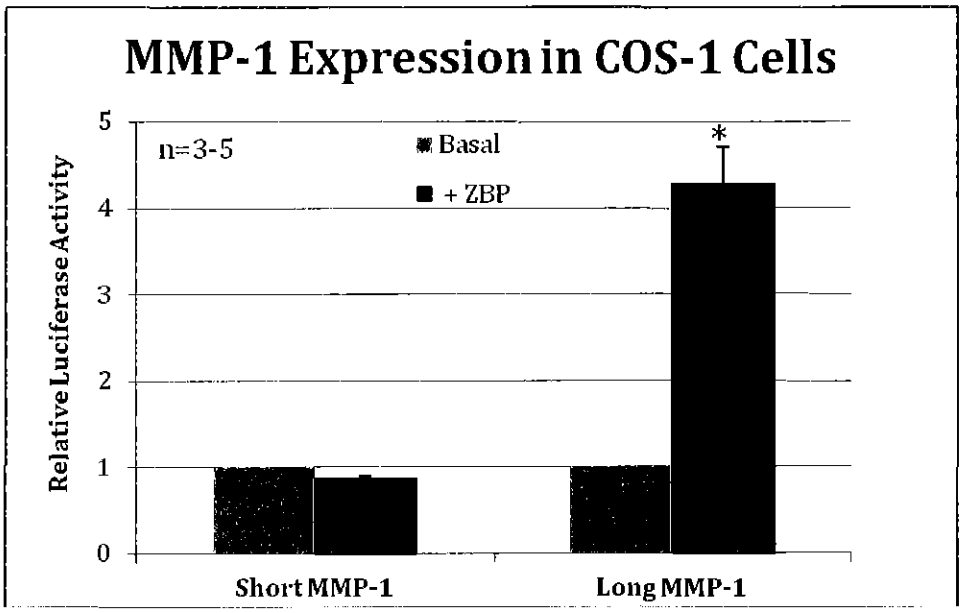

Figure 10: Short and Long MMP-1 expression in COS-1 cells with ZBP-89 and without (basal). COS-1 cells were transiently transfected with luciferase reporter plasmids containing 2 kb (Long) and 1.1 kb (Short) fragments of the human MMP-1 promoter, in the presence and absence of a ZBP-89 expression vector. Results are from 3 independent experiments performed in triplicate, expressed as fold increase over no ZBP-89 controls. Results are statistically different as determined by t-test. * $p<0.05$. (from Borghaei *et al.*, 2016)

The long MMP-1 plasmid (pGL3-MMP1) and short MMP-1 plasmid (pLightswitch-MMP1) were transfected in COS-1 cells, either with or without the ZBP-89 expression vector. As Figure 10 indicates, addition of ZBP-89 increased expression from the longer version of the MMP-1 promoter by approximately 4 fold ($p<0.05$). Addition of ZBP-89 had no effect on expression from the shorter version of the MMP-1

promoter.

3.2 Combined effect of ZBP-89 and NF-kB on long MMP-1 expression in COS-1

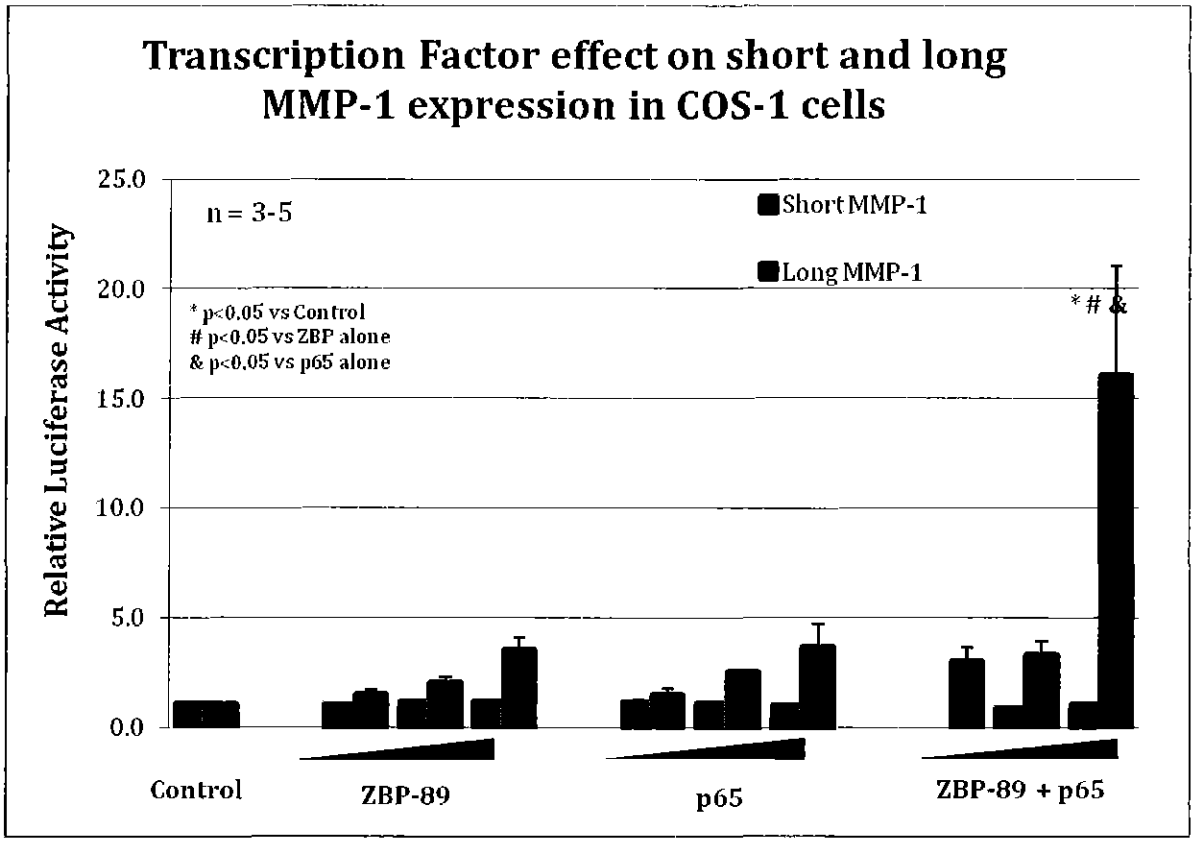

Figure 11: Transfection study demonstrating the effects of increasing amounts of ZBP-89, p65, and both transcription factors together on transcription from the MMP-1 promoter. COS-1 cells were transiently transfected with luciferase reporter plasmids containing 2 kb (Long) and 1.1 kb (Short) fragments of the human MMP-1 promoter, in the presence and absence of a ZBP-89 and/ or a p65 expression vector. Results are from at least 3 independent experiments performed in triplicate, expressed as fold increase over basal controls. Symbols indicate statistical significance determined by one-way ANOVA with post-hoc Bonferroni.

Figure 11 shows the cumulative data for experiments using short and long MMP-1 plasmids in COS-1 cells. The data shows the change of MMP-1 expression with increasing amounts of ZBP-89, increasing amounts of NF-kB (p65), and combined

effects of increasing amount of ZBP-89 and NF-kB (p65). As the graph shows, long MMP-1 plasmid expression increased with increasing amounts of transcription factor. On the other hand, the addition of transcription factors had no effect on short MMP-1 plasmid expression. Moreover, ZBP-89 and NF-kB seem to have a synergistic effect on expression from the long MMP-1 promoter. The highest dose of the combined transcription factors increased expression more than either transcription factor alone ($p<0.05$).

3.3 Combined effect of ZBP-89 and NF-kB on short MMP-1 expression in A549 NC and A549 KD

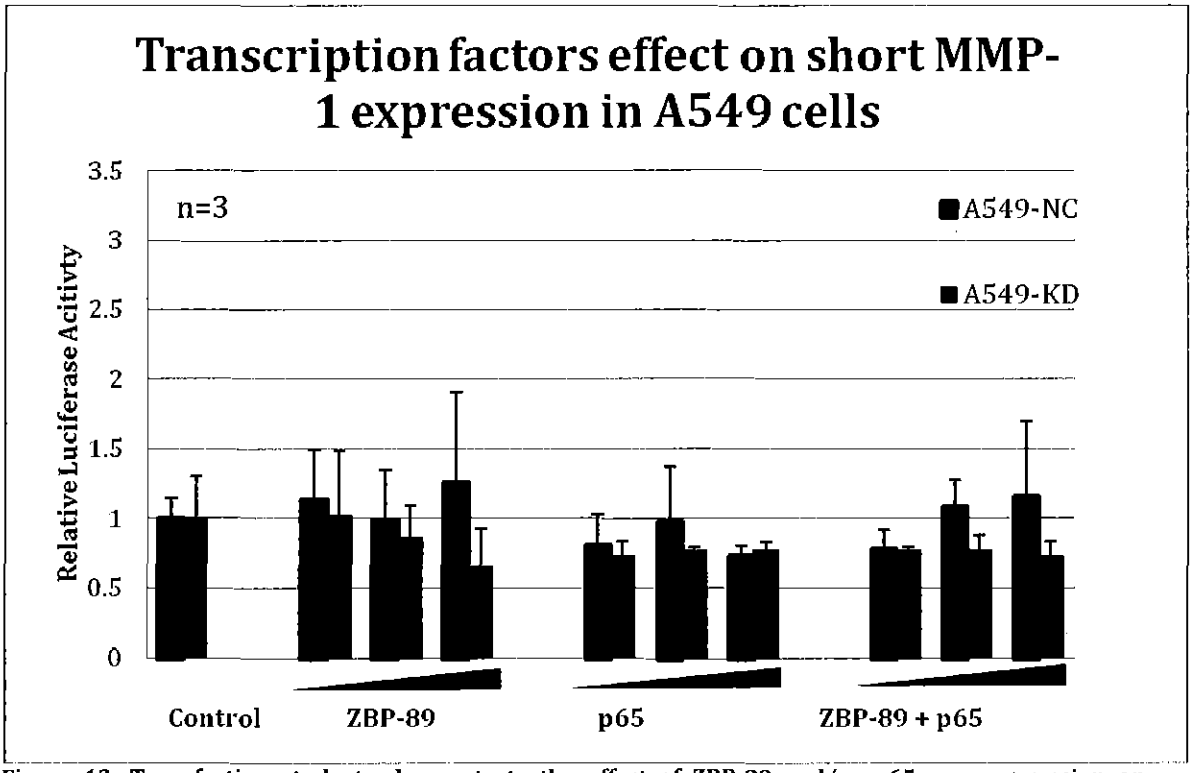

Figure 12: Transfection study to demonstrate the effect of ZBP-89 and/or p65 over-expression on transcription from the short MMP-1 promoter. Results are from at least 3 independent experiments performed in triplicate, expressed as fold increase over basal controls. No statistical difference was found within or between cell lines.

Figure 12 depicts the effects of transcription factor ZBP-89 and NF-kB on short MMP-1 plasmid in both negative control A549 cells and the ZBP-89 knock down cell line. The graph shows the relative change in MMP-1 expression with the addition of ZBP or NF-kB as compared to control (overexpressed MMP-1 expression without the addition of transcription factor). MMP-1 expression did not significantly change in either A549 cell line with the addition of transcription factor. This experiment was repeated once with A549-NC and twice with A549 KD with the addition of IL-1 cytokine and no effect was seen (data not shown).

3.4 Cytokine induced MMP-1 expression in human lung carcinoma A549 negative control and ZBP-knock down cell lines.

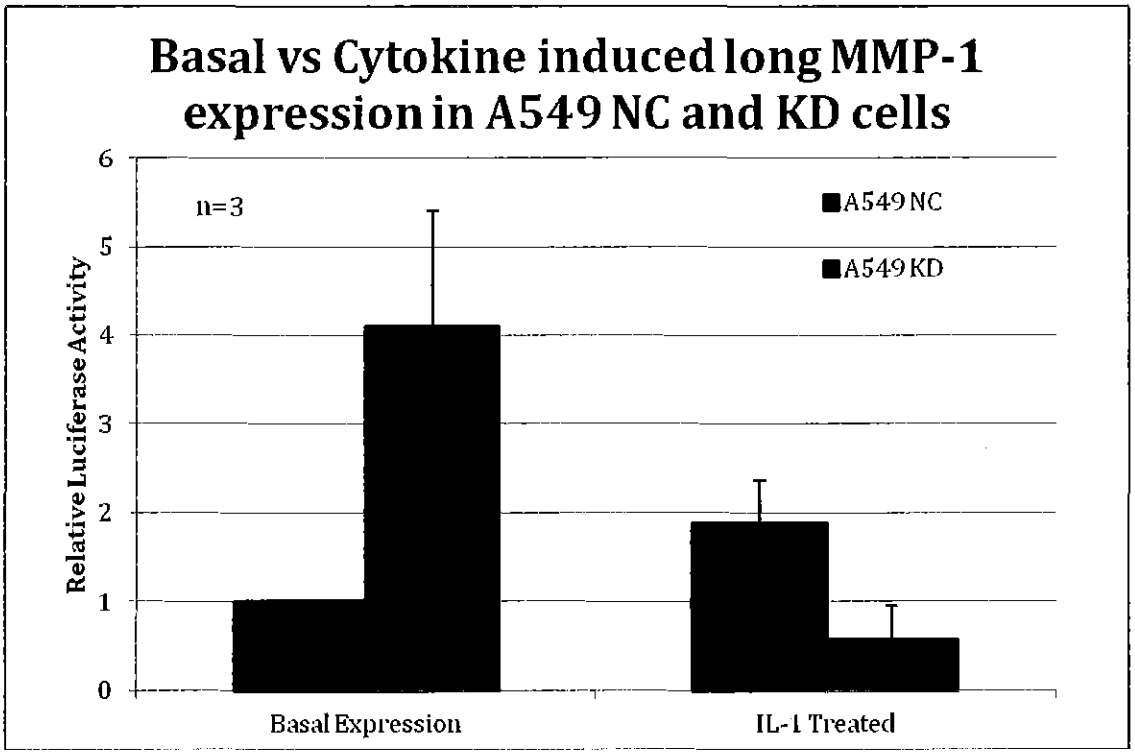

Figure 13: Effect of ZBP-89 knock-down on basal and IL-1 induced expression from the long MMP-1 promoter. A549 cells (NC or KD) were transiently transfected with luciferase reporter plasmids containing 2 kb (Long) fragments of the human MMP-1 promoter, with and without IL-1 (10ng/ml) treatment. Results are represented in arbitrary units of relative luciferase activity.

Figure 13 shows the results of basal and IL-1 induced expression of long MMP-1 plasmid in A549 negative control and ZBP-knock-down cell lines. The data was not statistically significant, however a trend indicated that long MMP-1 basal expression is higher in the knock-down cell line than in the negative control. This suggests ZBP-89 represses MMP-1 expression under basal conditions. Cytokine IL-1 induced expression of long MMP-1 is slightly increased in the negative control cell lines, but repressed in the ZBP-knock down cells. This suggests that ZBP-89 acts as an activator under cytokine conditions.

4. Discussion

The matrix metalloproteinase (MMP) family digests the macromolecules of the extracellular matrix, and thereby helps to regulate important biological processes such as the cell cycle, apoptosis, and tissue repair. However, the unregulated catabolism of collagen by MMP-1 can yield negative symptoms in a number of diseases. It can cause tooth loss in patients with periodontitis, joint damage in patients with arthritis, and metastasis in patients with cancer, which is often fatal. Considering the widespread and devastating effects of cancer and chronic inflammatory disease, researchers have identified MMP inhibitors as a potential therapeutic target. To date, most of the clinical trial results have been disappointing, with only one MMP inhibitor currently on the market. Understanding the mechanisms involved in balanced expression of MMPs might provide critical information for drug design. This study's findings can help guide the development of MMP inhibitors that can be used to treat pervasive and painful diseases.

To better understand MMP expression, we asked the following questions: does ZBP-89 affect MMP-1 expression, and if so, in what capacity? MMP-1 gene expression is regulated by cytokines and strong transcription factors such as AP-1, ETS, and NF-kB. Preliminary data shows that cytokine-induced expression of MMP-1 is decreased by gene knock-down of ZBP-89 in MG-63 osteosarcoma cells (Figure 7). This is the first evidence that suggests ZBP-89 may have a role (direct or indirect) in regulating MMP-1 gene expression. The results of ChIP assays demonstrated that ZBP-89, together with NF-kB, can bind to the endogenous MMP-1 promoter following stimulation with TNFα

(Figure 8). This suggests that MMP-1, together with NF-kB, has a direct role in gene regulation. This was further supported by a co-immunoprecipitation experiment that showed p65 and ZBP-89 can physically interact, or can at least be parts of a larger protein complex (Borghaei, *et al.* 2016). In MG-63 osteosarcoma cells, it appears that ZBP-89 and NF-kB are most likely working together to increase transcription of both MMP-1 and MMP-3 in response to inflammatory cytokines. This indicates that ZBP-89 has a larger role in the regulation of MMPs than was previously known (Borghaei *et al.* 2016). However, ZBP-89 and especially NF-kB are known to be affected by cellular context, and MG-63 cells are deficient in p53 (Mills *et al.* 2009), which is known to interact with both factors (Jeong *et al.* 2004; Bai *et al.*, 2001). To account for this limitation, new ZBP-89 knock-down and negative control cell lines were established using A549 lung adenocarcinoma cells (Gorski, unpublished data).

The goal of this research study was to investigate two questions: 1) does ZBP-89 play a direct role in MMP-1 gene regulation; 2) what is the relationship between ZBP-89 and NF-kB in regulation of MMP-1 expression under basal and cytokine induced conditions. Our hypothesis was that ZBP-89 has a direct role in MMP-1 gene expression by binding to the upstream site at -1969bp of the promoter, in cooperation with NF-kB, and that together these transcription factors increase cytokine-mediated transcription.

Two different luciferase reporter constructs were used in transfection studies. Each contained different fragments of the MMP-1 promoter – the "long" construct contains 2.2kb of the human MMP-1 5' flanking sequence, including the putative ZBP-89/NF-kB site at -1976, while the "short" construct contains a 1.12kb fragment that lacks the putative ZBP-89/NF-kB site but retains the proximal AP-1 and ETS sites. The fact

that overexpression of ZBP-89 was able to increase transcription from the long plasmid, but had no effect on the shorter version (Figure 10) is consistent with a direct and positive role for ZBP-89 in regulation of MMP-1 expression through the putative site, rather than an indirect effect mediated through the AP-1/ETS sites. However, since the two reporter constructs differ by 1kb, these data do not definitively show that ZBP-89 is necessarily working directly through the putative site. Site-directed mutagenesis of the putative site in the context of the larger construct would allow a more definitive conclusion to be drawn regarding the putative binding site. Unfortunately, our attempts to effectively mutate the binding site were unsuccessful.

Over-expression of NF-kB p65 also modestly increased expression from the longer, but not the shorter MMP-1 reporter plasmid, suggesting that it too might have a direct role through the putative site. Most importantly, the two transcription factors showed a synergistically positive effect on the longer promoter construct when over-expressed together. These results suggest that the relationship between ZBP-89 and NF-kB as related to regulation of MMP-1 expression is cooperative rather than competitive.

This confirms our prediction that the two transcription factors on MMP-1 expression would have a positive additive or synergistic effect. These results differ somewhat from similar experiments performed with the MMP-3 promoter, in which ZBP-89 seemed to activate while NF-kB inhibited transcription (Borghaei, 2009). Thus, although ZBP-89 and NF-kB have been shown to bind together and affect transcription from two different MMP promoters, their effects on the two genes are not identical.

COS-1 cells are an easily transfected and commonly used immortalized fibroblast-like cell line derived from monkey renal epithelial cells. They do not, however,

exhibit increased expression of MMPs in response to cytokines, which make them a suboptimal candidate for studies of inflammatory responses. To address this limitation, we attempted to transfect the ZBP-89 knock-down and negative control cell lines derived from MG-63 osteosarcoma cells, used in preliminary experiments as described in the introduction. These attempts failed to generate sufficient transfection efficiency to provide interpretable data. Therefore, we derived another pair of knock-down and negative control cell lines from A549 human lung adenocarinocma cells to repeat this experiment as well as compare the effects of ZBP-89 knock-down on MMP expression in a different cellular context (Gorski, unpublished). .

The A549 negative control (NC) and ZBP-knock down (KD) cells were successfully transfected with the shorter version of the MMP-1 promoter, but addition of ZBP-89 and/or NF-kB p65 had no effect on expression from that promoter fragment (Figure 12). This is consistent with both our results in COS cells and our hypothesis that ZBP-89 and NF-kB act through an upstream binding site missing in the truncated MMP-1 plasmid. Transfection studies using the longer MMP-1 promoter proved to be technically more challenging due to difficulties in detecting and measuring renilla luciferase production using the system currently available. This resulted in more variability than expected. However, although the results are not statistically significant, the trends suggest that ZBP-89 knockdown might increase basal expression of MMP-1 in A549 cells, and decrease induced expression in the presence of IL-1 (Figure 13). If these results can be replicated in a more reliable system, they would establish that ZBP-89 acts as a repressor under basal conditions, which appears to contradict our findings in the

COS-1 cells, in which overexpression increased expression from the long MMP-1 promoter.

The presence of a transcriptional repressor to limit MMP-1 expression under basal conditions is physiologically plausible. The function of MMP-1 is to catabolize the extracellular matrix, which is vital for wound healing or tissue remodeling. Yet, under most normal conditions, the body needs less MMP-1 to preserve the extracellular matrix. Under basal conditions, MMP-1 expression is expected to be repressed, and our experimental findings in the A549 cells suggest that ZBP-89 contributes to this down regulation. The negative control cell line, which has functional ZBP-89, shows less MMP-1 basal expression. When ZBP-89 is removed by gene knock-down, long MMP-1 expression increases.

Why does ZBP-89 seem to activate MMP-1 expression in COS cells, but repress it in A549 cells? One key difference between these two experiments is the source of ZBP-89. Exogenous ZBP-89 was added at supra-physiologic levels to induce expression in the COS-1 cells, whereas, in the negative control A549 cells, ZBP-89 is expressed at low normal levels from the endogenous gene. Another difference is the cellular environment, potentially including different basal levels of NF-kB and/or coactivators, which could influence the effects of ZBP-89. For example, ZBP-89 binds to a site in the MMP-3 promoter that functions as a repressor element in some cells but not in others (Borghaei *et al.* 1999; Ye *et al.* 1996; Borghaei *et al.* 2009), and the role of that polymorphic binding site in determining levels of MMP-3 protein in vivo is different in tissue compared to serum (Holiday *et al.* 2007; Samnegard *et al.*, 2005).

Although the exact roles of ZBP-89 and NF-kB in regulation of MMP-1 expression under inflammatory conditions could not be determined, these results do support a direct role for ZBP-89, in cooperation with NF-kB, in regulation of MMP-1 gene expression. To our knowledge, this is the first report of a role for ZBP-89 in regulating this important gene. Taken together with existing data demonstrating complicated and cell-specific effects of these two transcription factors in regulating expression of MMP-3 (Borghaei *et al.* 1999, 2009; Bond *et al.* 2001), a more general role for ZBP-89 in regulating remodeling of the extracellular matrix under normal and pathological conditions is suggested. Additionally, the synergistic effects we observed between ZBP-89 and NF-kB suggests the possibility of a broader role for this lesser known transcription factor in modulating gene expression during inflammation.

5. Conclusion

ZBP-89 is an important zinc finger transcription factor involved in regulating genes that influence cell growth, apoptosis, and other important biological processes. NF-kB is a protein complex that regulates gene transcription and is a key influencer of the human immune system. This research shows that these two important transcription factors can cooperatively increase expression of MMP-1. MMP-1 is one of many proteinases that help build and rebuild tissue and the extra-cellular matrix. MMP-1 also plays a key role in cancer metastasis and inflammatory disease. Elucidating the roles of regulators of MMP-1 gene expression, such as ZBP-89 and NF-kB, in mediating balanced expression under various conditions will aid in the development of therapies for various pathologies.

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