

University of New Hampshire

## University of New Hampshire Scholars' Repository

---

Doctoral Dissertations

Student Scholarship

---

Spring 2019

### THE ROLE OF THE TUMOR MICROENVIRONMENT IN WALDENSTRÖM MACROGLOBULINEMIA

Weiguo Han

*University of New Hampshire, Durham*

Follow this and additional works at: <https://scholars.unh.edu/dissertation>

---

#### Recommended Citation

Han, Weiguo, "THE ROLE OF THE TUMOR MICROENVIRONMENT IN WALDENSTRÖM  
MACROGLOBULINEMIA" (2019). *Doctoral Dissertations*. 2447.

<https://scholars.unh.edu/dissertation/2447>

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact [Scholarly.Communication@unh.edu](mailto:Scholarly.Communication@unh.edu).

THE ROLE OF THE TUMOR MICROENVIRONMENT IN  
WALDENSTRÖM MACROGLOBULINEMIA

BY

WEIGUO HAN

B.S. IN ECOLOGY, OCEAN UNIVERSITY OF CHINA, CHINA, 2008

M.S. IN ZOOLOGY, OCEAN UNIVERSITY OF CHINA, CHINA, 2011

DISSERTATION

Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the requirement for the Degree of

Doctor of Philosophy  
in  
Biochemistry

May 2019

This dissertation was examined and approved in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry by:

Dissertation Director, Sherine F. Elsawa,

Assistant Professor

Arturo Andrade, Assistant Professor

Martin Fernandez-Zapico, Professor, Mayo Clinic

Kelley W. Thomas, Professor

Don Wojchowski, Professor

On April 25, 2019

Approval signatures are on file with the University of New Hampshire Graduate School.

## ACKNOWLEDGEMENTS

I would like to first express my deepest appreciation to my advisor, Dr. Sherine Elsawa, for the opportunity she offered to me to prove myself, for the guidance and support she provided during my entire Ph.D. studies, and for the kind help she gave over the past years. Her encouragement made me overcome the troubles in my academic life since I joined the lab. Without the opportunity she offered, I would not be able to start or proceed so far in my Ph.D. studies. Without her rigorous guidance and teachings, I would not be able to achieve any scientific accomplishment. Without her kind help, good advice and patience, I would not be able to get my dissertation successfully completed. I deeply consider the Ph.D. program as a milestone in my life, and I would say, without Dr. Elsawa, this change would not happen for me. I would also like to express my gratitude to my current committee members, Dr. Arturo Andrade, Dr. Martin Fernandez-Zapico, Dr. Kelley Thomas and Dr. Don Wojchowski, and also my previous committee members, Dr. Barrie Bode, Dr. Linda Yasui and Dr. Yanbin Yin from Northern Illinois University, for all their kind help, advice and suggestions for my research.

This research was supported by grant CA175872-01A1 from the National Institutes of Health and a grant from the International Waldenström macroglobulinemia foundation. I appreciate the travel funds that allowed me to attend different national conferences and present my research, which were very valuable experiences for me. I also appreciate the graduate teaching assistantship provided by the University of New Hampshire Department of Molecular, Cellular and Biomedical Sciences.

I want to thank my family and friends for their support. The unconditional support from my wife Wendy helped me fully focus on my research and studies. I want to thank Wendy for being by my side and taking care of everything, so I could concentrate on writing my dissertation. I would like to thank my parents and my sister for their unlimited understanding, supporting and encouragement all the time. I really appreciate their unconditional love and support when I made the decision to pursue my Ph.D. I'm extremely grateful to my friends for their help, trust and support no matter what happened, and for believing I would eventually succeed and never doubting it. I'm grateful and lucky to have them in my life.

I also would like to express my gratitude to all the members of Dr. Elsawa's lab. Thanks for all the kind help they provided, for all the troubles they helped me overcome, for the joyful time we spent together, and for all their contributions to my project.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS .....	iii
TABLE OF CONTENTS .....	v
LIST OF FIGURES .....	vii
ABSTRACT.....	viii
INTRODUCTION .....	11
Introduction to the tumor microenvironment.....	11
The general roles of the TME in cancer biology.....	12
Cells in the TME .....	19
Inflammation is a hallmark of cancer .....	25
Waldenström macroglobulinemia (WM) .....	33
CHAPTER ONE TARGETING IL-6 RECEPTOR REDUCES IGM LEVELS AND TUMOR GROWTH IN WALDENSTRÖM MACROGLOBULINEMIA .....	49
Introduction .....	49
Materials and methods .....	51
Cells and reagents .....	51
Mice .....	52
IgM ELISA.....	52
Statistical analysis.....	53
Results .....	53
Targeting IL-6/IL-6R signaling in the WM TME does not affect mice survival .....	53
Targeting IL-6/IL-6R signaling with Tocilizumab reduces tumor growth and IgM secretion .....	54
Targeting IL-6 receptor with Tocilizumab does not induce toxicity .....	55
Discussion .....	56
CHAPTER TWO NOVEL MOLECULAR MECHANISM OF REGULATION OF CD40 LIGAND BY THE TRANSCRIPTION FACTOR GLI2.....	69
Introduction .....	69
Materials and Methods .....	71
Cell culture and reagents .....	71
Plasmid constructs and cell transfections.....	72
RNA Isolation and quantitative PCR (qPCR).....	73
Cytokine arrays .....	74
Proliferation assay .....	74

Coculture experiments .....	74
Enzyme-linked immunosorbent assay (ELISA) .....	75
Immunoblotting .....	75
Luciferase reporter assay.....	76
Chromatin immunoprecipitation (ChIP) assay .....	76
Mutations of GLI binding site.....	77
Flow cytometry.....	77
Statistical analysis.....	78
Results .....	78
Screening for novel GLI2 target cytokines identifies CD40L as a candidate GLI2 target gene .....	78
GLI2 regulates the expression of CD40L .....	79
GLI2 directly binds and regulates the CD40L promoter .....	80
CCR3-PI3K-AKT signaling modulates GLI2-CD40L axis.....	81
CCR3-GLI2-CD40L axis is important in the TME .....	82
Discussion .....	83
CHAPTER THREE IDENTIFICATION OF NOVEL GLI2 TRAGET GENES IN HUMAN BONE MARROW STROMAL CELLS.....	99
Introduction .....	99
Materials and Methods .....	101
Cell culture and reagents .....	101
Plasmid constructs and cells transfections .....	101
RNA Isolation and quantitative PCR (qPCR).....	102
RNA-Seq.....	102
Data analysis .....	102
Results .....	103
GLI2 knockdown in HS-5 and L88 cells.....	103
RNA sequencing.....	103
Discussion.....	104
DISCUSSION.....	116
LIST OF REFERENCES .....	125
APPENDIX.....	152

## LIST OF FIGURES

Figure 1- 1. Mouse survival in the presence of Tocilizumab therapy.....	59
Figure 1- 2. Targeting the TME with Tocilizumab reduces tumor growth.....	61
Figure 1- 3. Tocilizumab reduces human IgM secretion in mice xenografted with BCWM.1 cells and stromal cells. ....	63
Figure 1- 4. Tocilizumab is not toxic to mice.....	65
Figure 1- 5. Histology of tumors post euthanasia. ....	67
Figure 2- 1. GLI2 modulates cytokines genes.....	87
Figure 2- 2. GLI2 induces CD40L expression. ....	89
Figure 2- 3. GLI2 binds and modulates CD40L promoter.....	91
Figure 2- 4. CD40L promoter activity with mutated GLI2 binding site and GLI2 inhibition. ....	93
Figure 2- 5. CCR3-PI3K signaling modulates CD40L. ....	95
Figure 2- 6. CCR3 modulates GLI2-CD40L axis in the TME. ....	97
Figure 3- 1. GLI2 knockdown in HS-5 bone marrow stromal cells.....	110
Figure 3- 2. GLI2 knockdown in L88 bone marrow stromal cells.....	112
Figure 3- 3: Top 10 genes that are regulated by GLI2 identified by RNA-sequencing. ....	114



## ABSTRACT

### THE ROLE OF THE TUMOR MICROENVIRONMENT IN WALDENSTRÖM MACROGLOBULINEMIA

By

Weiguo Han

University of New Hampshire

The tumor microenvironment (TME) plays an important role in the initiation, progression and maintenance of cancer cells and is implicated in cancer cell resistance to therapy. In Waldenström macroglobulinemia (WM), a B-cell malignancy characterized by the overproduction of a monoclonal IgM protein, the TME plays an important role in disease biology by secreting cytokines that promote the malignant phenotype. In previous work, we have shown that the transcription factor GLI family zinc finger 2 (GLI2) regulates interleukin-6 (IL-6) secretion in the WM TME. IL-6, in turn, promotes WM growth and IgM secretion by malignant B cells. Tocilizumab/Actemra is an anti-IL-6R antibody, which can competitively block IL-6 binding to the IL-6R. In this dissertation, we investigated the efficacy of targeting the TME using Tocilizumab in a preclinical mouse model of WM that considers the role of the TME in disease biology. Furthermore, we screened for novel GLI2 target genes in the TME and identified cluster of differentiation 40 ligand as a novel GLI2 target gene in the TME. Our data shows that

Tocilizumab therapy results in a reduction in tumor growth rate and IgM secretion in mice sera. In addition, there was no significant change in mice weight suggesting Tocilizumab induced no toxicities to the mice. Taken together, our data suggests that administration of Tocilizumab to tumor bearing mice, results in a significant reduction in tumor volume and IgM secretion. Therefore, the evaluation of the role of Tocilizumab alone or in combination with other therapies that target malignant cells in WM patients may provide therapeutic efficacy. As a single agent, Tocilizumab may alleviate symptoms associated with IgM. In combination, it may reduce IgM levels and slow the rate of tumor growth, thereby allowing therapeutic agents that target cancer cells survival to better induce cell death. Further screening of the role of GLI2 in the TME identified CD40 ligand (CD40L) as a novel GLI2 target gene. We provide evidence of a novel pathway controlling the transcriptional activation of CD40 ligand in bone marrow-derived stromal cells. CD40L plays an important role in normal and malignant B cell biology and we found increased Erk phosphorylation and cell growth in malignant B cells co-cultured with CD40L expressing stromal cells. Further analysis indicated that GLI2 overexpression induced increased CD40L expression, and conversely, GLI2 knockdown reduced CD40L expression. We demonstrate that GLI2 directly binds and regulates the activity of the CD40L promoter. Additionally, we found that the CCR3-PI3K-AKT signaling modulates GLI2-CD40L axis and GLI2 is required for CCR3-PI3K/AKT mediated regulation of CD40L promoter. Finally, co-culture of malignant B cells with cells stably expressing human CD40L, results in increased Erk phosphorylation and increased malignant B cell growth indicating CD40L in the TME promotes malignant B cell activation. Therefore, our studies identify a novel molecular

mechanism of regulation of CD40L by the transcription factor GLI2 in the TME and suggests that targeting GLI2 may be therapeutically beneficial.

## INTRODUCTION

### **Introduction to the tumor microenvironment**

#### **General introduction of the tumor microenvironment**

These are the eight acquired hallmarks of cancer - sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011). Besides these hallmarks, there is another very critical capability that tumors exhibit: the tumor microenvironment (TME). In addition to our increased understanding of cancer cell biology over the past decade, cancers are now being recognized as organs or tissues more and more, instead of being simply defined as just an individual cell type. From this view point, it is not enough to just study a specialized cell type, but it is equally important to study the related tumor microenvironment, to better understand the biology of cancer as a tissue.

The tumor microenvironment is defined as the environment around or in a tumor, composed of cellular and soluble factors. The cellular factors include cells such as fibroblasts, a variety of immune cells (regulatory T cells, myeloid-derived suppressor cells, natural killer/NK-cells, dendritic cells, monocytes, mast cells, T lymphocytes and B lymphocytes), endothelial cells, stromal cells, as well as cells that comprise the blood vessels (Balkwill et al. 2012, Hanahan and Weinberg 2011, Hanahan and Coussens 2012). The soluble (non-cellular) factors include the extracellular matrix and the

signaling molecules produced by all the cells that support the growth and survival of cancer cells in the tumor such as a variety of growth factors and cytokines (Joyce and Fearon 2015, Spill et al. 2016).

The effect between the tumor and the tumor microenvironment is bidirectional: the tumor cells recruit the cells to surround and form its microenvironment, so they can change the surrounding microenvironment to better favor their survival and growth. On the other hand, the microenvironment can affect how the tumor grows and spreads, while also providing support and protection for the tumor cells.

### **The general roles of the TME in cancer biology**

Among the eight hallmarks of cancer, seven involve contributions by the tumor microenvironment, except for enabling replicative immortality (Hanahan and Coussens 2012). This suggests that the tumor microenvironment is involved in almost every aspect of cancer cell growth, survival, disease progression and resistance to therapy. Over the recent decades, there have been significant advances in our understanding of the contribution of the tumor microenvironment to cancer cell biology, yet many aspects of the microenvironment remain poorly understood.

The most fundamental trait of cancer cells relates to their ability to sustain chronic proliferation (Hanahan and Weinberg 2011). Although oncogenic mutations drive this trait in most forms of human neoplastic cells, the TME has the ability to support hyperproliferation of cancer cells (Hanahan and Coussens 2012). Both cellular and non-cellular factors contribute to this ability of the TME. Among the cellular factors,

the outlined cell type is the vascular cells (cells that line blood vessels); mainly endothelial cells, supporting pericytes, tumor-associated macrophages and vascular smooth muscle cells. The abnormal interactions between these cells in the regulation of vascular formation, stabilization, remodeling and function are heavily involved in tumor angiogenesis (Armulik et al. 2005). Studies in mice models have reported that the induction of angiogenesis increases the proliferation of cancer cells (Bergers et al. 1999, Hanahan and Folkman 1996). Both clinical trials (Carmeliet and Jain 2011, Ferrara and Alitalo 1999) and mice models (Parangi et al. 1996) also showed that inhibition of angiogenesis can reduce the proliferation of cancer cells. Besides the inhibition of cancer cell proliferation, inhibition of factors that promote angiogenesis also increased tumor cell and endothelial cell apoptosis (Shaheen et al. 1999).

Other cell types also play essential roles in promoting cancer cell growth and proliferation. This includes tumor-associated macrophages (TAMs), which are macrophages that are recruited into the hypoxic environment of cancers, by factors such as hypoxia inducible factor-1 (HIF-1)-dependent upregulation of the chemokine receptor CXCR4 (Balkwill et al. 2005, Bingle et al. 2002, Pollard 2004). In this case, cancer cells attract TAMs into the TME, and sustain their survival. In return, TAMs produce important mitogens as well as various growth factors and enzymes that stimulate angiogenesis, in responding to the hypoxic environment of the TME (Bingle et al. 2002). Cells in the TME favor the proliferation of cancer cells by supplying direct and indirect growth mediators (Balkwill et al. 2005, Hanahan and Coussens 2012); such as chemokines, epidermal growth factor (EGF), fibroblast growth factors (FGFs),

inflammatory cytokines, transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and a variety of interleukins (ILs), such as IL-1 $\beta$  and IL-6 (Balkwill et al. 2005).

The extracellular matrix (ECM) is a major component of the non-cellular factors in the TME. ECM remodeling is an important mechanism that regulates angiogenesis. Abnormal ECM dynamics can lead to restructure and dysfunction, both of which could result in tissue fibrosis and cancer promotion (Lu et al. 2011). Besides this, a variety of enzymes, such as cysteine proteinases, heparinases, metalloenzymes and serine proteases, are expressed by immune cells in the TME, which can selectively cleave cell-cell and cell-ECM adhesion molecules. This cleavage can disable growth suppressing adhesion complexes that maintain homeostasis (Hanahan and Coussens 2012, Lu et al. 2011, Mohamed and Sloane 2006, Pontiggia et al. 2012, Xu et al. 2009).

Both hypoxia and hypoxia-associated necrosis are involved in cancer cell metastasis (De Jaeger et al. 1998). This is a critical step for cancer cells to adapt to hypoxia during disease progression (Dang and Semenza 1999). In particular, this is regulated by the transcription factor hypoxia inducible factor-1 (HIF-1). Studies focusing on HIF-1 $\alpha$  indicate that the transcriptional activity of HIF-1 $\alpha$  is a significant positive regulator of tumor progression and metastatic potential (Liao et al. 2007). A follow-up study showed that hypoxia facilitates tumor cell migration, eventually leading to cancer cell metastasis (Haase 2009, Yang et al. 2008). Other factors like vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS) have also been found to be involved in angiogenesis and cancer cell progression (Ambs et al. 1998, Branco-Price et al. 2012, Ziche and Morbidelli 2009). The contribution of hypoxia in tumors and around blood vessels to cancer metastasis through the regulation by HIF-1, also

inducing VEGF and iNOS, by endothelial cells in the TME provides strong evidence that the TME plays an important role in promoting cancer cell migration and metastasis (Branco-Price et al. 2012, Chambers et al. 2002, Gupta and Massagué 2006, Hanahan and Coussens 2012, Takeda et al. 2010).

Another essential role for the TME in cancer biology is its role in promoting cancer cell resistance to therapy. Cancer treatment includes surgery, chemotherapy, radiation therapy, immunotherapy, targeted therapy and some other options depending on the location, type, as well as the stage of the cancer. Radiation therapy is a treatment that uses high doses of radiation to kill cancer cells and/or shrink tumors. Chemotherapy is a treatment that involves the use of drugs that target proliferating cells (including cancer cells) to kill cancer cells. More recently, immunotherapy and targeted therapies have emerged as promising strategies to treat cancer. Immunotherapy utilizes the patient's immune system to fight their cancer, while targeted therapy uses specific drugs, like small-molecule inhibitors or monoclonal antibodies, to target specific changes, molecules or signaling pathways in cancer cells that help them grow, divide and spread. Except for surgery, the rest of the treatments all happen in patients and require a more fundamental understanding of cancer biology to better benefit the therapeutic outcome of the patient. In all these treatments, the TME plays an important role in treatment outcome.

In the past, the ability of cancer cells to resist anticancer drugs was mostly considered to be due to genetic mutations or alterations in gene expression changes in cancer cells. Changes in gene expression can lead to the encoded protein being mutated which can affect the uptake and metabolism of anticancer drugs by cancer



cells, eventually leading to drug resistance. This type of resistance, acquired drug resistance, is a result of the long-term exposure to anticancer drugs, causing tumor cells to develop gene mutations or mechanisms that allow them to resist (Hanahan and Coussens 2012). Another type of resistance to anticancer drugs stems from the TME, which allows cancer cells to tolerate anticancer drug-induced stress during the initial exposure, and this is considered as the first step for cancer cells to survive and eventually develop acquired drug resistance (Li and Dalton 2006). For anticancer drugs to work well in the tumor, drugs need to pass through the structure of the tumor vasculature, the tumor tissue, as well as the blood vessel in the tumor, to reach and kill the tumor cells. In other words, before the drugs are efficiently delivered to the tumor cells, they need to pass the barriers imposed by the TME. Anticancer drug used to treat the same cells from a tumor showed high levels of drug resistance as in the tumor, while no significant resistance was observed when cancer cells were exposed to the same drugs *in vitro* (Teicher et al. 1990). This indicates that the resistance of the tumor to anticancer drugs can develop through mechanisms provided by the TME, not just the tumor cells themselves. Currently, more and more studies suggest that the TME involved around the tumor also mediates tumor resistance to therapy (Dalton 1999, Galmarini et al. 2007, Hazlehurst et al. 2002, Holohan et al. 2013, Li and Dalton 2006, Meads et al. 2008, Morin 2003).

TME-associated hypoxia is known to activate genes that are involved in angiogenesis and cancer cells survival through the HIF-1 (Haase 2009, Pouysségur et al. 2006, Yang et al. 2008). Several studies have shown that hypoxia can select cells that are insensitive to p53-mediated apoptosis and cells that are deficient in DNA

mismatch repair, leading to drug resistance (Graeber et al. 1996, Kondo et al. 2001). Other studies focused on the proliferation of tumor cells in the TME where they found that tumor cells that are more distant from blood vessels tend to have a low rate of proliferation, and anticancer drugs that target cell proliferation are more effective at targeting and killing proliferating cells more than quiescent cells, which exhibits more resistance to therapy (Galmarini et al. 2007, Ljungkvist et al. 2002).

Under normal conditions, immune cells recognize, target and kill the cancer cells, as they can recognize cancer cells' abnormal activities. However, cancer cells develop a variety of strategies to avoid being recognized by the immune system. This allows the tumor cells to evade being destroyed by immune cells. These strategies involve a variety of mechanisms that recruit different cells from the surrounding environment, in which the TME plays an essential role. In the TME, there are several types of cells that are involved in the immune escape (cancer cells escaping killing by T cells), including regulatory T cells, tumor-associated macrophages (TAMs), neutrophils and mast cells (Ruffell et al. 2010). These cells can block the activity of cytotoxic T lymphocytes and NK-T cell-mediated killing of neoplastic cells.

Tumor-associated macrophages (TAMs) can differentiate into activated TAMs, which have the ability to promote tumor escape (escape from being recognized and killed by CD8+ T cells), when exposed to several immunoregulatory cytokines and other signaling molecules, such as IL-13 and IL-14 (DeNardo et al. 2011, Doedens et al. 2010, Kryczek et al. 2006, Movahedi et al. 2010, Qian and Pollard 2010). Regulatory T cells suppress tumor-specific T cell immunity and contribute to the growth of human tumors *in vivo* (Curiel et al. 2004). In an indirect manner, tumor cells and tumor-

associated macrophages can recruit regulatory T cells to the tumor to suppress the immune response through the chemokine CCL22 in the TME (Curiel et al. 2004). In human breast cancer, tumor-associated macrophages and monocytes have been reported to be recruited into the TME by mammary epithelial cells via recruitment factors, such as colony stimulating factor 1 and interleukin-3. These factors together enhance colony stimulating factor receptor (CSF-R)-dependent macrophage infiltration. Blocking this macrophage recruitment can improve survival in a mouse model by slowing primary tumor development and reducing pulmonary metastasis (DeNardo et al. 2011). Regulatory T cells and myeloid-derived suppressor cells are also involved in immune tolerance induced by the chemokine CCL21, to prevent autoimmunity (Hanahan and Coussens 2012). CCL21 expression by melanoma tumors in mice is associated with an immunotolerant microenvironment, which contributes to the suppression of the immune response by shifting the host immune response from immunogenic to tolerogenic, to facilitate tumor progression (Shields et al. 2010).

Accumulation of mast cells in the TME can lead to tumor growth and the loss mast cells correlates with reduced tumor growth. The interaction between mast cells and the immune environment can mediate immune suppression and contribute to tumor escape from immune destruction (Wasiuk et al. 2009). This mediation is achieved by releasing cytokines that recruit cytotoxic T lymphocytes-suppressing myeloid-derived suppressor cells and regulatory T cells (Hanahan and Coussens 2012).

Cancer-associated fibroblastic cells can produce chemokines and other signaling molecules that recruit regulatory T cells, myeloid-derived suppressor cells and tumor-associated macrophages into the TME and prevent destruction of cancer cells by host

immunity (Hanahan and Coussens 2012). In prostate and breast cancer, cancer-associated fibroblasts support tumor growth and progression by mediating TGF- $\beta$  signaling, to inhibit cytotoxic T cell and NK-T cell responses (Hanahan and Coussens 2012, Stover et al. 2007).

### **Cells in the TME**

There are various cell types involved in the TME in cancer biology, including a variety of immune cells (such as Regulatory T cells, myeloid-derived suppressor cells, NK-cells, dendritic cells, monocytes/macrophages, mast cells, T lymphocytes and B lymphocytes), fibroblasts, endothelial cells, stromal cells, as well as cells that comprise the blood vessels. These cells play different or similar roles in the TME, mostly, their mechanisms favor cancer cell survival, growth, progression and resistance to therapy.

#### **Immune cells**

The immune cells that are recruited to the TME play different roles that apply to several hallmarks of cancer. These roles can be grouped into two types: cells or factors produced by cells that directly benefit and those that indirectly benefit cancer cells. The direct and indirect growth factors from the immune cells, as well as some types of stromal cells in the TME can stimulate the proliferation of cancer cells (Balkwill et al. 2005). Like the growth factors mentioned before: epidermal growth factor (EGF), fibroblast growth factors (FGFs), inflammatory cytokines, transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and variety kinds of interleukins (ILs, such as IL-1 $\beta$  and IL-6) (Balkwill et al. 2005). This is counted as direct benefit toward growth of

cancer cells. In addition, studies focused on regulatory T cells and myeloid-derived suppressor cells show that these two types of cells are major components of the immune suppressive tumor microenvironment, and they can suppress the natural killer cells' ability to carry on their normal function in the tumor, by regulating the TGF- $\beta$  signaling pathway (Lindau et al. 2013, Vitale et al. 2014, Yang et al. 2010). CD4+CD25+ regulatory T cells have been reported to be important contributors to the development of immune tolerance in the tumor and play a critical role in the suppression of anti-tumor immunity, through suppressive cytokines such as IL-10, IL-35 and TGF- $\beta$ , to dampen the NK cell immune responses and to benefit cancer cell growth and survival (Chen et al. 2016, Ghiringhelli et al. 2006).

Normal cells are "programmed" to die, typically through regulated apoptosis, so the body can control abnormal proliferation. For cancer cells to stay in their immortal status of proliferation, they must either develop intrinsic resistance to local cell death programs or coordinate development of cell extrinsic programs to support their survival (Hanahan and Coussens 2012). The immune cells in the TME contribute to this capacity. In breast cancer, vascular cell adhesion molecule-1 (VCAM-1) is reported to provide a survival advantage to cancer cells that infiltrate leukocyte-rich microenvironments like the lungs and binds metastasis-associated macrophages to cancer cells via counter-receptor  $\alpha 4$ -integrins. In addition, clustering of cell surface VCAM-1 can trigger Akt activation and protect cancer cells from apoptosis through Ezrin (which functions as a general cross-linker between plasma membrane proteins and the actin cytoskeleton) (Chen et al. 2011). Tumor-associated macrophages are also found to be involved. Increased levels of tumor-associated macrophages and cathepsin

protease were found in mammary tumors when treated with Taxol chemotherapy, and cathepsin-expressing macrophages also protected against tumor cell death induced by several other chemotherapies, such as Etoposide and Doxorubicin (Shree et al. 2011). These studies show that the immune cells in the TME provide survival signals for cancer cells which allows them to resist therapy-induced cell death.

One of the hallmarks of the cancer is inducing angiogenesis. In the past, studies mainly focused on the regulation by cancer cell expression of proangiogenic factors. More recently, accumulating evidence suggests that cells in the TME are essential components of chronic angiogenesis in tumor (Hanahan and Coussens 2012). Tumor-associated macrophages are reported to regulate tumor angiogenesis through the vascular endothelial growth factor A (VEGF-A) in mice. The expression of VEGF-A by tumor-associated macrophages led to a massive infiltration, into the tumor, of leukocytes and indicated that macrophage-secreted VEGF regulates malignant progression through stimulating tumor angiogenesis, leukocytic infiltration and tumor cells invasion (Lin et al. 2007). Other studies in mouse various models of cancer show that tumor-associated macrophages also produce MMP-9 to provide an alternative mechanism to promote angiogenesis, which still dependent on VEGF (Bergers et al. 2000, Du et al. 2008, Giraudo et al. 2004). Beside VEGF, tumor-associated macrophages can also produce placental growth factor to stimulate tumor angiogenesis. The host-produced histidine-rich glycoprotein (HRG) can polarize the TAMs to promote antitumor immune responses and blood vessel normalization, to decrease tumor growth and metastasis and to enhance chemotherapy (Rolny et al. 2011). Colony stimulating factor 1 (CSF1) is reported to recruit monocyte/macrophage in response to cytotoxic

therapies, to enhance CSF1 receptor-dependent macrophage infiltration, and blockade of macrophage recruitment with CSF1 receptor-signaling antagonists can improve survival of mammary tumor-bearing mice by slowing primary tumor development, thus decreasing blood vessel density and reducing metastasis (DeNardo et al. 2011).

### **Endothelial cells and Stromal cells**

Endothelial cells and stromal cells are two major supporting cell types in the TME. They play different but essential roles in cancer biology; one of these roles is to favor cancer cell growth and survival. Endothelial cells have been reported to play an important role in the regulation of vascular formation, stabilization, remodeling and function, and the abnormal interaction between endothelial cells and mural cells (which are perivascular cells that include vascular smooth muscle cells and pericytes) can lead to tumor angiogenesis (Armulik et al. 2005). Furthermore, these cells are also involved in resisting cell death by cancer cells, conversely, increased apoptosis resulting from anti-cancer therapies can reduce the rate of angiogenesis (Hanahan and Coussens 2012). Angiogenesis can limit cell death for cancer cells resulting from hypoxia and lack of serum-derived nutrients and survival factors. This limitation can be blocked by vascular disrupting agents, like the drug OXi4503 (Daenen et al. 2009).

### **Fibroblasts**

Fibroblasts involved in the TME are mainly tumor- or cancer-associated fibroblastic cells (CAFs). There are several subtypes of CAFs based on the expression

of different surface binding proteins (Hanahan and Coussens 2012). CAFs can express and secrete different signaling proteins to support cancer cell proliferation, such as hepatocyte growth factor (Cirri and Chiarugi 2012). CAFs can also contribute to the proliferation of cancer cells by associating with epithelial-to-mesenchymal transition, via secretion of the cytokine TGF- $\beta$  (Chaffer and Weinberg 2011).

Studies on normal tissue fibroblasts indicate that fibroblasts can inhibit the growth of cancer cells by direct contact with each other, suggesting that normal fibroblasts play a role in suppressing the growth of cancer cells (Bissell and Hines 2011, Flaberg et al. 2011). Compared to this, cancer-associated fibroblasts did not suppress the growth of cancer cells, and conversely, favor the escape of cancer cells from the growth suppressors. The reason they have lost this suppressor ability may be caused by them being reprogrammed to CAFs, but the precise mechanism remains unresolved.

Studies on the CAFs have shown that CAFs have the ability to limit the effect of apoptosis on cancer cells (Kalluri and Zeisberg 2006, Loeffler et al. 2006). This mechanism involves the secretion of survival factors like insulin-like growth factor-1 and insulin-like growth factor-2. Furthermore, CAFs are related to the formation of the extracellular matrix, which contributes survival signals for cancer cells in the TME by synthesizing the extracellular matrix molecules and the extracellular matrix-remodeling proteases (Hanahan and Coussens 2012). Studies in breast cancer show that breast cancer cells co-cultured with mature adipocytes exhibit radioresistance; and this radioresistant phenotype was driven by the significant increase of the cytokine IL-6 in the tumor cells, which plays a protective role (Bochet et al. 2011).



When looking at angiogenesis as a hallmark of cancer biology, CAFs are also involved in benefiting tumor tissue angiogenesis. CAFs have been reported to produce different proangiogenic signaling molecules, such as VEGF, IL-8/CXCL8 and platelet-derived growth factor C (PDGF-C) (Hanahan and Coussens 2012). PDGF-C has been shown to be upregulated in CAFs from resistant tumors and this upregulation of PDGF-C can even help some tumors overcome inhibition of VEGF-mediated angiogenesis, which means PDGF-C can rescue angiogenesis (Crawford et al. 2009). In addition, CAFs can participate indirectly in angiogenesis by producing chemoattractant to attract other proangiogenic cells (Räsänen and Vaheri 2010, Vong and Kalluri 2011).

CAF's also modulate and contribute to cancer cell invasion and metastasis. CAFs-derived TGF- $\beta$  has been shown to play a role in activating epithelial to mesenchymal transition and is involved in cancer cell invasion and metastasis (Chaffer and Weinberg 2011). Mesenchymal stem cells are reported to localize to breast carcinomas and cause cancer cells to increase their metastatic potency. When bone-marrow-derived human mesenchymal stem cells are mixed with weakly metastatic human breast cancer cells, the chemokine CCL5 from mesenchymal stem cells, was found to enhance motility, invasion and metastasis (Karnoub et al. 2007). CAFs have also been found at the invasive front in some tumors, indicating that the normal tissue fibroblasts may have been reprogrammed by signaling molecules released by cancer cells during the process of invasion (Hanahan and Coussens 2012).

## Inflammation is a hallmark of cancer

Tumor-promoting inflammation is one of the enabling characteristics in cancer besides the eight hallmarks (Hanahan and Weinberg 2011). Inflammation plays an essential role in cancer cell initiation, proliferation, invasion and metastasis. Indeed, an inflammatory microenvironment is now considered an essential component of the majority of cancers (Mantovani et al. 2008). The activation of transcription factors such as NF- $\kappa$ B, STAT3, and AP-1, by tumor-promoting cytokines which are produced by immune/inflammatory cells, induce genes that stimulate cell proliferation and survival and is considered a major tumor-promoting mechanism (Grivennikov et al. 2010). NF- $\kappa$ B has also been reported as a key molecule that is involved in the relationship between inflammation, tumor promotion and progression (Karin 2006, Karin and Greten 2005). NF- $\kappa$ B can regulate the expression of many genes which can suppress tumor cell death, stimulate cycle progression, enhance epithelial-to-mesenchymal transition (which plays an important role in tumor invasiveness), and provide newly emerging tumors with an inflammatory microenvironment that supports cancer cells progression, invasion of surrounding tissues, angiogenesis and metastasis (Lin and Karin 2007). There are variety of cytokines produced by immune cells that are involved in tumor development and progression. Among those cytokines, TNF- $\alpha$ , TNF-related apoptosis-inducing ligand (TRAIL), IL-1 $\beta$ , IL-6, IL-10, IL-12, IL-17, IL-23, TGF- $\beta$  and Interferon- $\gamma$  (IFN- $\gamma$ ) have been reported as outlined below (Grivennikov et al. 2010, Lin and Karin 2007).

TNF- $\alpha$  produced by tumor cells or inflammatory cells in the TME can promote tumor metastatic growth and this promotion depends the NF- $\kappa$ B activation in tumor cells

(Luo et al. 2004). TNF- $\alpha$  has also been reported to be involved in tumor initiation by stimulating the production of molecules that can lead to DNA damage and mutations (Hussain et al. 2003). Studies on skin carcinogenesis in mice with a deficiency in TNF- $\alpha$  show that TNF- $\alpha$  deficient mice were resistant to development of benign and malignant skin tumors, while there was a much higher percent of wild type mice developed skin tumors. Furthermore, deletion of a TNF- $\alpha$  inducible chemokine also showed some resistance to skin tumor development (Moore et al. 1999). Subsequent studies have shown that the TNF- $\alpha$  receptors, TNFR1 and TNFR2, also have protumor activity in mice. Tumor multiplicity, the average number of tumors per mouse, was significantly reduced in mice deficient in TNFR1 and TNFR2 compared to wild type mice. The fact that TNFR1 deficient mice showing more resistance to the development of skin tumors than TNFR2 deficient mice indicates that TNFR1 is the major mediator of TNF- $\alpha$ -induced tumor formation (Arnott et al. 2003). Studies on the Mdr2-knock-out mice, which develop hepatocellular carcinoma (HCC), show that the inflammatory process triggers hepatocyte NF- $\kappa$ B activation through upregulation of TNF- $\alpha$  in adjacent endothelial and inflammatory cells. Furthermore, anti-TNF- $\alpha$  treatment of I $\kappa$ B-super-repressor in later stages of tumor development to suppress NF- $\kappa$ B signaling resulted in apoptosis and finally failure to progress to HCC (Pikarsky et al. 2004). Another study in a murine cancer metastasis model, stimulated with bacterial lipopolysaccharide (LPS), showed that LPS-induced metastatic growth depends on TNF- $\alpha$  production by host hematopoietic cells and NF- $\kappa$ B activation in tumor cells and stimulates tumor growth in the lung (Luo et al. 2004). TNF- $\alpha$  can also cooperate with other proinflammatory cytokines, and together, they contribute to tumor promotion. It has been reported that

the enhanced production of the tumor promoting cytokines IL-6 and TNF- $\alpha$ , cause hepatic inflammation and activation of STAT3, to promote HCC development in mice (Park et al. 2010). These studies report that TNF- $\alpha$ , as a proinflammatory cytokine produced by both host and tumor cells, plays an important role at different stages of cancer including cancer initiation, growth, angiogenesis and metastasis.

IL-12 and IL-23 are tumor-promoting proinflammatory heterodimeric cytokines and belonging to the IL-12 family of proinflammatory cytokines (Langowski et al. 2006, Watford et al. 2004). These two cytokines share a common subunit and bind to a common receptor chain, IL-12R $\beta$ 1. The IL-12R is composed of IL-12R $\beta$ 1 and IL-12R $\beta$ 2 while IL-23 binds to a receptor composed of IL-12R $\beta$ 1 and IL-23R (Watford et al. 2004). Their receptors are mainly described to be expressed on T cells, NK cells and NKT cells (Lin and Karin 2007). Binding of these cytokines to their receptors will activate TYK2 and JAK-STAT (STAT1, STAT3, STAT4 and STAT5) signaling pathways, which eventually lead to effects on tumor development (Lin and Karin 2007, Watford et al. 2004). IL-12 can be produced by a variety of cells including monocytes, neutrophils and B cells, and the major producers of IL-12 are macrophages and dendritic cells. In addition, IL-12 is also produced in a T-cell-dependent manner through the engagement of antigen-presenting cells with CD40 ligand on the surface of T cells (Watford et al. 2004). IL-23 is produced by activated monocytes, activated antigen-presenting cells, dendritic cells, macrophages, T cells, B cells and endothelial cells (Lee et al. 2004, Oppmann et al. 2000, Pirhonen et al. 2002, van Seventer et al. 2002). IL-23 is mainly produced by tumor-associated macrophages (Kortylewski et al. 2009). Despite the fact

that IL-12 and IL-23 share a receptor subunit and are produced from several similar cells, they have different effects on tumor development.

IL-12 has been reported to induce the production of interferon- $\gamma$ , which favors the differentiation of T helper 1 cells and forms a link between innate and adaptive immunity (Trinchieri 2003). In mouse models, IL-12 has a strong anti-tumor activity and induces regression of tumors depending on its ability to promote Th1 cells and cytotoxic T lymphocytes (CTLs) response. Furthermore, the Th1 cells promoted by IL-12 can produce IFN- $\gamma$ , which in turn contributes to the antitumor activity of IL-12 (Trinchieri 2003). Evidence has shown that there is an increase in the expression of IL-23 in human tumors, and this increased expression regulates local inflammatory responses in the TME and infiltration of intra-epithelial lymphocytes (Langowski et al. 2006). IL-12 can promote infiltration of cytotoxic T cells and the resultant antitumor activity, while IL-23 promotes inflammation and increases angiogenesis, but reduces CD8 T-cell infiltration (Langowski et al. 2006). Furthermore, genetic deletion or antibody-mediated elimination of IL-23 leads to increased infiltration of cytotoxic T cells into the transformed tissue, which shows a protective effect against chemically induced carcinogenesis (Langowski et al. 2006). In mice, depletion of IL-23 or IL-23R, results in transplanted tumor cell growth-restriction (Langowski et al. 2006). IL-23 can also enhance the proliferation of memory T cells and the production of IFN- $\gamma$ , IL-12 and TNF- $\alpha$  from activated T cells, and the level of INF- $\gamma$  being produced by IL-23 is lower than that of IL-12 (Hao and Shan 2006). In murine models of cancer, IL-23 can also act directly on dendritic cells and possesses potent anti-tumor and anti-metastatic activity (Hao and Shan 2006). Moreover, IL-23 can also induce IL-17 secretion from activated

CD4+ T cells and stimulate the proliferation of memory CD4+ T cells. This regulation is mediated by the activation of JAK2, PI3K/AKT, STAT3 and NF- $\kappa$ B signaling pathways (Hao and Shan 2006). Taken together, these studies show that IL-23 plays complicated roles in cancer biology and may act as alternative and safer therapeutic agent for cancer, as IL-12 administration can lead to severe toxic side effects due to the extremely high levels of IFN- $\gamma$  it induces (Hao and Shan 2006).

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is another powerful cytokine in the TME. The role of TGF- $\beta$  in the TME is complicated and paradoxical. It has immune-suppressing and anti-inflammatory properties, and paradoxically, it can also modulate processes such as cell invasion, immune regulation, and microenvironment modification that can favor cancer cell development (Massagué 2008). TGF- $\beta$  signals mainly through activation of SMAD transcription factors, and can lead to MAPK activation; Signals from TGF- $\beta$  will cause phosphorylation of SMAD proteins, and their eventual translocation into the nucleus to regulate target gene expression (Massagué 2008). This contributes to different cellular functions, such as cytostatic effects, cell growth, invasion, extracellular matrix synthesis, cells cycle arrest and migration (Landskron et al. 2014, Matsuzaki 2013).

The role of TGF- $\beta$  in cancer biology is complicated and paradoxical. TGF- $\beta$  plays different roles in cells depending on cell type and stage of tumorigenesis. In early stages, TGF- $\beta$  acts as a tumor suppressor, inhibiting cell cycle progression and promoting apoptosis (Landskron et al. 2014). TGF- $\beta$  can suppress T cell-mediated autoimmune inflammation and anti-tumor immunity. Furthermore, TGF- $\beta$  is involved in the suppressive activity mediated by regulatory T cells (Becker et al. 2006). In colon

cancer mouse model, both TGF- $\beta$ 1- and SMAD3-deficient mice show increased colon carcinogenesis (Engle et al. 2002, Maggio-Price et al. 2006). Studies from a TGF- $\beta$  receptor II-deficient mouse model found that epithelia, such as rectal and genital epithelia, developed spontaneous squamous cell carcinomas. Moreover, this progression was associated with a reduction in apoptosis and could be accelerated by Ras mutations, suggesting that deficient TGF- $\beta$  signaling pathway contributes to tumorigenesis (Guasch et al. 2007). Despite the role of TGF- $\beta$  in anti-inflammatory and growth inhibition of early stages of cancer, it is also involved in enhancing tumor progression. In developing and progressing carcinomas, carcinoma cells emerge from the epithelial-mesenchymal transition (EMT) stimulated by TGF- $\beta$  providing a selective advantage to growing carcinoma cells, such as enhanced cell migration and invasion, heightened resistance to cytotoxic agents, targeted chemotherapeutic and radiation treatments, and boosted expansion of cancer-initiating and stem-like cells populations that underlie tumor metastasis and disease recurrence (Morrison et al. 2013). In addition, TGF- $\beta$  has been shown in mouse models of skin carcinomas to favor tumor cells invasion (Derynck et al. 2001). There is evidence that TGF- $\beta$  inhibits tumor-specific CD8+ T cells to favor angiogenesis and promote tumor development (Chen et al. 2005). Increased expression of TGF- $\beta$  mRNA and protein has also been observed in gastric carcinoma, non-small cell lung cancer and prostate cancer (Massagué 2008). Taken together, these studies indicate that the role of TGF- $\beta$  in the TME is complicated and paradoxical, and special therapy targeting this cytokine or related signaling pathway may advance cancer patients.

The inflammatory cytokine interleukin-6 (IL-6) can mediate the differentiation of lymphocytes, promote cell proliferation, and cell survival despite apoptotic signals (Heinrich et al. 1998, Kamimura et al. 2004). IL-6 plays a key role in promoting cell proliferation and inhibition of apoptosis via activation of the JAK/STAT signaling pathway upon binding to its receptor (IL-6R $\alpha$ ) and coreceptor gp130 (Hodge et al. 2005). Activation of gp130 triggers the phosphorylation of the proteins STAT1 and STAT3 for by the Janus associated kinase 1 (JAK1) (Lin and Karin 2007). This molecular activity eventually leads to activation of specific target genes in the nucleus (O'Shea et al. 2002). Studies on IL-6 target genes show that most of them are involved in cell cycle progression and suppression of apoptosis (Haura et al. 2005). This highlights the important role of IL-6 in tumorigenesis.

In the plasma cell cancer multiple myeloma (MM), IL-6 signaling through IL-6R/STAT3 contributes to the pathogenesis of MM, and blockage of the IL-6R/STAT3 signaling pathway leads to the induction of apoptosis (Chatterjee et al. 2004). It is also suggested that IL-6 plays a pivotal role in the pathogenesis of MM (Bommert et al. 2006) and IL-6 secreted by bone marrow stromal cells in MM can enhance the interaction between these two cells and lead to the promotion of proliferation of MM cells (Chauhan et al. 1996). In addition, IL-6 stimulation induces the phosphorylation of insulin-like growth factor-I and high expression of IL-6R $\alpha$  in human myeloma cells. Both of these colocalize to lipid rafts to promote tumorigenesis in response to IL-6 in human myeloma cells (Abroun et al. 2004). In bone marrow stromal cells, IL-6 has been reported to be regulated by the chemokine CCL5, at the level of both gene expression and protein secretion in stromal cells. This in turn, induces immunoglobulin (Ig)



secretion by malignant B cells in the surrounding bone marrow microenvironment (Elsawa et al. 2011b). Subsequent studies show that the IL-6R $\alpha$  subunit, which binds IL-6 to activate the IL-6 signaling pathway, is a downstream target of the transcription factor GLI2. This regulation was found to promote IgM secretion in the B-cell lymphoma Waldenström macroglobulinemia (WM) (Jackson et al. 2015). In IL-6-deficient mice, loss of IL-6 resulted in complete resistance to plasma cell tumor development (Hilbert et al. 1995) and abnormal regulation of IL-6 was shown to increase the risk of development of Hodgkin lymphoma (Cozen et al. 2004). These studies indicate the essential role of IL-6 in the development and maintenance of B cell neoplasms.

IL-6 has also been found to play an important role in cancer stem cells (CSCs) where it promotes the conversion of noncancer stem cells into cancer stem cells. IL-6 secreted from noncancer stem cells cultured in ultra-low attachment plates, can regulate CSCs-associated OCT-4 gene expression through the IL-6-JAK1-STAT3 signal transduction pathway in non-CSCs, which promote the conversion of non-CSCs into CSCs; Inhibition of the IL-6-JAK1-STAT3 signaling pathway with anti-IL-6 antibody effectively prevented OCT-4 gene expression and prevented non-CSCs converting into CSCs (Kim et al. 2013).

In colon cancer, IL-6 was reported to be involved in TGF- $\beta$  signaling in T cells, thereby regulating STAT-3 activation in tumor cells and this signaling in tumor infiltrating T lymphocytes controls the growth of dysplastic epithelial cells in colon cancer; Moreover, IL-6 signaling requires cancer cell-derived soluble IL-6R rather than membrane bound IL-6R (IL-6 trans-signaling) (Becker et al. 2004). IL-6 trans-signaling is critically involved in the maintenance of disease state, by promoting the switch from

acute to chronic inflammation in colon cancer, as well as other types of cancers (Rose-John et al. 2006).

These findings along with other studies address the essential role of IL-6 as an inflammatory cytokine in promoting cancer cell growth, survival, progression and metastasis. It also provides the rationale to propose IL-6 as a therapeutic target in cancer. Clinical trials using Siltuximab (CNTO 328), a monoclonal antibody against IL-6, have shown promising results for non-small cell lung cancer, ovarian cancer, prostate cancer, and MM (Landskron et al. 2014).

### **Waldenström macroglobulinemia (WM)**

Waldenström macroglobulinemia (WM) is a rare blood cancer characterized by an excess of abnormal white blood cells called lymphoplasmacytic cells in the bone marrow. It is also known as lymphoplasmacytic lymphoma (LPL), which belongs to the non-Hodgkin lymphoma (NHL) family. The World Health Organization (WHO) classifies it as a subset of a low-grade, non-Hodgkin lymphoma with unique pathologic characteristics such as monoclonal IgM secretion and infiltration of the bone marrow by clonal lymphoplasmacytic cells (Treon et al. 2009). WM is thought to be driven by genetic mutations. The most common somatic mutations are found in MYD88 (MYD88<sup>L256P</sup>) and CXCR4 WHIM-like mutations (CXCR4<sup>WHIM</sup>). MYD88 mutation is found in more than 90% of WM patients and CXCR4-WHIM like is mutated between 30% to 35% of patients (most of them also have the MYD88 mutation) (Treon et al. 2015b).

## Disease biology

WM accounts for approximately 2% of hematologic cancers, affecting approximately 1,500 Americans each year and is a slow-growing cancer (Dimopoulos and Alexanian 1994). The median age at diagnosis is approximately 65 years, with a slight male predisposition (Rajkumar et al. 2006). Splenomegaly and lymphadenopathy are uncommon at initial presentation, but at late stages, extramedullary disease is more common (up to 60% of patients) (Treon et al. 2015b). The typical symptoms for patients with WM at presentation are weakness, bleeding (usually in the form of chronic epistaxis and gingival oozing) and fatigue due to anemia (Ghobrial and Witzig 2004, Rajkumar et al. 2006). The fatigue is usually caused by anemia and the bleeding is caused by serum hyperviscosity (this is related to the excess IgM, which can thicken blood and impair circulation) (Ghobrial and Witzig 2004). Hyperviscosity resulting from high levels of IgM protein can lead to impairment of microcirculation in the central nervous system, and this may result in epistaxis, headache, blurring or loss of vision, dizziness and difficulty coordinating movements (Sahin et al. 2014). In some patients, the overproduced monoclonal IgM protein can clump together, causing a condition known as cryoglobulinemia, which causes the fingers and toes turn white or blue. The IgM protein can also circulate in the body and build up in organs like heart and kidneys, causing a condition called amyloidosis, which can lead to heart and kidney problems (Gertz et al. 1993, Ghobrial et al. 2003). Lymphadenopathy (enlarged lymph nodes) and splenomegaly (enlarged spleen) are found in around 25% of cases, which are not as high as in other non-Hodgkin lymphomas (Ghobrial and Witzig 2004). Other clinical symptoms may include constitutional symptoms (such as fever, night sweats and weight

loss), cryoglobulinemia and sensorimotor peripheral neuropathy (Ghobrial and Witzig 2004, Rajkumar et al. 2006). The reason for peripheral neuropathy is currently uncertain. Other features of WM are due to the accumulation of lymphoplasmacytic cells in different tissues. Accumulation of these cells can lead to an enlarged liver, spleen or lymph nodes. Patients with WM have an increased risk of developing other malignancies in the blood or other tissues. Some affected patients have elevated levels of IgM and lymphoplasmacytic cells, but there are no other symptoms. Criteria for diagnosis require a serum monoclonal IgM protein  $\geq 30$  g/L and  $>20\%$  bone marrow infiltration with malignant lymphocytes (García-Sanz et al. 2001). However, the IgM protein level is not sufficient for the diagnosis of WM, since there are other malignant cells that can produce IgM (Sahin et al. 2014). Therefore, in clinical practice, elevated expression of several cell surface markers, such as CD19, CD20 and Ig light chain, in association with non-paratrabeular pattern of bone marrow infiltration are used for WM diagnosis (Dimopoulos et al. 2005, Dimopoulos et al. 2000, Ghobrial and Witzig 2004).

### **Therapy for WM**

In the clinical setting, there is no standard-of-care for the treatment of WM. Initiation of therapy is not be based on the level of the IgM protein, but instead is based mainly on signs and symptoms (Dimopoulos et al. 2009, Ghobrial 2012). Patients with disease-related cytopenia, bulky adenopathy or organomegaly, symptomatic hyperviscosity, severe neuropathy, amyloidosis, cryoglobulinemia, cold agglutinin disease, or evidence of disease transformation should be considered for immediate therapy, and asymptomatic patients with WM do not require therapy (Dimopoulos et al.

2009). Treatment decisions are based on the presence of symptoms, patient factors (such as age and functional status) and disease factors including the presence of cytopenia, rate of disease progression, the level of IgM protein, the presence of neuropathy, cryoglobulinemia and hyperviscosity (Sahin et al. 2014).

Current treatments for WM include the use of alkylating agents, nucleoside analogs, the monoclonal antibody rituximab, the proteasome inhibitor bortezomib and the Bruton tyrosine kinase (BTK) inhibitor Ibrutinib (Dimopoulos et al. 2009, Treon et al. 2009). Ibrutinib is the first drug approved specifically for WM by the Food and Drug Administration after a clinical trial showed impressive responses in previously treated WM patients (Chakraborty et al. 2015). Another treatment is plasma exchange, to alleviate the hyperviscosity associated with WM. When IgM exceeds 4000 mg/dl, symptoms of hyperviscosity are generally present and include epistaxis, gingival bleeding and visual change (Sahin et al. 2014).

Rituximab is widely used for the treatment of WM, either as a single therapy or in combination with other chemotherapeutic agents. Rituximab is a monoclonal antibody that targets the molecule CD20 on the surface of B lymphocytes. In a phase II study that included untreated symptomatic patients with WM who received a combination of dexamethasone, rituximab and cyclophosphamide (DRC), results showed that 83% of patients achieved a response, including 7% complete, 67% partial and 9% minor responses. The median time to response was 4.1 months. The two-year progression-free survival rate was 90% (Dimopoulos et al. 2007). Another study that used the combination of rituximab and CHOP (Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone; R-CHOP) administered every 3 weeks at standard doses, showed that the

R-CHOP combination achieved a partial response in 91% of patients with a median response time of 1.6 months, which was much more rapid (Abonour et al. 2007). These studies indicate that combining rituximab with other chemotherapies is highly effective as first-line treatments.

Ibrutinib as the first FDA approved drug for the treatment of WM, has been shown to be highly active, and is associated with durable responses and is safe in pretreated patients with WM (Trean et al. 2015a). Trean et al performed a clinical trial with Ibrutinib with Initial ibrutinib dose at a daily dose of 420mg administered orally for 26 4-week cycles. After administration of ibrutinib, the median serum IgM decreased from 3520 to 880 mg/dL, median hemoglobin levels increased from 10.5 to 13.8 g/dL, and bone marrow involvement decreased from 60% to 25%. The overall response and major response rate were 90.5% and 73.0%. The highest overall response and major response were observed in patients with MYD88<sup>L256P</sup>CXCR4<sup>WT</sup> (100% overall response and 91.2% major response), followed by patients with MYD88<sup>L256P</sup>CXCR4<sup>WHIM</sup> (85.7% and 61.9%, respectively) and patients with MYD88<sup>WT</sup>CXCR4<sup>WHIM</sup> (71.4% and 28.6%, respectively). The estimated 2-year progression-free and overall survival rates among all patients were 69.1% and 95.2% (Trean et al. 2015a). Toxicities were moderate and grade 2 or higher were observed in WM, including neutropenia (22% of the patients) and thrombocytopenia (14%), which were more common in heavily pretreated patients; atrial fibrillation (5%) in patients associated with a history of arrhythmia, epistaxis (3%) associated with the use of fish-oil supplements and postprocedural bleeding (3%) (Trean et al. 2015a).

## **The role of bone marrow (BM) TME in WM**

The tumor microenvironment facilitates the growth, survival, progression and metastasis of tumor cells, through different mechanisms. The interaction between tumor cells and stromal cells, tumor and tumor cells, and tumor cells and the extracellular matrix, all contribute to disease biology. Additionally, soluble molecules provide further signals for tumor cell growth and survival (Li and Dalton 2006).

For hematologic malignancies, such as acute and chronic leukemias, lymphomas, multiple myeloma and WM, they occur in cells from blood-forming tissue, such as the bone marrow (BM). Therefore, the TME in these malignancies mostly would be refer as bone marrow tumor microenvironment (BM TME). WM is characterized by specific homing and tumor growth within the bone marrow niches, and indeed, the BM TME is involved in tumor cell growth, survival and drug resistance (Dimopoulos et al. 2005, Dimopoulos et al. 2000, Elsawa et al. 2011a, Elsawa et al. 2006, Elsawa et al. 2011b, Ghobrial et al. 2011, Ghobrial and Witzig 2004, Owen et al. 2003).

There are a growing number of studies that focus on understanding the role of BM TME in WM disease biology, specifically on BM cells, cytokines and growth factors that are involved. The interactions between BM TME and WM cells not only facilitates WM cell growth, but also provides a protective role against drug treatment for WM cells.

The BM TME has been specialized into two distinct niches, the “endosteal niche” and “vascular niche” (Mendelson and Frenette 2014, Sipkins et al. 2005, Yin and Li 2006). The endosteal niche is localized at the interface of the trabecular bone with the BM, and cells of endosteal niche including osteoblasts maintain the self-renewal

capacity of the hematopoietic stem cells (HSCs) and regulate their function, which plays an essential role to sustain the quiescent state of the HSCs (Jalali and Ansell 2016). The vascular niche supports proliferation and differentiation of the HSCs (Jalali and Ansell 2016, Sugiyama and Nagasawa 2012). The interaction between BM cells and the HSCs allows the HSCs to develop, egress into the peripheral blood, home and further localize into the BM niches at distant sites (Ghobrial et al. 2011).

In the bone marrow TME, the cellular factors consist of a variety of immune cells and non-immune cells, which include T lymphocytes, B lymphocytes, dendritic cells, myeloid-derived suppressor cells, mast cells, bone marrow stromal cells and endothelial cells. Although the functions and contributions of these cells to WM biology have not been fully investigated, more studies have focused on understanding the contributions of these cells to WM biology.

Of particular interest in the BM TME, bone marrow stromal cells have been shown to play an important role in WM cell growth, survival and resistance to therapy. In this dissertation, we found that coculture of WM cells with CD40L-expressing stromal cells increased Erk phosphorylation and ultimately cell growth in WM cells. Coculture of WM cells with stromal cells leads to resistance to various chemotherapies (Leleu et al. 2007, Ngo et al. 2009, Roccaro et al. 2010). Moreover, in bone marrow malignancies, such as multiple myeloma and WM, bone marrow stromal cells play an important role in malignant cell biology by secreting a variety of cytokines that are used by malignant cells to survival and proliferate (Azab et al. 2012a, Burger and Gandhi 2009, Burger and Peled 2009, Cheung and Van Ness 2001, Elsawa et al. 2006, Elsawa et al. 2011b, Kurtova et al. 2009b).



Mast cell hyperplasia is one of the characteristics of WM disease (Jalali and Ansell 2016). Mast cells are commonly found associated with lymphoplasmacytic cells in WM patients and provide growth and survival signals for lymphoplasmacytic cells (Santos et al. 2006). A study on bone marrow mast cells in WM found that CD52 is widely expressed on bone marrow mast cells, and leads to high levels of alemtuzumab-mediated, antibody-dependent, cell-mediated cytotoxicity against bone marrow mast cells from patients with WM, indicating that bone marrow cells can be targeted in the treatment of WM and other mast cell-related disorders, as bone marrow mast cells play a role in supporting growth and survival of WM cells (Santos et al. 2006). Another study on bone marrow mast cells in WM showed that coculture of sublethally irradiated autologous bone marrow mast cells along with bone marrow lymphoplasmacytic cells from WM patients resulted in mast-cell dose-dependent tumor colony formation and proliferation (Tournilhac et al. 2006). Furthermore, CD40 ligand (CD40L), a potent inducer of B cell expansion, was found to be expressed on bone marrow mast cells from WM patients while mast cells from healthy donors did not express CD40L. Moreover, mast cells induced-expansion of lymphoplasmacytic cells was blocked using a CD40L blocking protein in a dose dependent manner by. This indicates that in WM, mast cells support WM cell expansion by activating CD40-CD40L signaling (Tournilhac et al. 2006). In a novel *in vivo* model of human WM using severe combined immunodeficient (SCID) mice, and human fetal bone chips, from which WM cells from patient bone marrow are engrafted directly into the human bone chips to mimic the bone marrow microenvironment (termed SCID-hu mice), human monoclonal IgM protein, which is produced by WM cells in SCID-hu mice, was detectable in mice sera, and mast cells

were observed infiltrating the BM TME, further indicating that mast cells play an essential role in promoting WM cell growth (Tassone et al. 2005).

Endothelial cells have also been shown to interact with WM cells and play a critical role in the bone marrow microenvironment in WM. Endothelial cells are mainly involved in angiogenesis and angiogenesis represents an essential step in cell proliferation and expansion in several hematologic malignancies, including multiple myeloma (Vacca and Ribatti 2005) and WM (Terpos et al. 2009). Indeed, in WM, the bone marrow microvessel density is increased in 30%-40% of patients, and angiogenic cytokines, such as angiogenin, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF) are increased in the serum of WM patients. In addition, both macrophages and mast cells, which have angiogenic properties, are increased in the WM bone marrow microenvironment (Terpos et al. 2009). Studies using primary WM endothelial cells show that endothelial cells in WM patients are present and express high levels of the ephrin receptors (Eph), which are important regulators of cell adhesion, cell proliferation, cell cycle and tumor progression, indicating the important role of endothelial cells in WM. Eph-B2 receptor was found to be activated in WM patients compared with controls and activate cell adhesion signaling. Furthermore, Eph-B2 ligand was highly expressed on endothelial cells and bone marrow stromal cells isolated from WM patients and induced signaling in endothelial cells promoting adhesion and angiogenesis. Finally, blocking ephrin-B2 or Eph-B2 mediated signaling inhibited cell adhesion, cytoskeletal signaling, proliferation and cell cycle in WM cells, which was induced by coculture with endothelial cells. This decreased WM progression

*in vivo* (Azab et al. 2012b). Taken together, these studies indicate the importance of endothelial cells as a component of the TME in WM.

Another important component of the bone marrow microenvironment is cytokines that are present and/or produced in the BM microenvironment. Cytokines are known to regulate many biological processes in normal lymphocyte development including immunoglobulin production, and the presence of cytokines within the BM microenvironment of WM is likely to contribute to malignant cell growth, survival and immunoglobulin production, indicating that cytokines are key regulators in the BM microenvironment of WM (Elsawa and Ansell 2009). Several cytokines are known to be key regulators of B-cell homeostasis and play an important role in normal B-cell development. One of the cytokines with an established role is IL-21. B cells development and function are regulated by IL-21 and IL-21 can induce B cell proliferation, differentiation into Ig-producing plasma cells, or apoptosis in both mice and humans. Moreover, IL-21 alone and in combination with Th cell-derived cytokines, can regulate class switch recombination (CSR) to IgG, IgA, or IgE isotypes, indicating its important role in shaping the effector function of B cells (Konforte et al. 2009). Another cytokine, IL-7, has been reported to play an important role in B-cell development (Nagasawa 2006). IL-7 signaling can drive B-cell specification and commitment in the BM (Elsawa and Ansell 2009). Cytokines are required to maintain the normal functions of B-cells and the overexpression of many of these cytokines in may stimulate dysregulated B-cell proliferation or immunoglobulin production and results in malignant B cells growth.

The proinflammatory cytokine IL-6, plays an essential role in normal B-cell proliferation and maturation and also induces Ig secretion by these normal B cells (Kishimoto and Tanaka 2015). Stimulation of WM cells with IL-6 has been reported to increase IgM secretion, and in combination with B-lymphocyte stimulator, IL-6 induced even higher levels of IgM secretion by WM cells (Elsawa et al. 2006). Elsawa et al. used a multiplex-bead based array assay to screen cytokines from healthy and WM patients' sera and bone marrow biopsies, and identified several different dysregulated cytokines including CCL5, granulocyte colony-stimulating factor (G-CSF), soluble IL-2 receptor, and IL-6, which were significantly elevated in WM patients whereas IL-8 and epidermal growth factor (EGF) levels were significantly lower in these patients compared to healthy controls. CCL5 is correlated with features of WM aggressiveness such as elevated IgM levels and bone marrow involvement with lymphoplasmacytic cells. Furthermore, functional analysis revealed a correlation between CCL5 levels and IL-6 levels (Elsawa et al. 2011b). CCL5 was found to stimulate IL-6 secretion in WM bone marrow stromal cells resulting in increased IgM secretion by WM cells via the JAK/STAT signaling pathway. These studies define a novel signaling network in the WM BM microenvironment that regulates IgM secretion by malignant B cells (Elsawa et al. 2011b). IL-6 is a proinflammatory cytokine with a well-defined role in normal and malignant B-cell biology. IL-6 has been shown to be involved in several B-cell malignancies including diffuse large B-cell lymphoma, Hodgkin lymphoma and multiple myeloma (Aldinucci et al. 2004, Arendt et al. 2002, French et al. 2003, Pedersen et al. 2005). Subsequent studies about the role of IL-6 in promoting IgM secretion in WM cells demonstrated that the oncogenic transcription factor GLI2 in the WM BM

microenvironment mediates the regulation of IL-6 by CCL5 (Elsawa et al. 2011b). In the malignant cells, GLI2 was found to modulate the transcription and surface expression of the IL-6 receptor (IL-6R) (Jackson et al. 2015). Since IL-6 regulates Ig secretion, it was found that inhibition of GLI2 and/or IL-6R reduces IgM secretion by WM cells. This study identified IL-6R $\alpha$  as a downstream target of GLI2 mediating the regulation of IgM secretion (Jackson et al. 2015). Taken together, these studies indicate that cytokines within the WM BM microenvironment contribute to malignant B cells activation, growth, survival, as well as IgM production.

These studies led us to investigate the role of cytokines in the WM TME and how they mediate the interaction between WM cells and cells in the surrounding BM microenvironment. Furthermore, this led us to investigate the molecular mechanisms by which GLI2 can mediate these events.

### **Hedgehog and GLI2 in WM**

Several transcription factors play a variety of roles in the biology of WM. Among them is the transcription factor GLI2. GLI2, GLI family zinc finger 2, is a member of the GLI family of transcription factors and modulates gene expression directly or through interaction with other proteins (Ruiz i Altaba et al. 2007). There are three members of the GLI family of transcription factors (GLI1-3) that act together in response to signaling from Hedgehog (HH) and other signaling inputs, resulting in the regulation of target gene expression and ultimately lead to modulated cellular activities. There are two transmembrane proteins that are involved in and control the HH signaling pathway;

Patched-1 (PTCH1), which acts as HH signaling receptor, and Smoothed (SMO), which is the signal transduction component. In the absence of HH ligands, PTCH1 receptor blocks the function of SMO and maintains it in an inactive state. However, upon binding of any of the three HH ligands (Desert, Indian and Sonic HH) to PTCH1, the inhibition of SMO by PTCH1 is lifted, thereby allowing SMO to become activate and initiates and transduces signaling, resulting in the activation of GLI transcription factors (di Magliano and Hebrok 2003, Ingham and McMahon 2001, Varjosalo and Taipale 2007, Varjosalo and Taipale 2008).

Three GLI genes have been identified: GLI1, GLI2 and GLI3. These possess distinct repressor and activator functions in HH signaling. As the downstream component of HH signaling, GLI proteins are post-translationally modified and are normally suppressed/sequestered in the cytoplasm and prevented from entering the nucleus. This inactivation is achieved by interaction with cytoplasmic proteins, including Fused and Suppressor of Fused (Sufu). Activation of the HH signaling pathway initiates a signaling cascade that leads to cleavage of the GLI proteins, resulting in an N-terminal-truncated activator and C-terminal-truncated repressor fragments, and active forms of GLI proteins are generated, released from the GLI-Fused-SUFU complex and translocated into the nucleus. Nuclear GLI proteins proceed along the transcription factor function and active target gene expression, including PTCH and GLI genes. Some other target genes are regulated including those that are involved in controlling cell proliferation, such as cyclin D, cyclin E, Myc and components of the EGF pathway, in angiogenesis, such as components of the platelet-derived-growth-factor and vascular-epithelial-growth-factor pathway (di Magliano and Hebrok 2003). Cytokines in

the BM microenvironment are also targets of GLI and regulate malignant cell growth and survival (Elsawa et al. 2011a, Jackson et al. 2015, Mauviel et al. 2010, Zhao et al. 2006). HH-GLI signaling pathway is also involved in cancer development. Mutations in Sonic HH has been reported to cause basal cell carcinomas in mice, suggesting that HH may have a role in human tumorigenesis (Oro et al. 1997). Constitutively active mutations of SMO have been found in basal cell carcinoma, and GLI1 was originally identified in human glioma (Kinzler et al. 1987). Moreover, ectopic expression of transcription factor GLI1 in the embryonic frog epidermis results in tumor development and induces basal cell carcinoma formation (Dahmane et al. 1997). Similarly, overexpression of GLI1 in mice induces basal cell carcinoma as well as other follicle-derived neoplasias, such as trichoepitheliomas, cylindromas and trichoblastomas (Nilsson et al. 2000). In addition, overexpression of GLI2 in the skin also induces basal cell carcinomas in mice (Grachtchouk et al. 2000).

The regulation and transcriptional role of GLI2 have been widely studied. Studies on TGF- $\beta$  show that TGF- $\beta$  induces the expression of GLI2 in various human cell types, including normal fibroblasts and keratinocytes, as well as various cancer cell lines. This induction is rapid, independent of HH signaling and requires a functional SMAD pathway (Dennler et al. 2007). This finding indicates that GLI2 can be activated without signaling from HH receptors and identifies TGF- $\beta$  as potent transcriptional inducer of GLI2 (Dennler et al. 2007). Interestingly, GLI2 was also found to modulate TGF- $\beta$ 1 expression in CD4+ T cells by regulating the promoter of TGF- $\beta$ 1 (Furler and Uittenbogaart 2012). In bone marrow stromal cells, GLI2 was found to be activated through CCR3-PI3K-AKT signaling pathway without signaling from HH (Elsawa et al.

2011a). In WM B cells, GLI2 was found to regulate IgM secretion via regulation of IL-6Ra expression, again in a HH independent mechanism (Jackson et al. 2015). These studies indicate that GLI2 can be activated even in the absence of HH signaling, through TGF- $\beta$  and CCR3. Moreover, GLI2 has also been reported to play a critical role in maintaining the tumorigenic properties of prostate cancer cells (Thiyagarajan et al. 2007).

In melanoma cell lines, increased GLI2 expression was associated with loss of E-cadherin expression and with an increased capacity to invade Matrigel and form bone metastases in mice, indicating that GLI2 is directly involved in driving melanoma invasion and metastasis (Mauviel et al. 2010). The role of GLI2 in the BM microenvironment has also been investigated. In bone marrow stromal cells, signaling initiated by CCL5 in the TME leads to up-regulation of Ig production by malignant B cells. CCL5 promotes IL-6 expression and secretion in stromal cells via GLI2, and IL-6 in turn, induces Ig secretion by malignant B cells (Elsawa et al. 2011a). Work from this dissertation research identified CD40L as a novel downstream target of GLI2 in bone marrow stromal cells. Signaling through the CCR3-PI3K-AKT pathway can modulate the GLI2-CD40L axis, and GLI2 was found to be required for CCR3-PI3K-AKT-mediated regulation of the CD40L promoter (Han et al. 2017). These studies report a biological effect of TME-mediated GLI2 on WM cells and reveals the contribution of GLI2 to WM biology via regulating cytokines in the BM microenvironment, further supporting a role for GLI2 in promoting WM cell growth, survival as well as IgM production.

Collectively, there is evidence to support a role for targeting the TME as a therapeutic strategy in cancer. Since the bone marrow microenvironment provides a



protective role for WM cells, and promotes WM cell activation, growth, survival and IgM production, targeting BM microenvironment or the cytokines involved in the BM microenvironment may provide opportunities for therapeutic advances.

## CHAPTER ONE

# TARGETING IL-6 RECEPTOR REDUCES IGM LEVELS AND TUMOR GROWTH IN WALDENSTRÖM MACROGLOBULINEMIA

### Introduction

Waldenström macroglobulinemia (WM) is a subtype of non-Hodgkin lymphoma (NHL) characterized by infiltration of the bone marrow with lymphoplasmacytic cells (Kapoor et al. 2015). Despite WM being an indolent lymphoma, WM cells secrete very high levels of a monoclonal immunoglobulin M (IgM) protein, which is associated with symptoms such as anemia, serum hyperviscosity syndrome and peripheral neuropathy (Ansell et al. 2010). At initial diagnosis, many WM patients do not require immediately clinical intervention unless disease symptoms, mostly associated with hyperviscosity syndrome are evident (Ansell et al. 2010, Gertz 2018a, b). In the past decade, several significant enhancements were made in our understanding of WM biology. This has led to the evaluation and introduction of several new therapeutic options for WM patients. Current therapies used for the treatment of WM patients mainly focus on targeting cancer cells directly using combination therapies. Rituximab-containing therapies are a standard of care in the United States. This includes therapies such as R-CHOP (Cyclophosphamide, doxorubicin, vincristine and prednisone plus rituximab). Ibrutinib, a BTK inhibitor, is the only FDA approved therapy for WM. However, it is administered indefinitely and can cause adverse reactions such diarrhea, thrombocytopenia, rash,

atypical bleeding, among other symptoms (Gertz 2018b). However, despite evidence for a role of the tumor microenvironment (TME) in promoting WM cell growth, survival and IgM secretion (Jalali and Ansell 2016), there have been little studies investigating targeting the TME as a therapeutic strategy for WM patients.

Despite the progress made to understand disease biology, like most other neoplasms, WM remains an incurable disease and ultimately patients succumb to disease progression. The TME plays an important role in the development and progression of WM and has been shown to play a protective role in resistance to therapy (Jalali and Ansell 2016, Leleu et al. 2007, Roccaro et al. 2010). In fact, the cross-talk between malignant cells and cells in the TME favors disease progression and promotes IgM secretion. In previous studies, we and others have shown that IL-6 from the TME promotes IgM secretion and cell growth via binding to the IL-6R on WM cells (Elsawa et al. 2011a, Elsawa et al. 2011b, Hatzimichael et al. 2001, Jackson et al. 2015). Therefore, targeting IL-6/IL-6R signaling may provide therapeutic benefit for WM patients, particularly those with high levels of IgM in their serum.

Tocilizumab/Actemra is an anti-IL-6R antibody, which can competitively block IL-6 binding to the IL-6Ra. Tocilizumab has been administered or investigated in several clinical settings in patients with several inflammatory-mediated diseases including Castleman's disease, rheumatoid arthritis, systemic juvenile idiopathic arthritis, crohn's disease, giant cell arteritis, systemic sclerosis, systemic lupus erythematosus and multiple sclerosis (Hunsucker et al. 2011, Tanaka et al. 2011). These studies indicate that administration of Tocilizumab can be used as a novel therapy to block IL-6 in these chronic inflammatory conditions and suggest its potential role in other diseases in which

IL-6 plays a role. Furthermore, several studies have investigated the addition of anti-IL-6 therapy in multiple myeloma (MM) with enhanced results over chemotherapeutic regimens (Hunsucker et al. 2011, Rossi et al. 2005, Shah et al. 2016). However, despite investigations of anti-IL-6 therapy in autoimmune and malignant disorders, no studies to date have investigated its therapeutic efficacy in WM.

In this study, we report the efficacy of targeting the TME with Tocilizumab in a preclinical mouse model of WM that considers the role of the TME in disease biology. We show that Tocilizumab reduced tumor growth and IgM secretion and suggest it may provide therapeutic benefit to WM patients.

## **Materials and methods**

### **Cells and reagents**

The BCWM.1 cells (Ditzel Santos et al. 2007, Drexler et al. 2013) were kindly provided by Dr. Steve Treon (Dana Farber Cancer Institute, Boston, MA), the RPCI-WM1 cells (Chitta et al. 2013, Drexler et al. 2013) were kindly provided by Dr. Chanan-Khan (Mayo Clinic, Jacksonville, FL) and HS-5 cells were purchased from ATCC (Manassas, VA). WM cells were maintained in RPMI and HS-5 cells in DMEM, all supplemented with 10% FBS and antibiotics/antimycotics as previously published (Elsawa et al. 2011a, Elsawa et al. 2008, Elsawa et al. 2011b, Han et al. 2017, Jackson et al. 2015).

## Mice

Hairless SCID mice (male; 6-8 weeks old; Charles River, Wilmington, MA) were purchased and allowed to acclimate for 2 weeks. Mice were then subcutaneously implanted with BCWM.1 cells ( $10 \times 10^6$ ) and HS-5 cells ( $2 \times 10^6$ ) (5:1 ratio) as previously published (Elsawa et al. 2011a). Mice were cared for and handled in accordance with institutional and National Institutes of Health guidelines, after obtaining IACUC approval. Mice were treated with 100 µg/mouse either Tocilizumab or control antibody (Genentech, South San Francisco, CA) in a total volume of 100 µl injected via intraperitoneal route every other day for a total of 5 weeks (**Figure 1-1A**). Tumors were measured (using digital calipers; Fisher Scientific, Waltham, MA) and mice were weighed 3 times/week. Upon euthanasia, tumor samples and sera were harvested and stored for later use.

## IgM ELISA

Human IgM in mice sera was quantified using human IgM ELISA (Bethyl laboratories, Inc., Montgomery, TX) following manufacturer's recommendations, as previously published (Elsawa et al. 2011a) using ELISA plates (Nunc Maxisorp, Fisher Scientific). ELISA plates were developed using the Turbo TMB-ELISA (Fisher Scientific) and the reaction was stopped by addition of 2N H<sub>2</sub>SO<sub>4</sub>. Results were quantified using a plate reader (Molecular Devices, Palo Alto, CA) and data was analyzed using SoftMax Pro 7.0.2 software.

## Statistical analysis

The last observed outcome data for actual tumor volume, tumor volume relative to baseline and mice weights were compared between treatment groups using a Student's t-test with the assumption of unequal variance. The mixed effect model with the assumption of random intercepts and slopes for the trajectory lines for each mouse were also fitted to compare the slope of the growth of the outcome measures between treatment groups. The fixed interaction term of the number of days from baseline by treatment indicator were tested for the difference of two arms. All analysis were conducted within tumor type (BCWM.1 or RPCI-WM1).

## Results

### Targeting IL-6/IL-6R signaling in the WM TME does not affect mice survival

Because bone marrow stromal cells in the WM TME are an important source of IL-6, we subcutaneously implanted hairless SCID mice with BCWM.1 cells or RPCI-WM1 cells and HS-5 stromal cells at a ratio of 5:1 onto the right flank of mice. This allowed us to examine the role of paracrine IL-6 from bone marrow stromal cells, which have been shown to play an important role in malignant B cells in WM cells (Elsawa et al. 2011a, Elsawa and Ansell 2009, Elsawa et al. 2011b, Han et al. 2017, Jalali and Ansell 2016) *in vivo*. Upon tumor appearance, we treated mice with either Tocilizumab or IgG control antibody every other day for 5 weeks (**Figure 1-1A**) and examined the effect of therapy on mice survival. In accordance with Institutional IACUC, mice were euthanized when tumors reached 2 cm in any dimension or when tumors became

ulcerated. We found that Tocilizumab treatment did not affect overall survival in mice xenografted with BCWM.1 or RPCI-WM1 and stromal cells (**Figure 1-1B**).

### **Targeting IL-6/IL-6R signaling with Tocilizumab reduces tumor growth and IgM secretion**

Tumor growth was monitored and recorded 3 times/week. When we examined the rate of tumor growth (tumor growth relative to the size of the first recorded tumor), we found a significant reduction in tumor growth rate in mice implanted with RPCI-WM1 and stromal cells and treated with Tocilizumab ( $p=0.0394$ ) (**Figure 1-2B**). Although the overall tumor growth rate was not statistically significant in mice implanted with BCWM.1 cells and stromal cells, the rate of tumor growth was slower in Tocilizumab treated mice (**Figure 1-2A**). Interestingly, when we examined actual tumor volume and tumor growth rate in mice implanted with BCWM.1 and stromal cells at day 14 (when all mice were alive), we found a significant reduction in tumor growth rate ( $p=0.0306$ ) (**Figure 1-2C**). We also found a reduction in the actual tumor volume in this group at day 14, although this did not reach statistical significance ( $p=0.057$ ) (**Figure 1-2C**). This is consistent with our previous reports on the effect of IL-6 on malignant cell growth in WM, where IL-6 induced a modest increase in WM cell proliferation in this indolent lymphoma (Elsawa et al. 2011a, Elsawa et al. 2011b).

The role of IL-6 in normal and malignant B cell biology is well established (DuVillard et al. 1995, Elsawa et al. 2011b, French et al. 2002, Hatzimichael et al. 2001, Hirano 1991, Kishimoto 2005). IL-6 has been shown to promote immunoglobulin (Ig) secretion in normal B cells (Kishimoto 2005) and malignant B cells (DuVillard et al.

1995, ElSawa et al. 2011b, French et al. 2002, Hatzimichael et al. 2001, Hirano 1991, Jackson et al. 2015). We have previously shown that IL-6 promotes IgM secretion in WM (ElSawa et al. 2011a, ElSawa et al. 2011b). Therefore, we examined the effect of IL-6 therapy on human IgM secretion in mice sera. Consistent with our previous reports, we found a significant reduction in human IgM secretion in mice sera in groups of mice xenografted with BCWM.1 cells and stromal cells, treated with Tocilizumab ( $p=0.0029$ ) (**Figure 1-3**). However, in mice xenografted with RPCI-WM1 cells and stromal cells, there was no reduction in IgM secretion with Tocilizumab treatment (**Figure 1-3**). The RPCI-WM1 tumors were significantly larger ( $928.8 \pm 599.6 \text{ mm}^3$  in control mice) than BCWM.1 xenografted mice ( $115.1 \pm 73.23 \text{ mm}^3$  in control mice) prior to euthanasia of mice in either group. Furthermore, of the 3 WM cell lines that are currently available, RPCI-WM1 cells secrete the highest levels of IgM (data not shown).

### **Targeting IL-6 receptor with Tocilizumab does not induce toxicity**

To examine potential therapy-induced toxicities, we monitored the weights of mice three times/week. Tocilizumab treated mice did not differ from control mice in weight in RPCI-WM1 xenografted mice (**Figure 1-4A**). In BCWM.1 xenografted mice, there was a significant ( $p=0.005$ ) difference in mice weights between Tocilizumab treated mice and control mice (**Figure 1-4A**). However, when we examined individual mice within each group, the data indicates that control mice consistently increased their weight, while the majority (7/10 mice) of Tocilizumab-treated mice either maintained their weight or increased it (**Figure 1-4B**). Taken together, these results suggest that



targeting IL-6 in the TME reduced tumor growth rate and IgM secretion while having no toxic effects on mice.

We performed immunohistochemical staining of tumor biopsies with H&E and found a similar cellular morphology composed of small round cells in the 2 mice treatment groups (**Figure 1-5**). This is consistent with B cell morphology and suggests growth of WM cells to form these tumors.

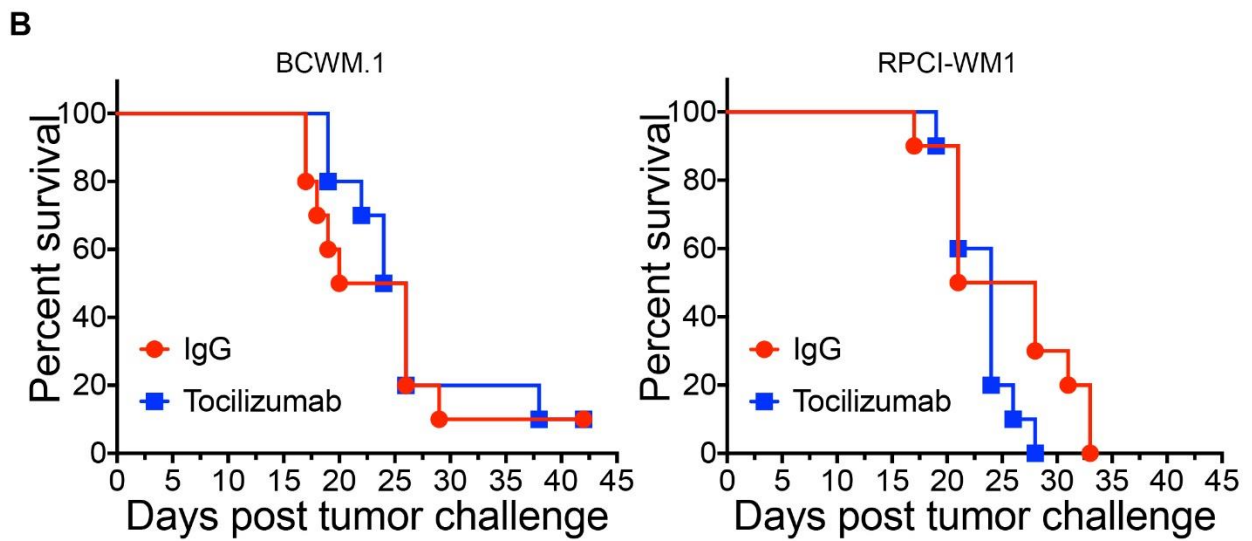
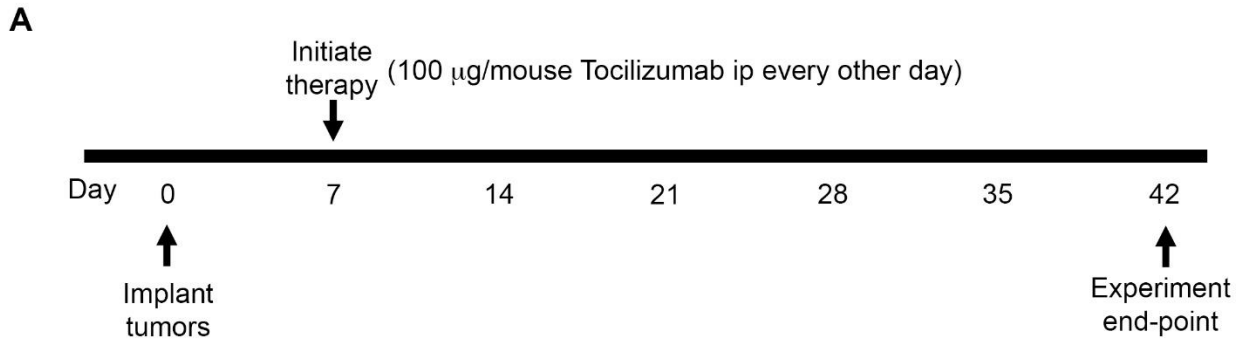
## Discussion

The role of the bone marrow TME in WM and other B cell malignancies is well documented (Burger et al. 2009, Burger and Stewart 2009, Elsawa and Ansell 2009, He et al. 2004, Leleu et al. 2007). Interactions between malignant B cells and stromal cells in the TME play an essential role in regulating malignant B cell biology including cell growth, cell survival and Ig secretion (Burger et al. 2009, Elsawa et al. 2011a, Jalali and Ansell 2016). In previous work, we have shown that IL-6 levels are elevated in WM patients and IL-6 from human bone marrow stromal cells can promote WM cells growth and IgM secretion (Elsawa et al. 2011a, Elsawa and Ansell 2009). This finding led us to investigate the efficacy of blocking IL-6 to block the interaction between the TME and WM cells. Our results show that targeting IL-6 in the WM TME did not affect the survival in two WM cell line models investigated (**Figure 1-1B**). This finding is not surprising, as IL-6 does not induce apoptosis of WM cells (Elsawa et al. 2011b). Rather, the role of IL-6 in WM was shown to promote IgM secretion and WM cell growth (Elsawa et al. 2011a, Elsawa et al. 2011b). Consistent with this role, we did find a reduction in tumor growth rate when tumor bearing mice were treated with Tocilizumab (**Figure 1-2**). These

results indicate that targeting IL-6 in the TME in WM may slow the rate of tumor growth. Since WM remains an incurable disease, a reduction in IgM levels may provide a much needed symptomatic relief for patients. Future studies combining targeting of IL-6 with therapies that induce apoptosis of WM cells, may prove to be effective; with IL-6 therapy slowing tumor growth and another therapy targeting the malignant cells. Currently, Ibrutinib is the only drug approved by the Food and Drug Administration (FDA) for WM patients. However, several other therapies are used as monotherapies or in combination and include Bendamustine, Rituximab, Dexamethasone, Fludarabine, Chlorambucil, Everolimus, among others (Kapoor et al. 2015). An examination of the role of Tocilizumab therapy in combination with these therapies may allow a dose reduction in these therapies and therefore alleviate some of the side effects associated with each therapy.

One of the hallmarks of WM is the overproduction of monoclonal IgM (Elsawa et al. 2011a, Elsawa et al. 2006, Elsawa et al. 2011b, Han et al. 2017, Jackson et al. 2015). Our results show that tumor-bearing mice treated Tocilizumab had reduced levels of human IgM secretion in mice serum (**Figure 1-3**). Interestingly, RPCI-WM1 tumor-bearing mice treated with Tocilizumab had similar levels of IgM as control mice. The 2 WM cell lines used in this study are derived from different WM patients (Chitta et al. 2013, Ditzel Santos et al. 2007). Interestingly, RPCI-WM1 cells secrete the highest levels of IgM among the available WM cell lines (data not shown), raising the possibility that the rate of increase in IgM levels in patient may correlate with the efficacy of Tocilizumab monotherapy. Therefore, an examination of the efficacy of IL-6 therapy in WM patients is necessary to evaluate its effect on IgM levels and tumor growth.

Taken together, these data suggest that administration of Tocilizumab monotherapy to tumor bearing mice results in a reduction in tumor burden and IgM secretion, despite the presence of IL-6 from bone marrow stromal cells in the TME. Therefore, administration of an anti-IL-6 therapy such as Tocilizumab to WM patients may provide therapeutic efficacy by targeting the TME to reduce IgM production and slow (Burger et al. 2009, Burger and Peled 2009, Elsawa and Ansell 2009, He et al. 2004, Leleu et al. 2007) the rate of tumor growth.



**Figure 1- 1. Mouse survival in the presence of Tocilizumab therapy.**

**Figure 1-1:** (A) Hairless SCID mice (n=10 mice/group) were subcutaneously injected with  $10 \times 10^6$  BCWM.1 or RPCI-WM1 cells + HS-5 stromal cells (5:1 ratio). Upon tumor appearance (day 7), groups of mice were treated with either Tocilizumab or Control antibody (IgG) at 100 $\mu$ g/mouse i.p every other day for a total of 5 weeks. (B) Mice were monitored and survival was reported.

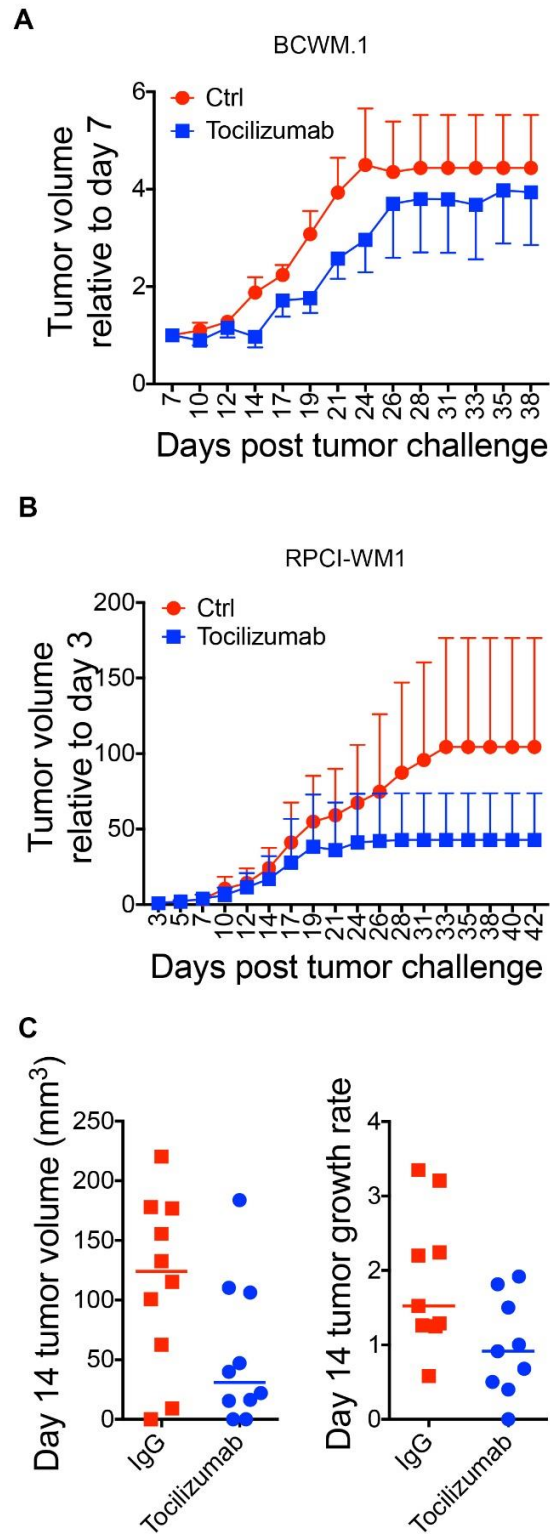
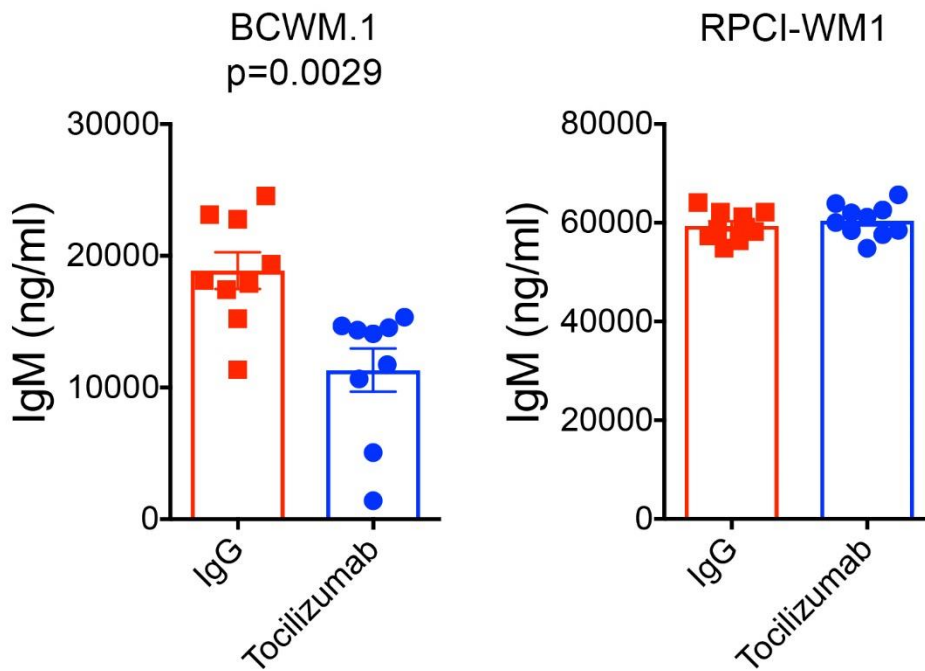


Figure 1- 2. Targeting the TME with Tocilizumab reduces tumor growth.

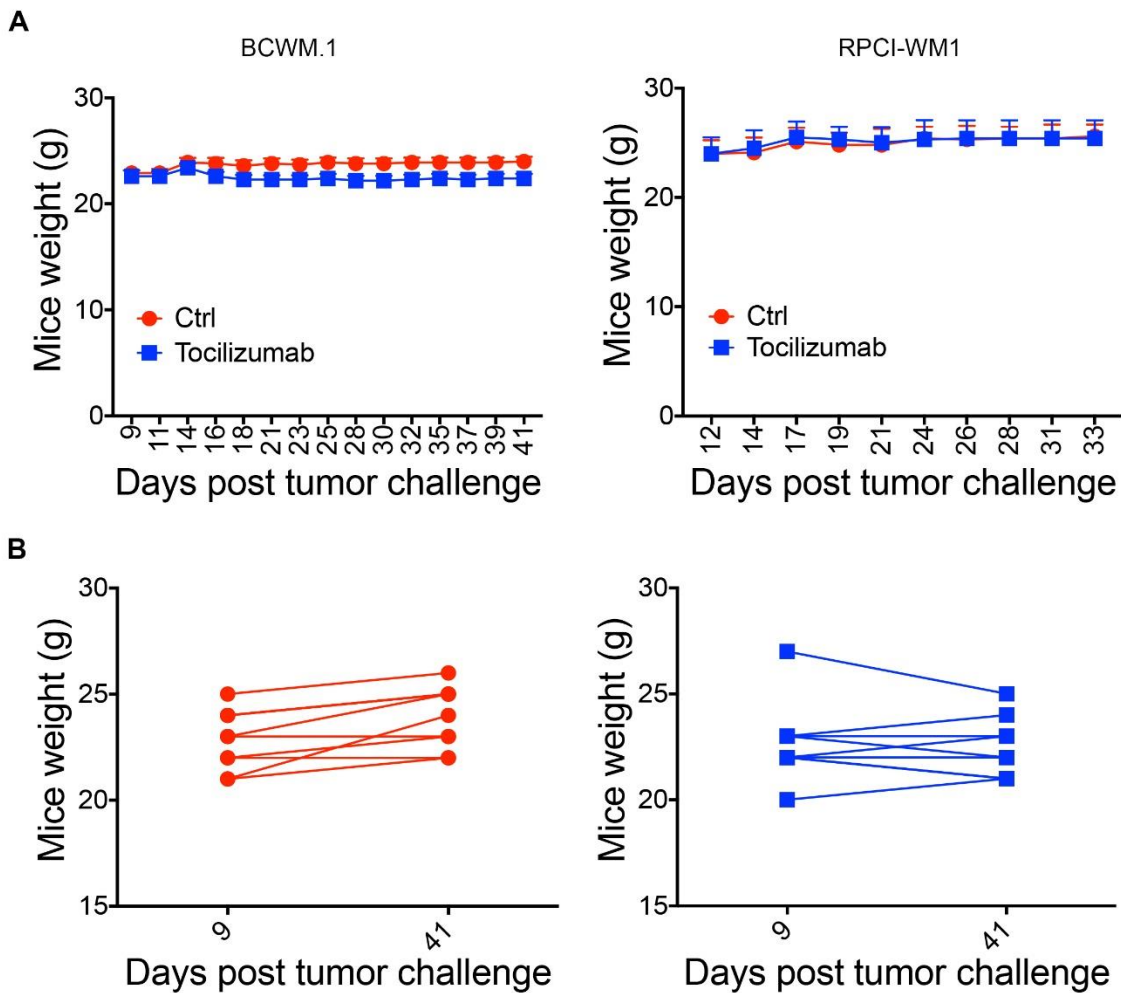
**Figure 1-2:** (A) Relative tumor growth rate in BCWM.1 (left; n=10/group) and RPCI-WM1 (p=0.0394) (right; n=10/group) (both with stromal cells) xenografted mice treated with either Tocilizumab or IgG control. The Y-axis indicates tumor volume relative to first recorded tumor size. (B) Tumor volume (left; p=0.057) and relative tumor growth (right, p=0.0306) on day 14 in mice xenografted with BCWM.1 cells and stromal cells. (C) Actual tumor volume (left, p=0.057) and tumor growth rate (right, p=0.0306) on day 14 in mice injected with BCMW.1 and stromal cells.



**Figure 1- 3. Tocilizumab reduces human IgM secretion in mice xenografted with BCWM.1 cells and stromal cells.**

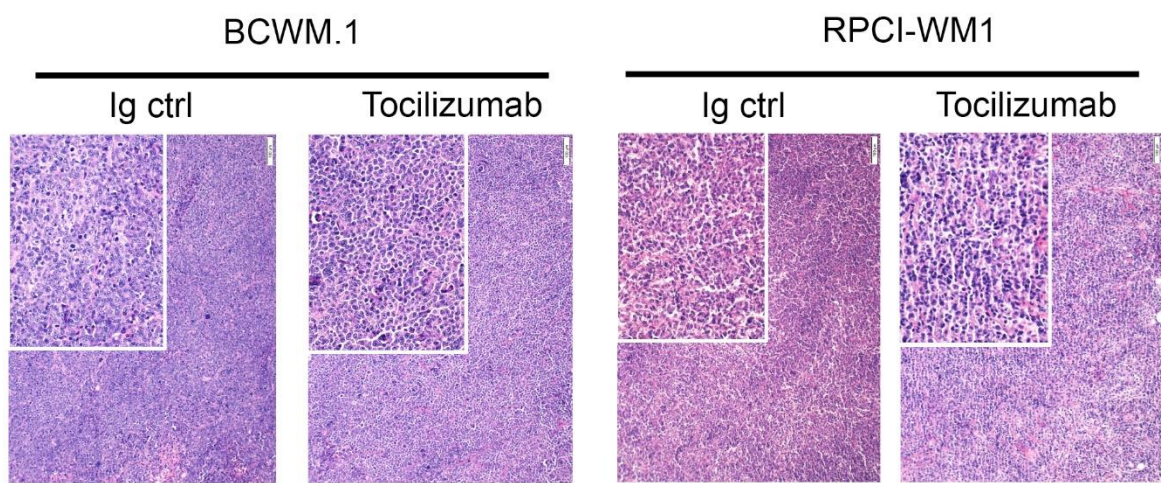


**Figure 1-3:** Serum was harvested from mice xenografted with BCWM.1 (left;  $p=0.0029$ ) and RPCI-WM1 (right) cells upon euthanasia and used to quantify human IgM levels in mice sera. IgM levels were determined using a human IgM ELISA.



**Figure 1- 4. Tocilizumab is not toxic to mice.**

**Figure 1-4:** (A) Mice weights were monitored 3x/week and recoded to determine potential treatment toxicity for BCWM.1 ( $p=0.005$ ) and RPCI-WM1 xenografted mice. (B) Individual mice weights on day 9 (first recorded weight) and day 42 (experiment end point) for mice xenografted with BCWM.1 cells and stromal cells.



**Figure 1- 5. Histology of tumors post euthanasia.**

**Figure 1-5:** H & E staining for tumors harvested from mice treated with Tocilizumab and control (BCWM.1 left, RPCI-WM1 right). Tumor samples were harvested and immediately fixed in 4% formaldehyde. Five  $\mu\text{m}$  sections were then stained with H & E as described in the methods.

## CHAPTER TWO

### NOVEL MOLECULAR MECHANISM OF REGULATION OF CD40 LIGAND BY THE TRANSCRIPTION FACTOR GLI2

#### **Introduction**

The tumor microenvironment (TME) plays an integral role in tumor cell biology and therefore it is difficult to dissociate the role of the TME from cancer cell biology. Signals from the TME have been shown to promote activation, proliferation, survival and cancer cell resistance to therapy (Briones et al. 2002, Burger et al. 2009, Cheung and Van Ness 2001, Elsawa et al. 2011a, Elsawa and Ansell 2009, Elsawa et al. 2006, Elsawa et al. 2011b, Finger and Giaccia 2010, Inácio Pinto et al. 2015, Kurtova et al. 2009b, Leleu et al. 2007, Moreaux et al. 2004, Pellegrino et al. 2005, Roccaro et al. 2010, Tournilhac et al. 2006). In bone marrow malignancies such as multiple myeloma (MM) and Waldenström macroglobulinemia (WM), bone marrow stromal cells play an important role in malignant cell biology by secreting a variety of cytokines that are used by malignant cells to survive and proliferate (Azab et al. 2012b, Burger et al. 2009, Burger and Peled 2009, Cheung and Van Ness 2001, Elsawa et al. 2011b, Kurtova et al. 2009a). Therefore, understanding the regulation of these cytokines in stromal cells is fundamental for our understanding of the role of the TME in malignant cell biology, and ultimately allows for the development of targeted therapies toward the TME.

Among the pathways controlling the bone marrow TME is CD40 signaling. This pathway is activated by its ligand (CD40L, as known as CD154), a 39 KDa type II transmembrane protein that belongs to the TNF gene superfamily (Schönbeck et al. 2000). This molecule is preferentially expressed by activated CD4+ T cells and activated platelets, although it can also be variably expressed by monocytes, NK cells, B cells, CD8+ T cells, mast cells, basophils and endothelial cells (Fiumara and Younes 2001, Mach et al. 1997, Schönbeck et al. 2000). CD40L can be cleaved from the cell surface to produce a soluble biologically active form (sCD40L) (Schönbeck et al. 2000). CD40L binds to its receptor CD40 on the surface of normal and malignant B cells and triggers a signaling cascade that leads to the activation of various transcription factors such as AP-1, NFAT and NF- $\kappa$ B (Srahna et al. 2001). In hematological malignancies such as chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL) and Waldenström macroglobulinemia (WM), CD40L/CD40 signaling induces increased growth and survival of malignant cells (Azab et al. 2012b, Burger and Peled 2009, Clodi et al. 1998, Fiumara and Younes 2001, Inácio Pinto et al. 2015, Kurtova et al. 2009a, Mach et al. 1997, Schönbeck et al. 2000, Srahna et al. 2001, Tournilhac et al. 2006). Therefore, disrupting the CD40L/CD40 pathway may offer therapeutic potential in malignant diseases. However, the mechanism controlling the regulation of the pathway remains poorly understood, thus limiting the targeting of this cascade in the clinical setting.

In this study, we identify CD40L as a novel target gene of the transcription factor GLI2 in the bone marrow microenvironment. GLI2, is an oncogenic member of the GLI family of transcription factors (Pasca di Magliano et al. 2006, Rohatgi and Scott 2007,

Ruiz i Altaba et al. 2007). Overexpression of GLI2 has been reported to play a role in oncogenic transformation in multiple tissues (Amarsaikhan and Elsawa 2013, Elsawa et al. 2011a, Furler and Uittenbogaart 2012, Jackson et al. 2015, Javelaud et al. 2011, Kumar et al. 2015, Takahashi et al. 1995). A screen for cytokines that are differentially regulated by GLI2 identified CD40L as a GLI2 target gene. Using quantitative RT-PCR and western blot, we found a strong induction of CD40L expression. We identified 2 candidate GLI binding sites in the promoter region of CD40L. We used a combination of luciferase and chromatin immunoprecipitation assays to show that GLI2 regulates CD40L expression by binding to its promoter and modulating its activity. Further analysis indicated that GLI2-mediated regulation of CD40L occurs downstream of the CCR3 signaling pathway. GLI2 was found to be required for CCR3-PI3K-AKT mediated regulation of CD40L. Using stable cell lines with CCR3 or GLI2 knockdown, we confirm the role of the CCR3-GLI2 signaling axis in the regulation of CD40L. Taken together, our studies identify a novel mechanism of regulation of CD40L via CCR3-GLI2 signaling in stromal cells as a novel transcriptional target of GLI2 and increases our understanding of the repertoire of cytokines that are modulated by this transcription factor in TME.

## **Materials and Methods**

### **Cell culture and reagents**

The bone marrow stromal cell line HS-5 and mouse embryonic fibroblasts NIH-3T3 were purchased from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin (P/S). L87 and L88 bone



marrow stromal cells were kindly provided by Dr. Chiara Corsini (European Institute of Oncology, Milan, Italy) and maintained in RPMI supplemented with 10% FBS and P/S. The Saka cell line was kindly provided by Dr. David Roodman (University of Pittsburgh, Pittsburgh, PA), and maintained in MEM $\alpha$  supplemented with 10% FBS and P/S as previously described (Elsawa et al. 2011a, Takahashi et al. 1995). BCWM.1 (Ditzel Santos et al. 2007) B cell line was a generous gift from Dr. Steven Treon (Dana Farber Cancer Institute, Boston, MA), MWCL-1 (Hodge et al. 2011) cells were a kind gift from Dr. Stephen Ansell (Mayo Clinic, Rochester, MN), and RPCI-WM1 (36) cells were from Dr. Asher Chanan-Khan (Mayo Clinic, Jacksonville, FL). All B cells were grown in RPMI 1640 with 10% FBS and P/S. The GLI1/GLI2 inhibitor, Gant61, was purchased from EMD Millipore (Billerica, MA). Antibodies against GLI2 and CD40L for western blot were both obtained from Abcam (Cambridge, UK). The PI3K inhibitor (LY294002) was obtained from Selleckchem (Houston, TX).

### **Plasmid constructs and cell transfections**

Short hairpin RNA (shRNA) targeting GLI2 was purchased from Origene Technologies (Rockville, MD) using plasmid vector backbone pGFP-V-RS. The shRNA targeting CCR3 was purchased from Santa Cruz Biotechnology (Dallas, TX) as previously described (Elsawa et al. 2011a). The GLI2 expression construct (in pCDNA3.1 6XHis) was previously described (Jackson et al. 2015). The CD40L promoter construct (in pGL3 basic vector), constitutively active (ca) PI3K and AKT, dominant negative (dn) PI3K and AKT were kindly provided by Dr. Martin Fernandez-Zapico (Mayo Clinic, Rochester, MN). CD40L expression construct was kindly provided

by Dr. Silvia Bruno (Genoa University, Italy). The dominant active pCS2-MT GLI2 delta N ( $\Delta$ N GLI2) plasmid (Roessler et al. 2005) was obtained from Dr. Erich Roessler through Addgene (plasmid # 17649) (Cambridge, MA).

For transfection experiments,  $4 \times 10^6$  cells were electroporated with the indicated concentrations of plasmid constructs at 250 V for 25 ms and harvested after 48 hours, unless otherwise noted. For overexpression experiments, 5  $\mu$ g of expression construct was used and 10  $\mu$ g of shRNA was used for knockdown studies. In luciferase experiments, 2  $\mu$ g of luciferase reporter construct was used.

### **RNA Isolation and quantitative PCR (qPCR)**

Total RNA was isolated using TRIsure reagent (Bioline, London, UK) following manufacturer's recommendations. Reverse transcription reactions were conducted by Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR (qPCR) was conducted using the ViiA 7 real-time PCR system (Life Technologies, Grand Island, NY). For expression of GLI2, CD40L or CCR3 relative to the expression of the housekeeping gene, GAPDH, the following primers were used: GAPDH, 5'-CTCGACTTCAACAGCGACA-3' (forward) and 5'-GTAGCCAAATTCGTTGTCATACC-3' (reverse); GLI2, 5'-CTCCGAGAAGCAAGAAGCCA-3' (forward) and 5'-GATGCTGCGGCACTCCTT-3' (reverse); CD40L, 5'-AACATCTGTGTTACAGTGGGCT-3' (forward) and 5'-AACGGTCAGCTGTTTCCCAT-3' (reverse); and CCR3, 5'-CTACTCCCCTGCTGCATGA-3' (forward) and 5'-CTGCTGTGGATGGAGAGACA-3' (reverse).

## **Cytokine arrays**

HS-5 stromal cells ( $4 \times 10^6$ ) were transfected with GLI2 overexpression construct. After 48 hours, cells were collected, RNA was isolated and reverse transcribed. Samples were then screened for GLI2 cytokine targets by qPCR using RT2 Profiler PCR Arrays kit from Qiagen (Hilden, Germany). Samples were analyzed on a Mx3000p Multiplex Quantitative PCR system (Agilent Technologies, Santa Clara, CA).

## **Proliferation assay**

Cells were serum starved in media with 0.5% FBS and then resuspended in medium with 0.5% FBS without phenol red at a concentration of  $0.25 \times 10^6$  cells/mL. 100  $\mu$ L of cell suspension were plated in triplicate wells of a 96-well plate and an additional 100  $\mu$ L of conditioned medium from stromal cells transfected with either empty vector or  $\Delta$ N GLI2 expression construct at a final concentration of 50% conditioned media in a final volume of 200  $\mu$ L/well. After 3 days, 50  $\mu$ L of XTT working solution (Trevigen, Gaithersburg, MD) was added to each well and incubated at 37°C for 3 hr then analyzed on an Epoch plate reader (Biotek, Winooski, VT).

## **Coculture experiments**

NIH-3T3 cells stably expressing CD40L or wild-type NIH-3T3 cells ( $0.25 \times 10^6$  cells/ml) were irradiated with 10Gy followed by plating 100  $\mu$ L/well in 96-well plates. Cells were allowed to adhere overnight followed by removal of media and addition of

serum-starved B cells ( $0.25 \times 10^6$  cells/ml) in 100  $\mu$ L/well (ratio of stromal cells: B cells is 1:1). Cocultures were incubated for an additional 3 days then 50  $\mu$ L of XTT working solution (Trevegin, Gaithersburg, MD) was added to each well and incubated at 37°C for 3 hr then analyzed on an Epoch plate reader (Biotek, Venoski, VT).

### **Enzyme-linked immunosorbent assay (ELISA)**

ELISA plates (Nunc maxisorp; Nalgene Nunc International, Rochester, NY) were used to quantify soluble CD40L (sCD40L) levels. Human CD40L ELISA (PeproTech, Rocky Hill, NJ) was used following manufacturer recommendations. Reactions were stopped by the addition of 1N H<sub>2</sub>SO<sub>4</sub> and the data was analyzed using an Epoch plate reader (BioTek).

### **Immunoblotting**

Total protein lysate was collected from stromal cell lines and protein concentration was determined by a BCA protein assay kit from Thermo Fisher Scientific (Waltham, MA). Protein was loaded into each well of a 7.5% SDS protein gel and a semidry transfer system (Bio-Rad, Hercules, CA) was used to transfer proteins to a nitrocellulose membrane. Ab specific for GLI2, CD40L or  $\beta$ -actin was used to detect protein. Densitometry was performed using ImageJ software, normalized to control and presented as an average of 3 independent experiments.

### **Luciferase reporter assay**

Cells ( $2 \times 10^6$ ) were transfected as described and plated in triplicate wells in 24-well plates. Following 2-day incubation with media containing 10% FBS, cells were harvested and assayed for luciferase activity following manufacturer's protocol (Promega Corporation, Madison, WI). To control for variations in transfection efficiency, luciferase readings were normalized to total protein for each sample using a BCA protein assay kit (ThermoFisher Scientific). Relative luciferase units (RLU) represents luciferase readouts/protein concentration relative to control samples in each experiment.

### **Chromatin immunoprecipitation (ChIP) assay**

Chromatin immunoprecipitation was conducted as previously described (Lo Re et al. 2012). Briefly, chromatin was digested with micrococcal nuclease (2 gel units/ul; New England Biolabs, Ipswich, MA) for 20 min at 37 °C. Chromatin was further sheared by sonication for 5 cycles of 1 min on/1 min off at 4 °C and 5 cycles of 30s on/30s off at 4 °C (Bioruptor; Diagenode, Denville, NJ). Aliquots of sheared chromatin were subjected to immunoprecipitation using an anti-GLI2 Ab (Novus Biologicals) or normal rabbit IgG (EMD Millipore). qPCR of the ChIP products and genomic input DNA was conducted using specific designed primers that amplify an area of the CD40L promoter containing GLI binding sites. The sequences of primers are as following: Binding site 1 (BS1), 5'-GCCTCTGACTTGACTGATCAAAG-3' (forward) and 5'-CCTGCTTTCTTCCCCTCCC-3' (reverse); Binding site 2 (BS2), 5'-GTGGCCACTTTGACAGTCTTC-3' (forward) and 5'-GCCGCAGATCGGGGAGAAG-3' (reverse). Quantitative PCR was performed using

SYBR Green at 65 °C in triplicate for each sample or control, and results are presented as a percentage relative to input.

### **Mutations of GLI binding site**

Mutations of GLI binding site in CD40L promoter was performed using QuikChange II XL Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) following manufacturer's recommendations. GLI2 binding site 1 (BS1) sequence from -1482 to -1478 bp was changed from CCACC to AAAAA using the following primers: 5'-GTAGCTTTTCACTACATCTGCCAAGTATTTTTCTTCGATGCACGTGATGAACTTTGATC-3' (sense) and 5'-GATCAAAGTTCATCACGTGCATCGAAGAAAAATACTTGGCAGATGTAGTGAAAAGCTAC-3' (antisense). Mutated plasmid was verified by sequencing.

### **Flow cytometry**

Fluorochrome-conjugated antibodies used for flow cytometry were obtained from BD Biosciences. To confirm/compare CD40L expression on wild-type or NIH-3T3 cells stably expressing CD40L,  $0.5 \times 10^6$  cells were stained with CD40L-APC or isotype control for 20 min followed by washing with FACS buffer and analysis on a FACS Caibur.

To determine B cell activation in response to NIH-3T3s stably expressing CD40L, serum starved B cells were co-cultured with NIH-3T3 cells for 15 minutes then collected in FACS tubes. Cells were then permeabilized and stained with the BD

Cytofix/Cytoperm Plus kit (BD Biosciences 555028) according to manufacturer recommendations with some modifications. Briefly, cells were spun down and resuspended in 100  $\mu$ l of Fix/Perm solution and incubated at 4°C for 30 min. Cells were then washed twice with Fix/Perm Wash Buffer and resuspended in 50  $\mu$ l Fix/Perm solution. Four  $\mu$ g of phosphor-Erk-PE (pErk-PE) antibody or PE-conjugated Isotype control were added and the tubes were incubated at 4°C for 30 min. Cells were washed twice with Fix/Perm Wash buffer and resuspended in 50  $\mu$ l Fix/Perm solution. Cells were resuspended in 500  $\mu$ l FACS Buffer and analyzed via Flow-Cytometry. Cells were analyzed on a FACSCalibur instrument and analyzed using FlowJo software with gating on viable cell populations.

### **Statistical analysis**

A two-tailed t test was used to determine statistical significance between two sets or ANOVA for more than two sets of data. A p value <0.05 was considered significant. Statistical analysis was carried out using GraphPad Prism software.

## **Results**

### **Screening for novel GLI2 target cytokines identifies CD40L as a candidate GLI2 target gene**

We hypothesized that GLI2 can target cytokine genes and modulate their expression. To address this hypothesis, we screened for cytokines that are important in the bone marrow tumor microenvironment that are potentially regulated by GLI2. We

identified several cytokine genes to be differentially regulated by this transcription factor (**Figure 2-1A**). Among those genes is the cytokine CD40L. Because CD40L plays a role in malignant B cell biology (Burger and Gandhi 2009, Fiumara and Younes 2001, Tournilhac et al. 2006), we co-cultured B cell lines from Waldenström macroglobulinemia (WM) with stromal cells stably expressing CD40L (**Figure 2-1B**) and found an increase in Erk phosphorylation (**Figure 2-1C**), indicating an increase in malignant B cell activation. Furthermore, coculture of CD40L-expressing stromal cells with WM cells induced an increase in WM cell proliferation (**Figure 2-1D**), further confirming the importance of the CD40L/CD40 signaling axis in WM B cell lines and WM biology.

### **GLI2 regulates the expression of CD40L**

To validate cytokine array data, we overexpressed GLI2 in several bone marrow-derived stromal cell lines (HS-5, L87, L88 and Saka) and determined the effect on CD40L mRNA expression by quantitative PCR. Overexpression of GLI2 resulted in a significant increase in CD40L expression (**Figure 2-2A**). This also resulted in increased CD40L protein by western blot (**Figure 2-2B**). Using RNAi to target GLI2, we found a significant reduction in CD40L mRNA expression in Saka and L88 cells (**Figure 2-2C**). This reduction in CD40L gene expression also resulted in reduced CD40L protein expression in these stromal cells (which expressed higher levels of CD40L under basal conditions) in the presence of GLI2 knockdown (**Figure 2-2D**). The effect of GLI2 knockdown on CD40L expression was partially rescued using RNAi-resistant GLI2 cDNA (**Figure 2-2E**).



CD40L can be cleaved from the cell surface to produce a soluble form (sCD40L) (Schönbeck et al. 2000). Using a dominant active GLI2 cDNA that lacks an N-terminal repressor domain ( $\Delta$ N GLI2), we found an increase in sCD40L by ELISA compared with cells transfected with empty vector (**Figure 2-2F**). Using supernatant from 2-day cultures of  $\Delta$ N GLI2 transfected cells ( $\Delta$ N GLI2 conditioned media), we found a significant increase in BCWM.1 and MWCL-1 cell proliferation ( $p < 0.0001$ ) compared to cells cultured with supernatant from cells transfected with empty vector (**Figure 2-2G**). Although this increase in proliferation was modest (12% in BCWM.1 and 13% in MWCL-1 cells), this may have biological implications for an indolent lymphoma such as WM (2005, Dimopoulos and Anagnostopoulos 2005, Fonseca and Hayman 2007, Ghobrial et al. 2003). Taken together, our data indicate that the transcription factor GLI2 regulates CD40L mRNA and protein expression.

### **GLI2 directly binds and regulates the CD40L promoter**

Bioinformatic analysis of the CD40L promoter region (~ 2000 bp) identified 2 candidate GLI2 binding sites (BS1 and BS2) (**Figure 2-3A**). To characterize the role of GLI2 in modulating the CD40L promoter, we utilized a combination of luciferase reporter and chromatin immunoprecipitation (ChIP) assays. We found that GLI2 overexpression induces CD40L promoter activity in stromal cells (**Figure 2-3B**). Conversely, GLI2 knockdown using shRNA targeting GLI2 results in significantly reduced CD40L promoter activity (**Figure 2-3C**).

Using ChIP assay, we confirmed that endogenous GLI2 binds to the distal GLI binding region of the CD40L promoter (BS1) from -1524 to -1397 using qPCR (**Figure**

**2-3D**). To demonstrate the specificity of GLI2 binding to BS1 on the CD40L promoter, we performed luciferase assay using stromal cells transfected with WT CD40L promoter sequence or mutant version of this plasmid lacking the GLI2 binding sequence. We found that GLI2 was not able to increase luciferase activity of the mutant CD40L promoter although it did increase WT CD40L promoter (**Figure 2-4A**). Next, we found that binding of GLI2 to the CD40L promoter was reduced in stromal cells treated with the GLI inhibitor Gant61 (**Figure 2-4B**). Treatment of stromal cells with Gant61 also resulted in a reduction in CD40L mRNA expression (**Figure 2-4C**). Finally, using HS-5 cells stably transduced with retrovirus containing either shScr or shGLI2 also confirmed that this binding of GLI2 to CD40L promoter is reduced with GLI2 knockdown (**Figure 2-4D**). Together, these results identify CD40L as a novel direct target of the GLI2 transcription factor and for the first time provide evidence for a potential role of GLI2 in the regulation of this signaling cascade.

### **CCR3-PI3K-AKT signaling modulates GLI2-CD40L axis**

We have previously identified the CCR3-PI3K-AKT signaling pathway as a regulator of GLI2(Elsawa et al. 2011a). Therefore, to define a potential mechanism driving this novel GLI2-CD40L axis we examined the role of the CCR3-PI3K-AKT signaling pathway on the regulation of CD40L. We utilized luciferase reporter and expression assays to investigate the role of this signaling axis on CD40L. We found that overexpression of CCR3 increased the mRNA expression of CD40L (**Figure 2-5A**). Furthermore, using constitutively active PI3K and AKT mutants, we found an increase in the expression of CD40L mRNA (**Figure 2-5B**). Overexpression of CCR3 also resulted

in an increased in CD40L promoter activity (**Figure 2-5C**). Conversely, CCR3 knockdown using shRNA results in a reduction in CD40L promoter activity (**Figure 2-5D**). Furthermore, we found that constitutively active PI3K (**Figure 2-5E**) and AKT (**Figure 2-5F**) increased CD40L promoter activity and dominant negative forms of PI3K and AKT that antagonize this pathway reduced CD40L promoter activity (**Figure 2-5G**). To confirm the role of PI3K/AKT, we treated stromal cells with the PI3K inhibitor (LY294002) and examined the downstream effect on CD40L expression. Similar to results obtained with genetic inhibition of PI3K/AKT, stromal cells treated with the pharmacological inhibitor of PI3K had a significant reduction in CD40L protein expression (**Figure 2-5H**). Finally, in the absence of GLI2, constitutively active AKT was not able to increase CD40L promoter activity to the extent that it does in the presence of an intact GLI2 (**Figure 2-5I**). Taken together, this data demonstrates that the CCR3-PI3K-AKT signaling pathway can modulate CD40L expression and promoter activity and this regulation of CD40L requires an intact GLI2.

### **CCR3-GLI2-CD40L axis is important in the TME**

To validate the role of GLI2 in the regulation of CD40L, we used stromal cells with stable GLI2 knockdown by retroviral transduction using 2 different shRNAs targeting GLI2 and found a significant reduction in CD40L expression by qPCR (**Figure 2-6A**). We also generated stromal cells with stable CCR3 knockdown and found a reduction in CD40L expression (**Figure 2-6B**). This reduction in CD40L expression as a result of CCR3 knockdown was rescued by the introduction of GLI2 cDNA (**Figure 2-6B**) downstream of CCR3 signaling. Taken together, these results confirm the role of

CCR3-GLI2 signaling axis in regulating CD40L expression in the TME and that GLI2 can rescue the effect of CCR3 knockdown on CD40L expression (**Figure 2-6A-C**).

## Discussion

Previous evidence has shown that the bone marrow tumor microenvironment is critical for the growth and persistence of malignant B cells (Burger et al. 2009). The interactions between tumor cells and their surrounding cells are essential for malignant cell biology (Burger et al. 2009, Podar et al. 2008). Our findings presented here have identified CD40L as a direct transcriptional target gene of GLI2 in stromal cells (**Figures 2-2 and 3**). Several studies have focus on the role of GLI proteins as the mediators of Hedgehog signaling pathway (Amakye et al. 2013, Bar et al. 2007, Dierks et al. 2008, Fernandes et al. 2009, Liu et al. 2006, Varnat et al. 2009). GLI2, one of the transcription factors from the GLI family, known as the effector of the Hedgehog signaling pathway, plays a major rule during cell development (Ruiz i Altaba et al. 2007). Here, we focused our studies on the molecular mechanism of cytokine regulation by the oncogenic transcription factor GLI2. The results from our initial screening of cytokines potentially modulated by GLI2 identified several cytokines that are differentially regulated by GLI2 (**Figure 2-1**). Among those, CD40L was identified as a new novel GLI2 target gene (**Figure 2-1**). CD40L is an important cytokine that has been shown to play an important role during normal B cell responses where it regulates B cell growth, survival, antibody secretion as well as class switch recombination (CSR) (Wykes 2003). In addition to its role in normal B cell functions, it has also been shown to modulate malignant B cells (Burger and Gandhi 2009, Fiumara and Younes 2001, Tournilhac et al. 2006).

Therefore, understanding the mechanism of its regulation is important to further our understanding of normal and malignant B cells responses. We found that the oncogenic transcription factor, GLI2, induces the expression of CD40L in stromal cells (**Figure 2-2**). We also show that GLI2 induces an increase in sCD40L and conditioned media from  $\Delta$ N GLI2 transfected stromal cells induced an increase in WM cell proliferation (**Figure 2-2F and G**). Because the increase in proliferation was modest (12-13%), this did not allow us to observe a statistical difference in WM cell proliferation in the presence of CD40L blocking antibodies, although the trend was consistent with at least a partial role for sCD40L (data not shown). This raises the possibility that in addition to sCD40L, other soluble mediators regulated by GLI2 may contribute to the increase in WM cell proliferation. A comprehensive understanding of GLI2 target genes is likely to shed light onto these mechanisms. Moreover, we found that GLI2 modulates CD40L expression by directly binding to its promoter (**Figures 2-3 & 4**). Our group has shown that GLI2 can directly target the IL-6 promoter in bone marrow stromal cells and lead to increased IL-6 expression and secretion (Elsawa et al. 2011a). We also demonstrated a direct role for GLI2 in regulating IgM secretion by WM cells (Jackson et al. 2015). Therefore, this study identifies CD40L as a novel GLI2 target gene and suggests a novel mechanism by which GLI2 modulates malignant B cell biology via modulation of the TME. Furthermore, we increase our understanding of the repertoire of cytokines that are regulated by this transcription factor.

Our data identified CD40L as a novel direct target of the GLI2 transcription factor in human bone marrow stromal cells (**Figures 2-1, 2 and 3**). Previous studies have shown that CD40L expression on mast cells in the TME promotes CD40 signaling on primary

WM B cells (Tournilhac et al. 2006). Our data support these studies showing GLI2-mediated CD40L increases WM B cell activation (as evidenced by Erk phosphorylation) (**Figure 2-1C**) and ultimately increased malignant B cell proliferation (**Figure 2-1D**) upon co-culture with stromal cells stably expressing CD40L. A role for CD40L has been described in mantle cell lymphoma (MCL) where CD40L promotes activation and proliferation of MCL cells (Andersen et al. 2000). Similarly, a role for CD40L/CD40 signaling has been described in Hodgkin lymphoma, B cell lymphoma, chronic lymphocytic leukemia and other hematological neoplasms (Aldinucci et al. 2012, Burger and Gandhi 2009, Fiumara and Younes 2001, Jin et al. 2004, Schattner 2000, Tong et al. 2000, Tournilhac et al. 2006, Tsirakis et al. 2012, Voorzanger-Rousselot et al. 1998, Younes 2001). Despite the importance of CD40L-CD40 signaling in normal and malignant B cells, little is known about the mechanisms that regulate CD40L expression. The identification of GLI2 as a regulator of CD40L promoter promotes our understanding of the regulation of this important cytokine.

The identification of GLI2 as a regulator of CD40L promoter promotes our understanding of the regulation of this important cytokine. In human T cells, several transcription factors including AP-1, NF-AT and NF- $\kappa$ B have been shown to modulate CD40L (Srahna et al. 2001). These transcription factors regulate a region closer to the transcription start site. The region containing the GLI2 binding site (BS1; -1478) is located further upstream from the regulatory regions of these transcription factors (**Figure 2-3A**). It will be interesting in future studies to address whether GLI2 in concert with AP-1, NF-AT and NF- $\kappa$ B regulates CD40L expression.

Previous studies have shown that GLI2 can modulate the expression of TGF- $\beta$  (Furler and Uittenbogaart 2012). Furthermore, we have shown that GLI2 modulates the expression and secretion of IL-6 and IL-6R (Elsawa et al. 2011a, Jackson et al. 2015). Here, we identify CD40L as a novel GLI2 target genes, therefore increasing our knowledge of the array of cytokine genes regulated by this transcription factor and further supporting a role for GLI2 in the regulation of inflammation. Furthermore, this regulation occurs downstream of CCR3-PI3K-AKT signaling in stromal cells.

In summary, our studies demonstrate a novel role for GLI2 in promoting malignant B cell activation and proliferation via regulation of CD40L expression in the TME and signaling through the CCR3-PI3K-AKT pathway can modulate CD40L expression and promoter activity and this regulation requires an intact GLI2 (**Figure 2-6C**). These studies for the first time provide evidence for the molecular mechanism of GLI2 regulating CD40L expression in human bone marrow stromal cells. Further investigation of the GLI2-CD40L axis has great potential in increasing our understanding of the mechanisms of GLI2 regulating cytokines in the TME.

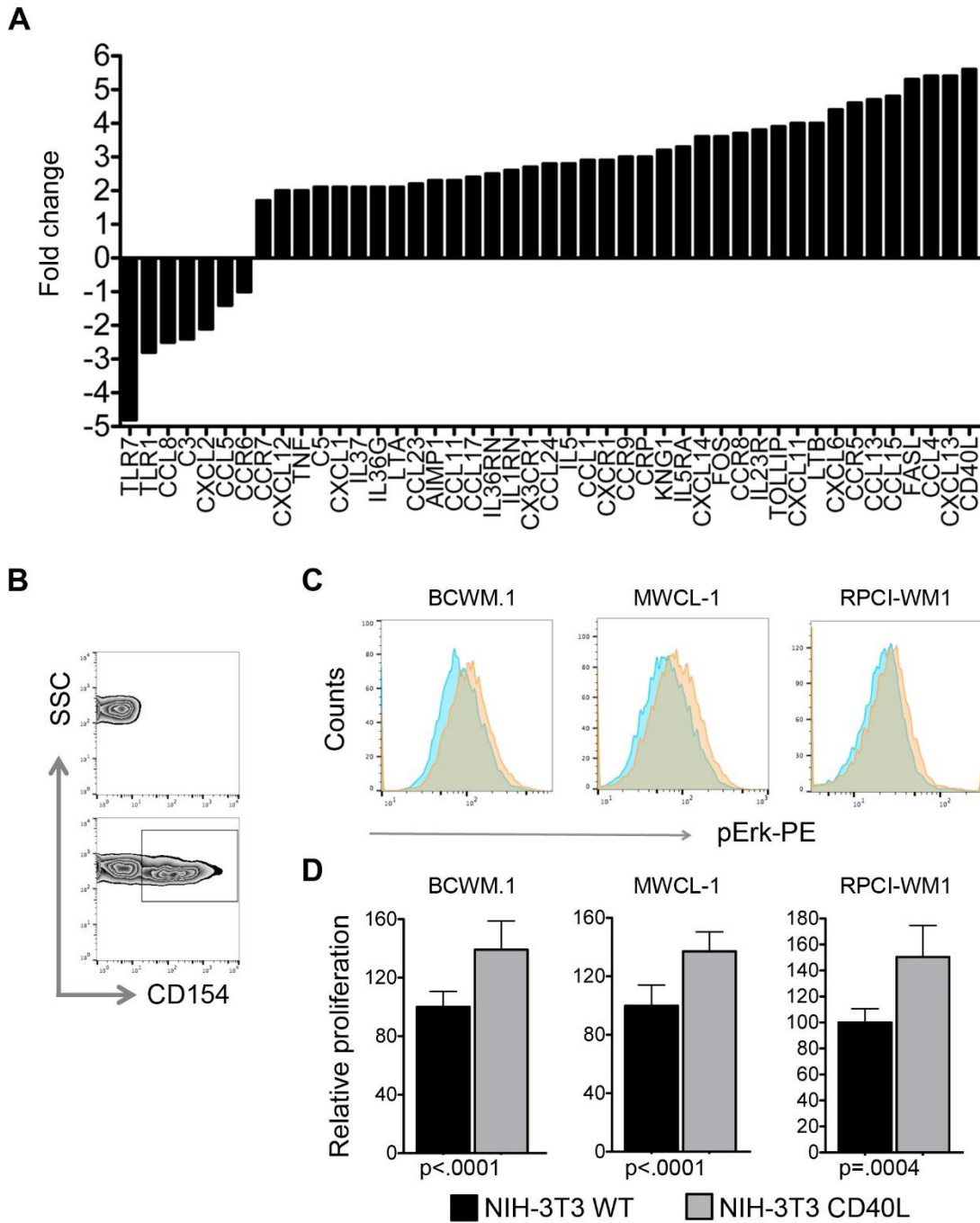


Figure 2- 1. GLI2 modulates cytokines genes.



**Figure 2-1:** (A) HS-5 stromal cells ( $4 \times 10^6$ ) were transfected with either empty vector (Ctrl) or GLI2 expression construct (GLI2). After 48 hours, cells were collected and followed by RNA extraction to determine gene expression by using PRT-PCR arrays as indicated in methods. (B) FACS analysis showing the expression of CD40L on NIH-3T3 cells stably transfected with human CD40L expression construct compared with wild type NIH-3T3. (C) NIH-3T3 cells stably expressing CD40L ( $0.5 \times 10^6$  cells/well) were allowed to adhere overnight in 6-well plates. Serum-starved malignant B cells (BCWM.1, MWCL-1 and RPCI-WM1) were then added (1:1 ratio) and incubated for 15 minutes. Non-adherent B cells were then collected and used to determine the level of ERK phosphorylation by FACS. (D) NIH-3T3 cells were irradiated with 10 Gy, resuspended at  $0.25 \times 10^6$  cells/ml, and 100  $\mu$ l were plated in 96-well plates and allowed to adhere overnight. Serum-starved B cells were then added at a ratio of 1:1 were added to NIH-3T3 cell cultures and incubated at 37°C. After 3 days, cell proliferation was determined by XTT assay as described in the methods. This experiment was repeated 3X and the data are presented as an average of 3 independent experiments +/- SEM.

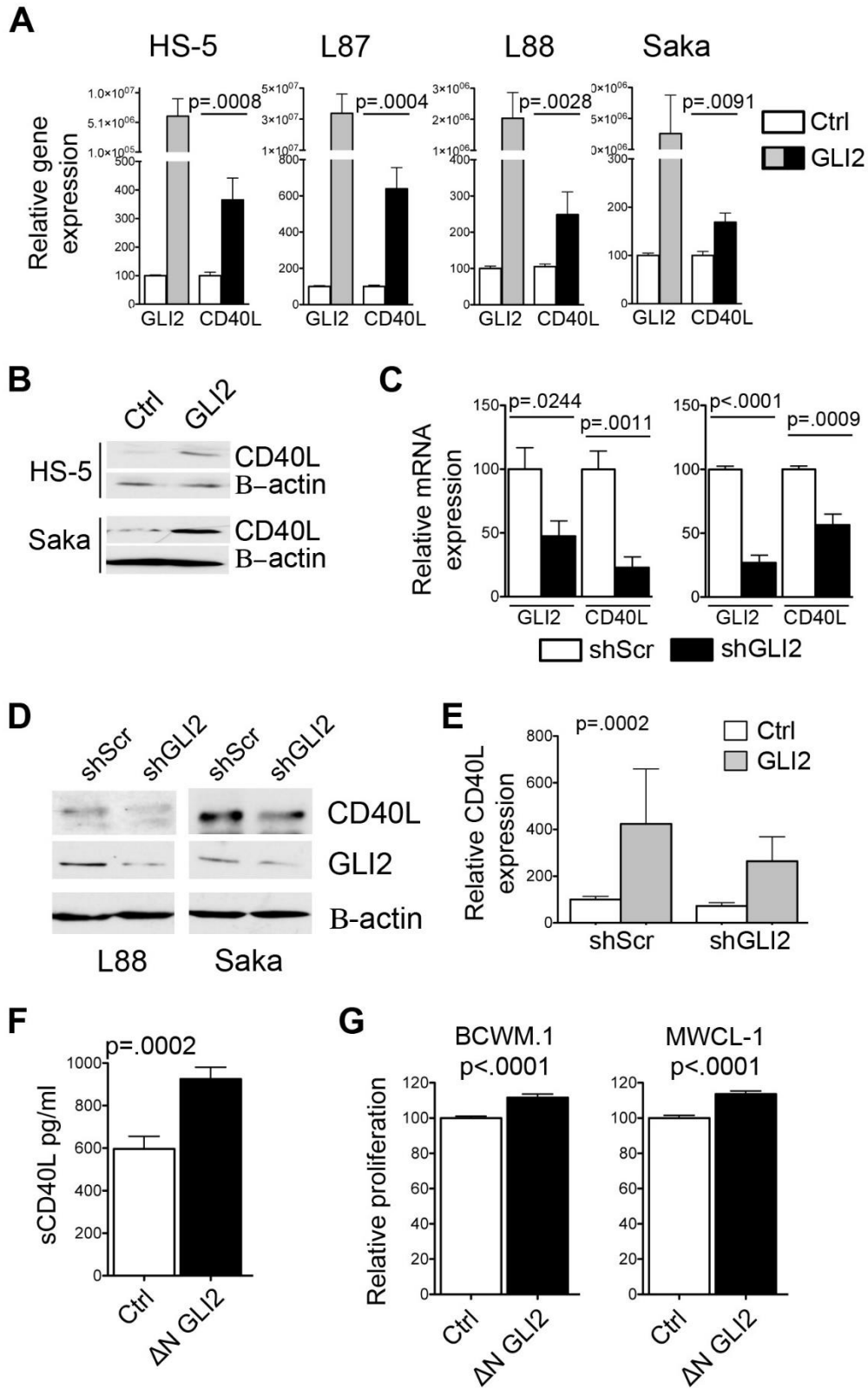
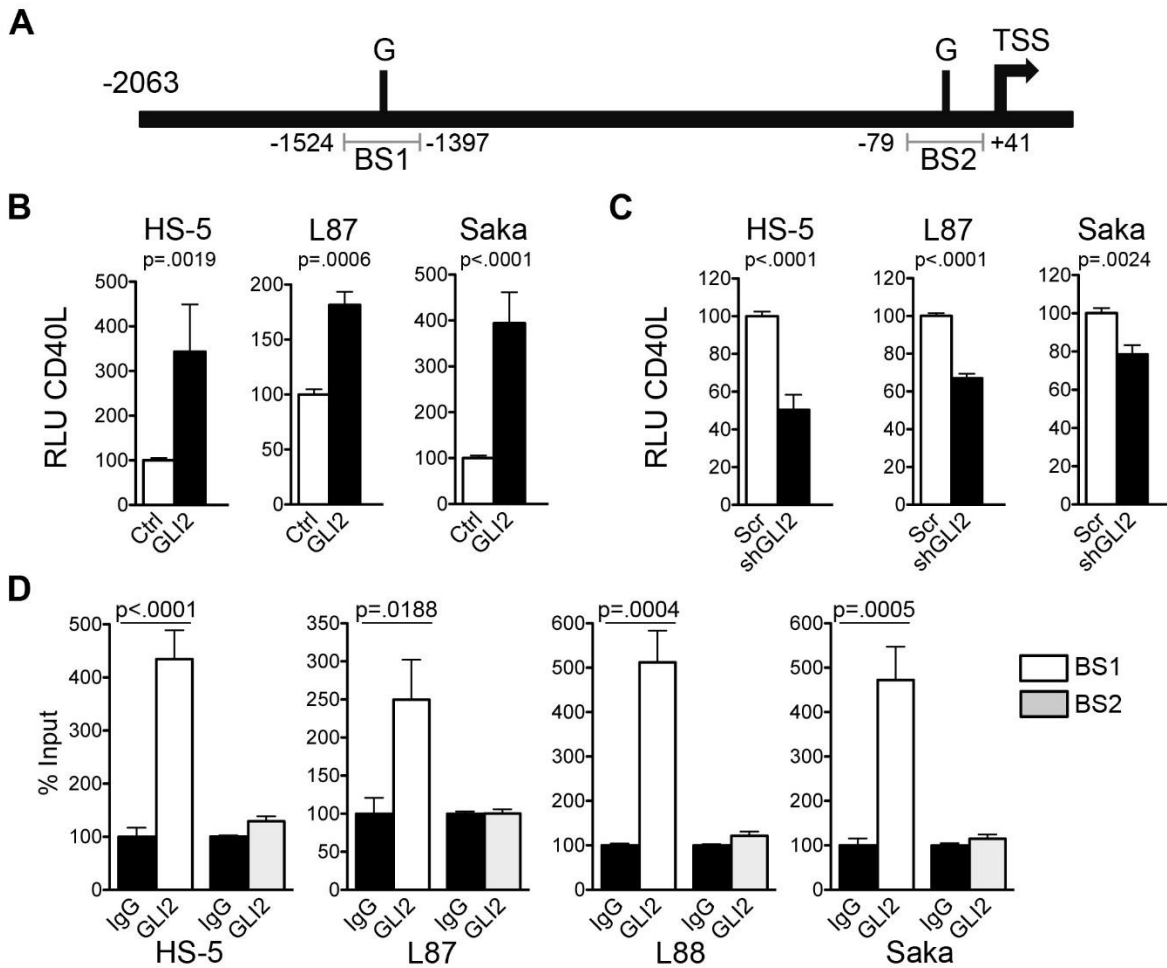


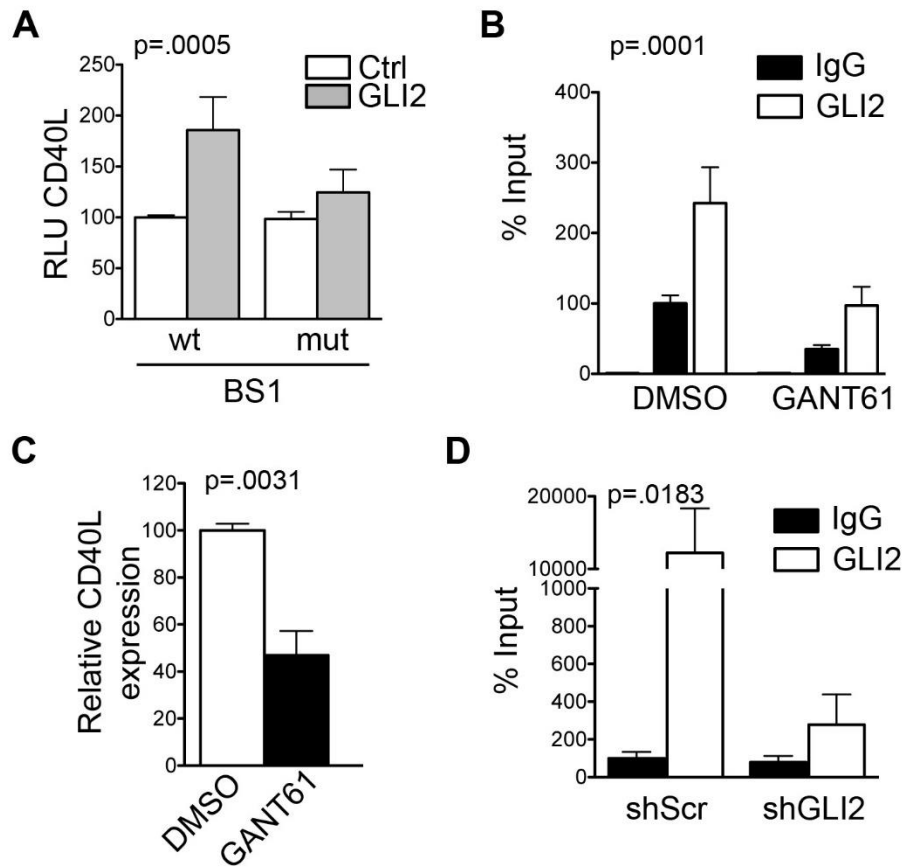
Figure 2- 2. GLI2 induces CD40L expression.

**Figure 2-2:** (A) Stromal Cells ( $4 \times 10^6$ ) were transfected with either empty vector (Ctrl) or GLI2 expression construct (GLI2) for 2 days followed by RNA extraction for use to determine gene expression by qPCR as indicated in methods. (B) Western blot for CD40L protein in HS-5 and Saka cells transfected with either empty vector (Ctrl) or GLI2 expression construct (GLI2) for 2 days. (C) qPCR for GLI2 and CD40L using Saka cells (left) and L88 cells (right) transfected with shScr or shGLI2. (D) L88 and Saka stromal cells were transfected with shRNA targeting GLI2 (shGLI2) or scrambled control (shScr). After 2 days, cells were lysed and used to determine protein expression by western blot. (E) Relative expression of CD40L in L88 stromal cells transfected with either Ctrl or RNAi-resistant GLI2 cDNA (GLI2) in the presence of either shScr or shGLI2. Gene expression was determined by qPCR. (F) L88 cells ( $4 \times 10^6$ ) were transfected with a dominant active form of GLI2 ( $\Delta N$  GLI2) or empty vector (Ctrl). After 2 days, supernatants were harvested and used to determine soluble CD40L (sCD40L) levels by ELISA. (G) Supernatants (50%) from L88 cells transfected with Ctrl or  $\Delta N$  GLI2 were used to culture B cells for 3 days followed by determination of cell proliferation by XTT assay. Each experiment was repeated at least 3 times and bars represent the mean  $\pm$  S.E. of triplicate experimental wells.



**Figure 2- 3. GLI2 binds and modulates CD40L promoter.**

**Figure 2-3:** (A) Bioinformatics analysis of the CD40L promoter identified candidate GLI (G) binding sites. Gray lines represent amplicons for the primer sets used that span the GLI binding sites. Forward and reverse primer start sequences are indicated. TSS, transcription start site. (B) Relative changes in luciferase activity in stromal cells transfected with CD40L promoter-luciferase reporter and with either empty vector (Ctrl) or GLI2 expression construct. Cells were harvested after 2 days and changes in luciferase activity were assayed as described in methods. (C) Relative luciferase activity in stromal cells transfected with CD40L promoter-luciferase reporter and with either shScr or shGLI2. Cells were harvested after 2 days and changes in luciferase activity were assayed. (D) Chromatin immunoprecipitation (ChIP) assay was performed on lysates from stromal cells using antibodies specific for GLI2 or an IgG isotype control. Quantitative PCR was performed using primers indicated above for 2 candidate GLI binding sites.



**Figure 2- 4. CD40L promoter activity with mutated GLI2 binding site and GLI2 inhibition.**

**Figure 2-4:** (A) Relative changes in luciferase activity in Saka stromal cells transfected with either wild-type (wt) CD40L promoter-luciferase reporter or CD40L promoter with mutated BS1 (mut) and with either empty vector (Ctrl) or GLI2 expression construct. Cells were harvested after 2 days and changes in luciferase activity were assayed as described in methods. (B) A ChIP assay was performed on L88 cell lysates from cells treated with either 20  $\mu$ M GANT61 or DMSO control for 24 hours. qPCR was performed to determine the effect of treatment on GLI2 binding to the CD40L promoter. (C) HS-5 cells were treated with either 20  $\mu$ M GANT61 or DMSO control for 24 hours. Cells were harvested and followed by RNA extraction for use to determine CD40L gene expression effected by GLI inhibitor GANT61 using qPCR. (D) A ChIP assay was performed on HS-5 stromal cells stably transduced with retrovirus containing either shScr or shGLI2. qPCR was performed to determine the binding of GLI2 to the CD40L promoter. Each experiment was repeated at least 3 times and Bars represent the mean  $\pm$  S.E. of triplicate experimental wells.

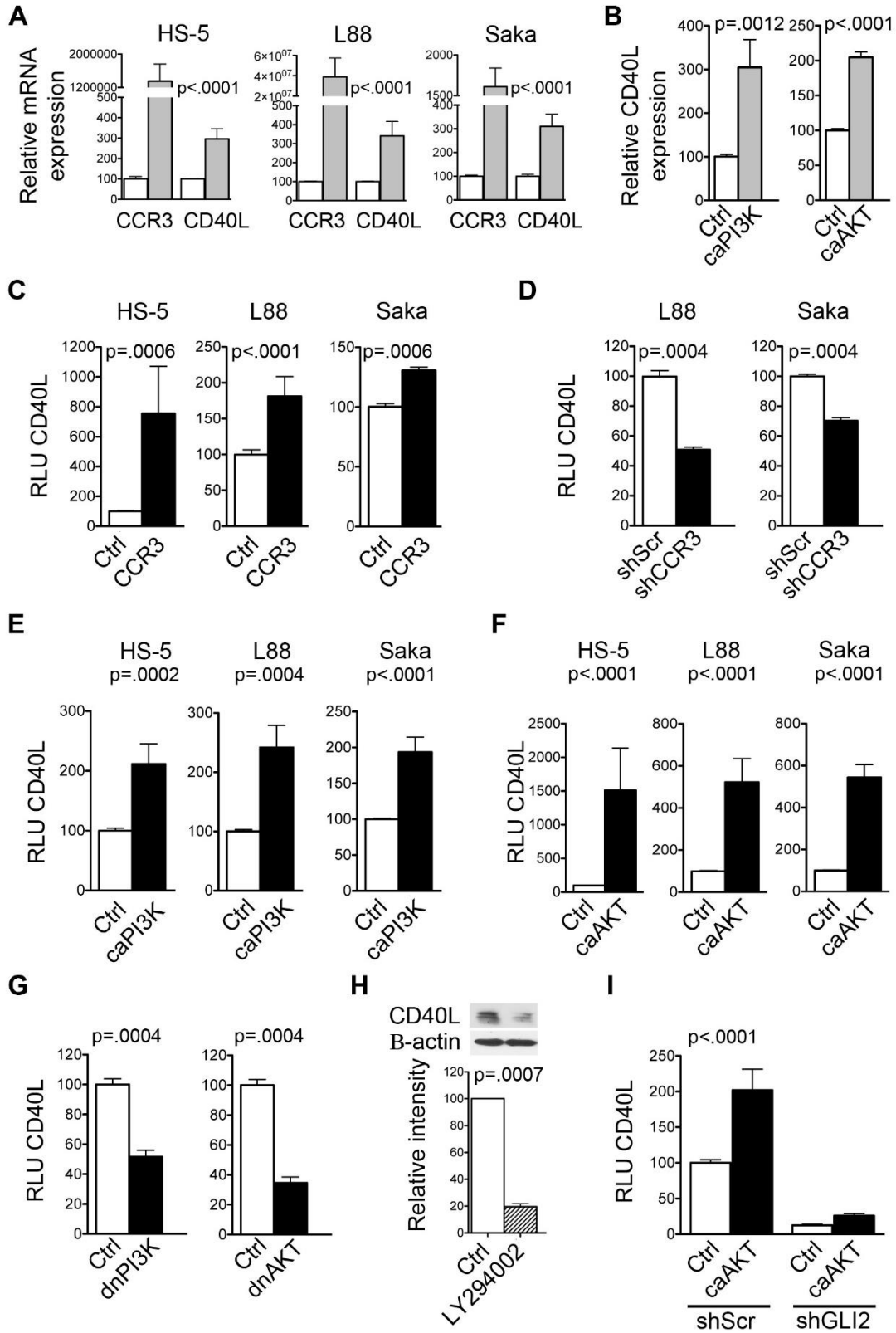


Figure 2- 5. CCR3-PI3K signaling modulates CD40L.



**Figure 2-5:** (A) Stromal cells were transfected with a CCR3 expression construct or empty vector (Ctrl). After 48 hours, RNA was isolated and used to determine CD40L expression by qPCR. (B) L88 cells were transfected with a constitutively active PI3K construct (caPI3K), a constitutively active AKT construct (Gruber Filbin et al.), or empty vector (Ctrl). After 48 hours, RNA was isolated and used to determine CD40L expression by qPCR. (C-D) Relative luciferase activity in stromal cells transfected with CD40L promoter-luciferase reporter and either CCR3 expression construct or empty vector (Ctrl) or shScr or shCCR3. Cells were harvested after 2 days and changes in luciferase activity were assayed. (E-F) Relative luciferase activity in stromal cells transfected with CD40L promoter-luciferase reporter and caPI3K, caAKT or empty vector (Ctrl). Cells were harvested after 2 days and changes in luciferase activity were assayed. (G) Relative luciferase activity in HS-5 cells transfected with CD40L promoter-luciferase reporter and dominant negative PI3K (dnPI3K), dominant negative AKT (dnAKT) or empty vector for 2 days. Cells were harvested and changes in luciferase activity were assayed. (H) HS-5 stromal cells were treated with the PI3K inhibitor (LY294002) or DMSO (Ctrl) for 24 hr followed by immunoblotting to determine CD40L protein levels. Graph represents densitometry on analyzed immunoblotting images from 3 independent experiments showing a reduction in CD40L protein expression. (I) Relative luciferase activity in HS-5 cells transfected with CD40L promoter-luciferase reporter and caAKT or empty vector in the presence or absence of GLI2 (shGLI2). Cells were harvested after 2 days and changes in luciferase activity were assayed. Each experiment was repeated at least 3 times and Bars represent the mean  $\pm$  S.E. of triplicate experimental wells.

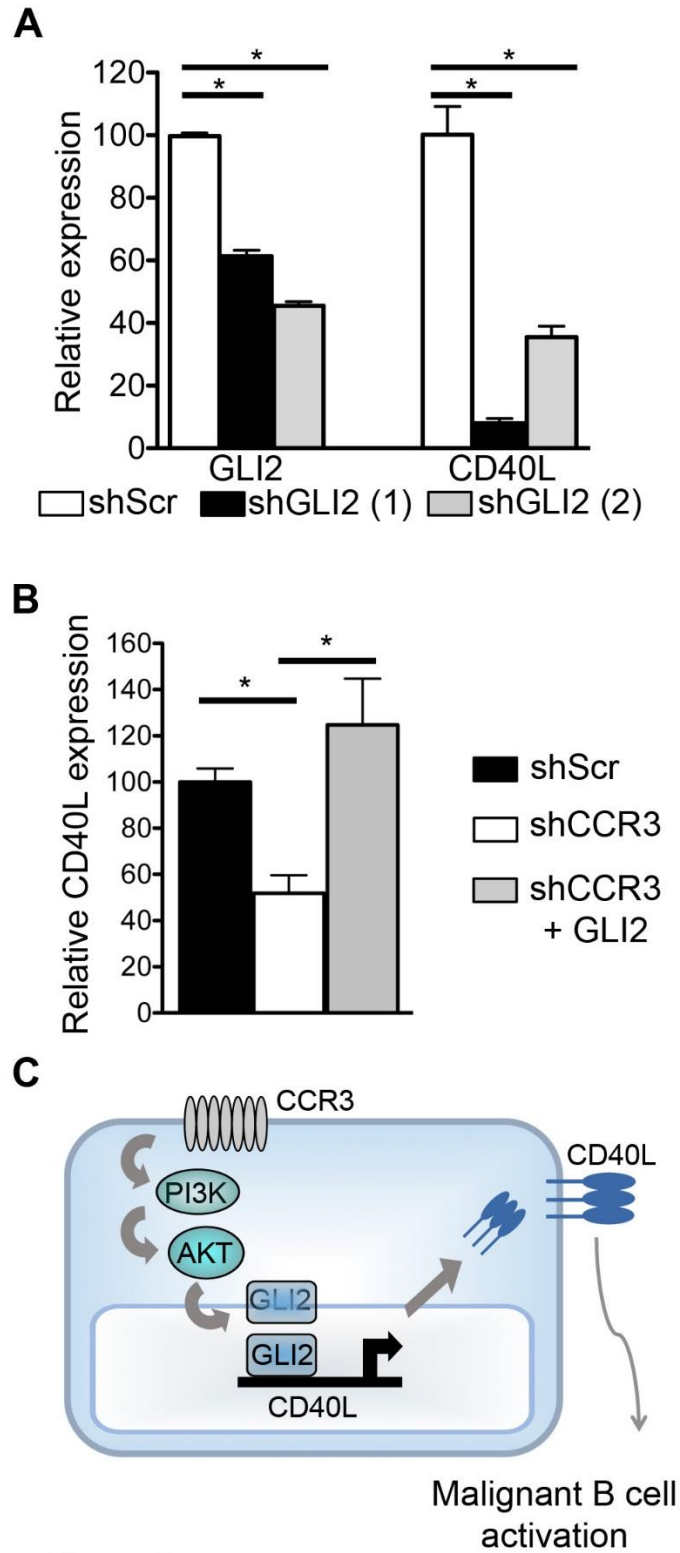


Figure 2- 6. CCR3 modulates GLI2-CD40L axis in the TME.

**Figure 2-6:** (A) Relative changes in GLI2 and CD40L mRNA expression in L88 stromal cells stably transduced with retrovirus containing either shScr or 2 different shRNA targeting GLI2 [shGLI2(In et al.) and shGLI2(In et al.)]. RNA was extracted from  $10 \times 10^6$  cells and used to determine gene expression by qPCR. (B) Relative changes in CD40L gene expression in L88 stromal cells stably transfected with either shScr or shCCR3. Stable cells ( $4 \times 10^6$  cells) were transfected with either GLI2 cDNA or empty vector. After 2 days, cells were collected and followed by RNA extraction to determine gene expression by qPCR. (C) Model of CCR3-GLI2-CD40L signaling axis in stromal cells.

## CHAPTER THREE

### IDENTIFICATION OF NOVEL GLI2 TRAGET GENES IN HUMAN BONE MARROW STROMAL CELLS

#### Introduction

GLI2, GLI family zinc finger 2, is a member of the GLI family of transcription factors that modulates gene expression directly or indirectly through interaction with other proteins (Ruiz i Altaba et al. 2007). GLI2, along with two other members of the GLI family of transcription factors (GLI1 and GLI3), act together in response to signaling from the Hedgehog (HH) receptor and other signaling inputs, resulting in the regulation of target gene expression. This ultimately leads to modulated cellular and biological functions. Subsequent studies have shown that GLI2 can be activated in the absence of signaling from HH suggested that other signaling pathways may modulate GLI activity. Indeed, TGF- $\beta$  has been reported as potent transcriptional inducer of GLI2 and can activate GLI2 without signaling from HH receptors (Dennler et al. 2007). In bone marrow stromal cells, GLI2 was found to be activated through CCR3-PI3K-AKT signaling pathway without signaling from HH (Elsawa et al. 2011a).

The regulation and transcriptional role of GLI2 have been widely studied. GLI2 was found to modulate TGF- $\beta$ 1 expression in CD4+ T cells by regulating the promoter of TGF- $\beta$ 1 (Furler and Uittenbogaart 2012). In WM B cells, GLI2 was found to regulate IgM secretion via regulation of IL-6Ra expression, again in a HH independent

mechanism (Jackson et al. 2015). In melanoma cell lines, increased GLI2 expression was associated with loss of E-cadherin expression and with an increased invasion and metastases capacity (Mauviel et al. 2010). In a model of the TME using bone marrow stromal cells, CCL5/CCR3 signaling in the TME modulates Ig secretion by malignant B cells. CCL5 promotes IL-6 expression and secretion in stromal cells via GLI2, and IL-6 in turn, induces Ig secretion by malignant B cells (Elsawa et al. 2011a). Work from this dissertation research identified CD40L as a novel downstream target of GLI2 in bone marrow stromal cells. Signaling through the CCR3-PI3K-AKT pathway was found to modulate the GLI2-CD40L axis, and GLI2 was found to be required for CCR3-PI3K-AKT-mediated regulation of the CD40L promoter. These studies report a biological role for GLI2 in the TME and reveals a novel contribution of GLI2 to WM biology via regulating cytokines in the BM microenvironment.

These studies also raise the possibility that GLI2 may modulate other genes in the TME that are important in cancer cell biology. Therefore, understanding the genes that are regulated by GLI2 will be important to enrich our understanding of the role of GLI2 in regulating inflammation in the TME.

RNA-sequencing (RNA-seq), also known as whole transcriptome shotgun sequencing, uses next-generation sequencing (deep sequencing) to reveal the presence and quantity of RNA in a biological sample (Chu and Corey 2012, Wang et al. 2009). Compared to previous Sanger sequencing- and microarray-based methods, RNA-Seq provides a better resolution to study the nature of the transcriptome and can be used to investigate polyadenylated message RNA (mRNA) transcripts. In addition, RNA-Seq can be applied to investigate different populations of RNA, including total

RNA, pre-mRNA, and noncoding RNA, such as microRNA and long non-coding RNA (lncRNA), as well as ribosomal profiling (Ingolia et al. 2012, Kukurba and Montgomery 2015). Maher et al employed long and short read transcriptome sequencing to discover novel gene fusions in cancer cells and established a robust pipeline for the discovery of novel gene chimeras (Maher et al. 2009).

Here, we knockdown GLI2 using shRNA in human bone marrow stromal cells and used these RNA samples to perform RNA-Seq to identify potential target genes of GLI2 for future investigation. These studies will enrich our understanding the role of GLI2 in regulating inflammation in the TME.

## **Materials and Methods**

### **Cell culture and reagents**

The bone marrow stromal cell line L88 was kindly provided by Dr. Chiara Corsini (European Institute of Oncology, Milan, Italy) and HS-5 cells were purchased from ATCC (Manassas, VA). L88 cells were maintained in RPMI and HS-5 cells in DMEM, all supplemented with 10% FBS and antibiotics/antimycotics as previously published (8-10, 17, 29 IL-6 paper).

### **Plasmid constructs and cells transfections**

Short hairpin RNA (shRNA) targeting GLI2 was purchased from Origene Technologies (Rockville, MD) using plasmid vector backbone pGFP-V-RS.

For transfection experiments,  $4 \times 10^6$  cells were electroporated with the indicated concentrations of plasmid constructs at 250 V for 25 ms and harvested after 48 hours, unless otherwise noted. 10  $\mu$ g of shRNA was used for knockdown studies.

### **RNA Isolation and quantitative PCR (qPCR)**

Total RNA was isolated using TRIsure reagent (Bioline, London, UK) following manufacturer's recommendations. Reverse transcription reactions were conducted by Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). Quantitative real-time PCR (qPCR) was conducted using the ViiA 7 real-time PCR system (Life Technologies, Grand Island, NY). For expression of GLI2 relative to the expression of the housekeeping gene, GAPDH, the following primers were used: GAPDH, 5'-CTCGACTTCAACAGCGACA-3' (forward) and 5'-GTAGCCAAATTCGTTGTCATACC-3' (reverse); GLI2, 5'-CTCCGAGAAGCAAGAAGCCA-3' (forward) and 5'-GATGCTGCGGCACTCCTT-3' (reverse).

### **RNA-Seq**

RNA-seq was performed at the Hubbard Center for Genome Studies at the University of New Hampshire under the supervision from Dr. Kelley Thomas.

### **Data analysis**

Bioinformatic analysis of RNA-seq data was performed by Dr. Christopher Pin at the University of Western Ontario, Canada.

## Results

### GLI2 knockdown in HS-5 and L88 cells

To investigate the genes that are potentially regulated by GLI2, we performed GLI2 knock down by shRNA targeting GLI2 in HS-5 and L88 bone marrow stromal cells. Reverse transcription quantitative real-time PCR (qPCR) was used to confirm the knockdown of GLI2 in our samples prior to RNA-seq experiments. Indeed, qPCR results confirmed that GLI2 was effectively knocked down in HS-5 (**Figure 3-1**) and L88 (**Figure 3-2**) cells. We performed 4 individual experiments and found a significant reduction in GLI2 expression (at least 50%) compared to scrambled control in both cell lines tested.

### RNA sequencing

At least 2.5 µg of RNA from each sample was used for RNA sequencing. Because of our interest in understanding the cytokines that are regulated by GLI2, we identified the top 10 genes that are positively regulated by GLI2. This identified genes with a reduction in gene expression in the absence of GLI2 (GLI2 knockdown). Our data using HS-5 cells yielded fewer reads than anticipated. These samples will be repeated in future studies. However, in L88 cells, we identified over 200 genes that are positively regulated by GLI2. Of those genes, we show the top 10 genes identified by the statistical analysis here (**Figure 3-3**), including Cep170, AGL, VGLL3, KIAA1109, AKAP11, ETNK1, PRUNE2, IL6ST and MIB1. These genes encode different molecules which are involved in various cellular activities, such as centrosomal protein 170 kDa



(Cep170; involved in mitosis); the glycogen debranching enzyme (AGL; involved in the breakdown of glycogen); transcription factor vestigial-like 3 (VGLL3; targets genes that have a strong association with multiple autoimmune diseases); KIAA1109 encoded protein (thought to be involved in the regulation of epithelial growth and differentiation); AKAP11 (encodes A-kinase anchor protein 11 which belongs to the group of proteins which binding to the regulatory subunit of protein kinase A); Ethanolamine kinase 1 (a kinase encoded by ETNK1; involved in the first step of phosphatidylethanolamine synthesis); PRUNE2 (encodes prune homolog 2 with BCH domain; belongs to an interacting protein family whose members play roles in many cellular processes including apoptosis, cell transformation, and synaptic function); IL6ST (gp130; the interleukin 6 receptor signal transduction subunit that is shared by many cytokines including IL-6); and MIB1 (encodes the mind bomb E3 ubiquitin protein ligase1; a protein that positively regulates Notch signaling by ubiquitinating the Notch receptors).

## Discussion

Previous studies have shown that GLI2 can modulate the expression of TGF- $\beta$  (Furler and Uittenbogaart 2012). Furthermore, we have shown that GLI2 modulates the expression of IL-6 and IL-6R (Elsawa et al. 2011a, Jackson et al. 2015). We also identified CD40L as a novel target of GLI2 (**Figure 2-6**). Furthermore, when targeting the IL-6 in the TME in WM, we found reduced tumor growth rate and IgM secretion (**Figure 1-1, 2 and 3**). These studies indicate that the interaction between malignant B cells and bone marrow stromal cells are essential for cancer cell biology (Burger et al. 2009, Podar et al. 2008). More specifically, cytokines that are regulated by GLI2 play an

important role in WM biology. This leads to an important finding and necessitates the identification of novel transcriptional targets of GLI2 in bone marrow stromal cells. This may lead to the selection of new target genes in the TME that are important in WM biology.

Although we previously identified novel GLI2 target genes using commercially available cytokine arrays that included 100 cytokines and their receptors, here, we utilized RNA sequencing to investigate a broader spectrum of genes that are regulated by GLI2 in human bone marrow stromal cells. Indeed, our preliminary analysis identified several GLI2 target genes in L88 stromal cells (**Figure 3-3**).

Cep170, is a gene that encodes a 170 KDa centrosomal protein, a component of the centrosome. Studies have shown that in human cells, Cep170 is constantly expressed throughout the cell cycle but phosphorylated during mitosis. Cep170 can interact with polo-like kinase 1 (Plk1) *in vivo* and can also be phosphorylated by Plk1 *in vitro*. Furthermore, Cep170 associates with centrosomes during interphase and with spindle microtubules during mitosis, suggesting a role of Cep170 in microtubule organization and cell morphology (Guarguaglini et al. 2005).

AGL gene is the gene that encodes the glycogen debranching enzyme (Amylo-1,6-glucosidase,4- $\alpha$ -glucanotransferase, AGL). AGL is mainly reported to associate with Glycogen Storage Disease Type III (GSDIII). The deficiency in activity of AGL in the liver and muscle causes GSD type IIIa or liver only (GSD type IIIb) (Goldstein et al. 2010). Most of the AGL mutations are nonsense mutations, but some rare missense mutations can affect AGL function leading to GSDIII. One study found that mutations in the carbohydrate-binding domain are more severe in nature, leading to a significant loss

of all enzymatic activities and carbohydrate binding ability, as well as enhancing targeting for proteasomal degradation, indicating that inactivation of enzymatic activity is sufficient to cause GSDIII disease (Cheng et al. 2009).

VGLL3, vestigial-like 3, is a transcription factor with target genes that have a strong association with multiple autoimmune diseases including lupus, scleroderma and Sjögren's syndrome and had a prominent transcriptomic overlap with inflammatory processes in cutaneous lupus (Liang et al. 2017). VGLL3 has also been reported as a putative transcription co-factor, involved in tumor suppressor pathway, a feature that is characterized by the absence of VGLL3 expression in high-grade serous ovarian carcinomas (Gambaro et al. 2013).

KIAA1109 encodes the protein KIAA1109, and its function is not yet fully understood. It has been reported that the KIAA1109-IL2-IL21 gene region associates with several autoimmune phenotypes (Bouزيد et al. 2014). Furthermore, the KIAA1109/Tenr/IL2/IL21 gene cluster associates with rheumatoid arthritis (Hollis-Moffatt et al. 2010, Teixeira et al. 2009).

AKAP11 is a gene that encodes A-kinase anchor protein 11. The A-kinase anchor proteins (AKAPs) are a group of structurally diverse proteins which have the common function of binding to the regulatory subunit of protein kinase A (Colledge and Scott 1999). As a member of this family, the role of AKAP11 hasn't been thoroughly investigated. It has been reported that AKAP11 gene polymorphism is considered as a factor affecting peak bone mass acquisition (Correa-Rodríguez et al. 2018).

ETNK1 gene encodes ethanolamine kinase 1 (ETNK1). ETNK1 is an ethanolamine-specific kinase that catalyzes the first step of phosphatidylethanolamine biosynthesis via the CDP-ethanolamine pathway (Lykidis et al. 2001). Gambacorti-Passerini et al performed whole exome sequencing on atypical chronic myeloid leukemia (aCML) patients and report the presence of recurrent somatic missense mutations in the ETNK1 gene, suggesting that the recurrent somatic ETNK1 mutations are important in myeloproliferative/myelodysplastic disorders (Gambacorti-Passerini et al. 2015). Furthermore, somatic missense ETNK1 mutations were found in systemic mastocytosis with eosinophilia and chronic myelomonocytic leukemia, in addition to aCML (Kosmider 2015, Lasho et al. 2015).

PRUNE2 encodes prune homolog 2 with BCH domain. In prostate cancer, PRUNE2 acts as a tumor suppressor and its expression is regulated by prostate cancer antigen 3 (PCA3), via a unique regulatory mechanism involving formation of a PRUNE2/PCA3 double-stranded RNA, so that expression of PRUNE2 decreases or is silenced in prostate cancer (Salameh et al. 2015). Furthermore, a whole-exome sequencing study identified somatic mutations in PRUNE2 in parathyroid carcinoma and recurrent mutations in kinase genes related to cell migration and invasion (Gan et al. 2015). Interestingly, PRUNE2 has also been reported to contribute to the maintenance of the mature nervous system (Iwama et al. 2011).

NEK7 (NIMA (never in mitosis gene A-related kinase 7) belongs to the NIMA related kinases which control the initiation of mitosis. NEK7 has been shown to be directly involved in cytokinesis and NEK7 knockdown can cause a prometaphase arrest of the cell cycle with monopolar or disorganized spindle. Moreover, NEK7 suppression

in cells causes a decrease in the centrosomal  $\gamma$ -tubulin levels and a reduction of the microtubule re-growth activity (Kim et al. 2007). Further studies show that depletion of NEK7 inhibits progression through the G1 phase via down-regulation of various cyclins and CDKs and also inhibits the earliest stages of procentriole formation, suggesting that NEK7 is involved in the timely regulation of G1 progression, S-phase entry and procentriole formation (Gupta et al. 2017). NEK7 has also been reported as an essential protein acting downstream of potassium efflux (which is a common step that is essential for NLRP3 inflammasome activation) to regulate NLRP3 inflammasome assembly and activation (He et al. 2016, Xu et al. 2016). In hepatocellular carcinoma (HCC), NEK7 was found to be significantly overexpressed and high NEK7 expression correlated with tumor numbers, tumor diameter, adjacent organs invasion, tumor grade and stage. Finally, NEK7 silencing resulted in an inhibition of HCC cell growth *in vitro* and tumor growth *in vivo* through decreasing cyclin B1 levels both *in vitro* and *in vivo*, suggesting that NEK7 could be a novel HCC therapeutic target (Zhou et al. 2016).

IL6ST (interleukin 6 signal transducer; also known as glycoprotein 130/gp130) or CD130 is a signal transducer shared by many cytokines, including IL-6, IL-11, IL-27 and leukemia inhibitory factor (LIF). In particular for IL-6 signaling, IL-6 receptor family all complex with gp130 for signal transduction and this is well established in previous studies (Heinrich et al. 1998, Kishimoto et al. 1995). Recently studies show that elevated intratumoral gp130 expression was associated with unfavorable survival in gastric cancer patients (Cao et al. 2016). Loss of gp130 function can cause a novel immunodeficiency with phenotypic similarities to STAT3 hyper-IgE syndrome (Schwerd et al. 2017). We also previously showed that GLI2 can modulate IL-6 (Elsawa et al.

2011b) and the IL-6R $\alpha$ /gp80 (Jackson et al. 2015). The RNA sequencing data identified gp130 as a potential target. This may be an interesting biological target for further investigation to explore the mechanism by which GLI2 regulates gp130 in normal and malignant B cells.

MIB1 (mind bomb E3 ubiquitin protein ligase 1) has been reported to interact with receptor-like tyrosine kinase (RYK, which function as a transmembrane receptor for the Wnt family of secreted protein ligands) to activate Wnt/ $\beta$ -catenin signaling (Berndt et al. 2011). Moreover, MIB1 has been reported to play an essential role in T cells and marginal zone B cell development (Song et al. 2008) and in pancreatic  $\beta$ -cell formation (Horn et al. 2012). Both occur through Notch signaling.

Our RNA-seq data indicate that there are other genes that are potentially regulated by GLI2. However, additional bioinformatic and cluster analysis of the data is needed in order to identify the most relevant candidates from a biological perspective. Further studies should be aimed at performing additional bioinformatic analysis of the data, validating the findings in multiple cell lines and primary cells in order to address the role of GLI2 as a regulator of these genes in the TME. Perhaps, chromatin immunoprecipitation (ChIP) assay followed by sequencing (ChIP-seq) experiments can be performed and analyzed in the context of the RNA-seq data in order to identify the novel GLI2 target genes that are directly bound by GLI2.

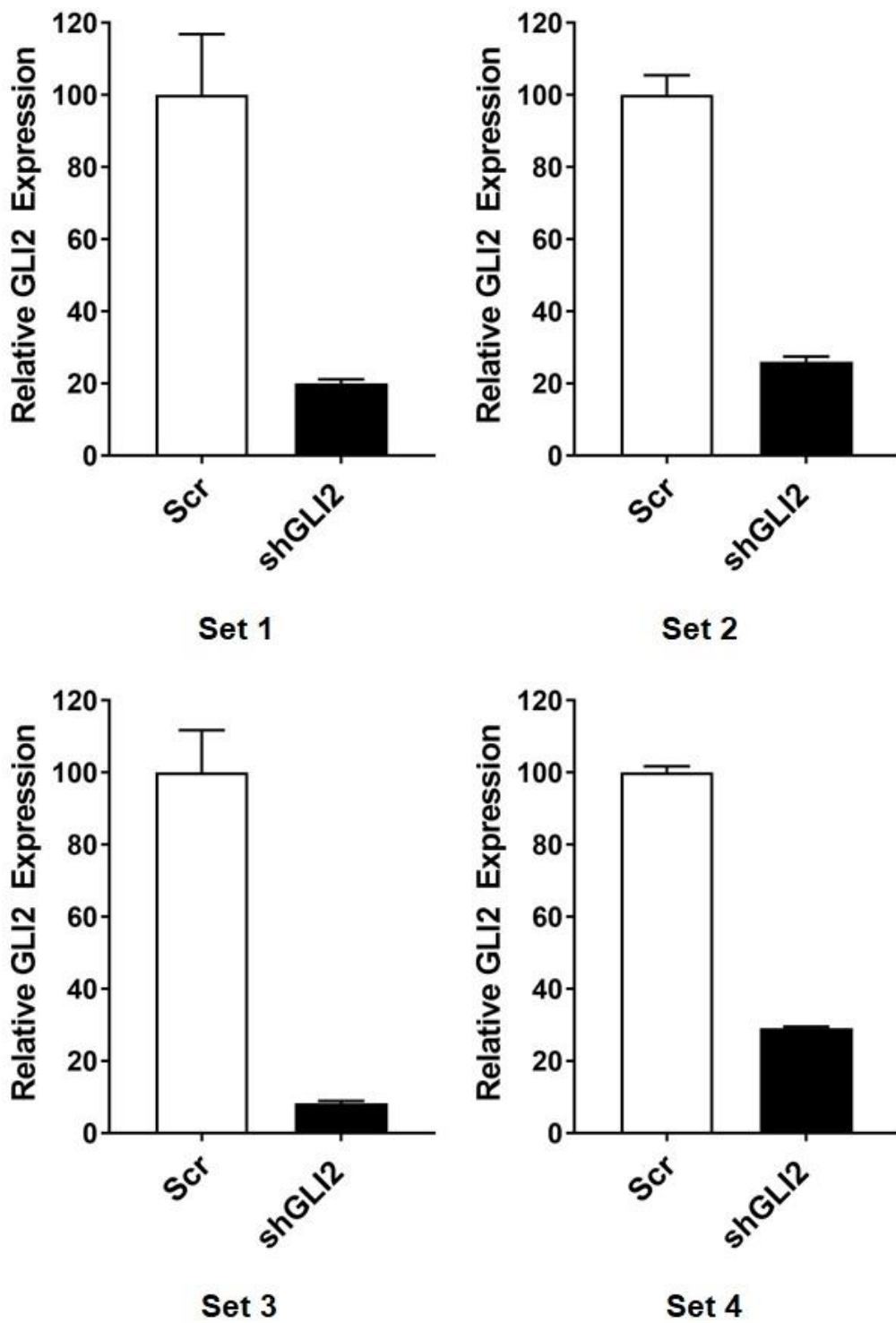


Figure 3- 1. GLI2 knockdown in HS-5 bone marrow stromal cells.

**Figure 3-1:** Bone marrow stromal cells HS-5 ( $4 \times 10^6$ ) were transfected with shRNA targeting GLI2 (shGLI2) or scrambled control (shScr) for 2 days followed by RNA extraction and cDNA synthesis for use to determine gene expression by qPCR as indicated in methods. Four sets of experiments were performed, each representing a biological replicate.



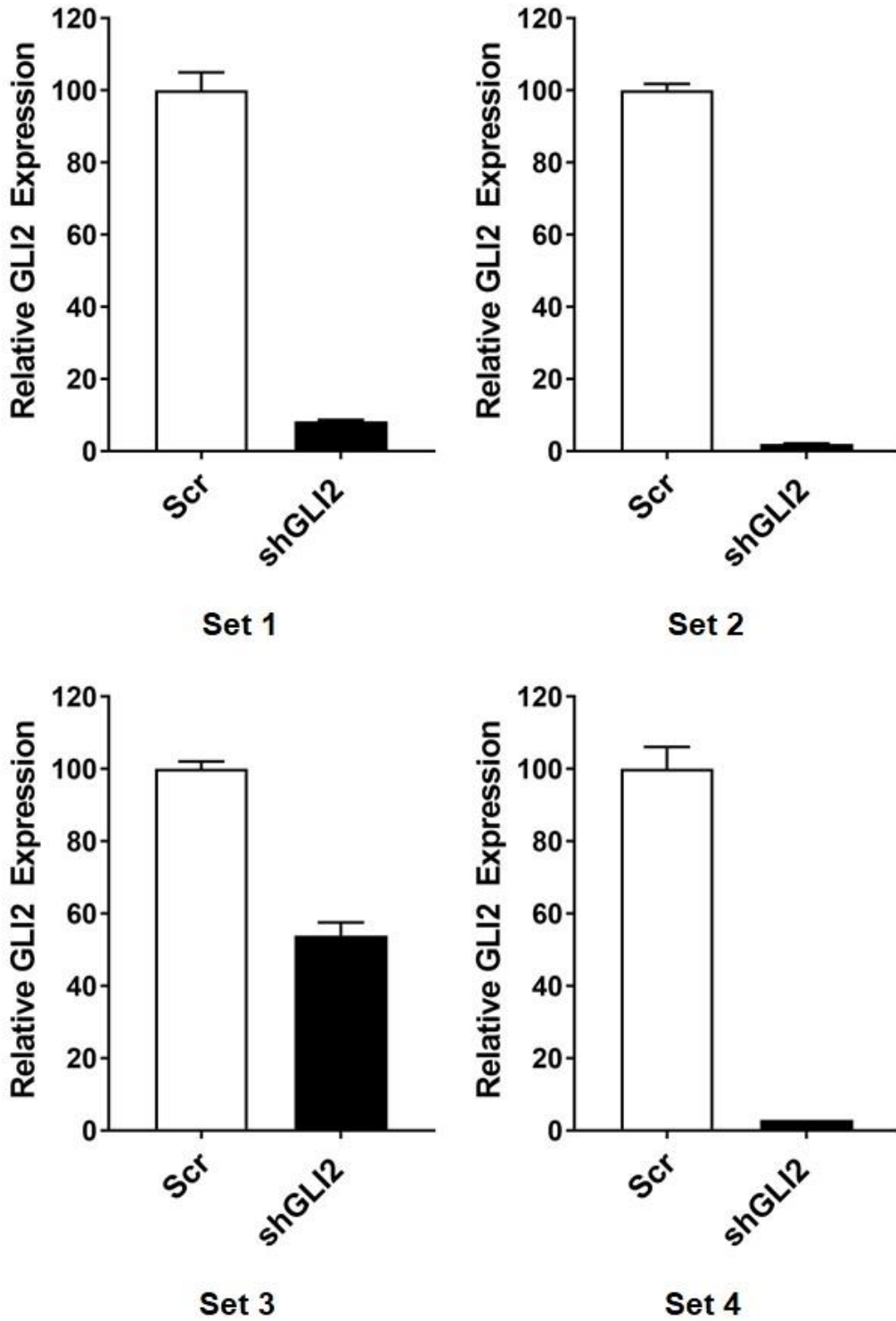


Figure 3- 2. GLI2 knockdown in L88 bone marrow stromal cells

**Figure 3-2:** Bone marrow stromal cells L88 ( $4 \times 10^6$ ) were transfected with shRNA targeting GLI2 (shGLI2) or scrambled control (shScr) for 2 days followed by RNA extraction and cDNA synthesis for use to determine gene expression by qPCR as indicated in methods. Each set was generated from a different experiment representing biological replicates.

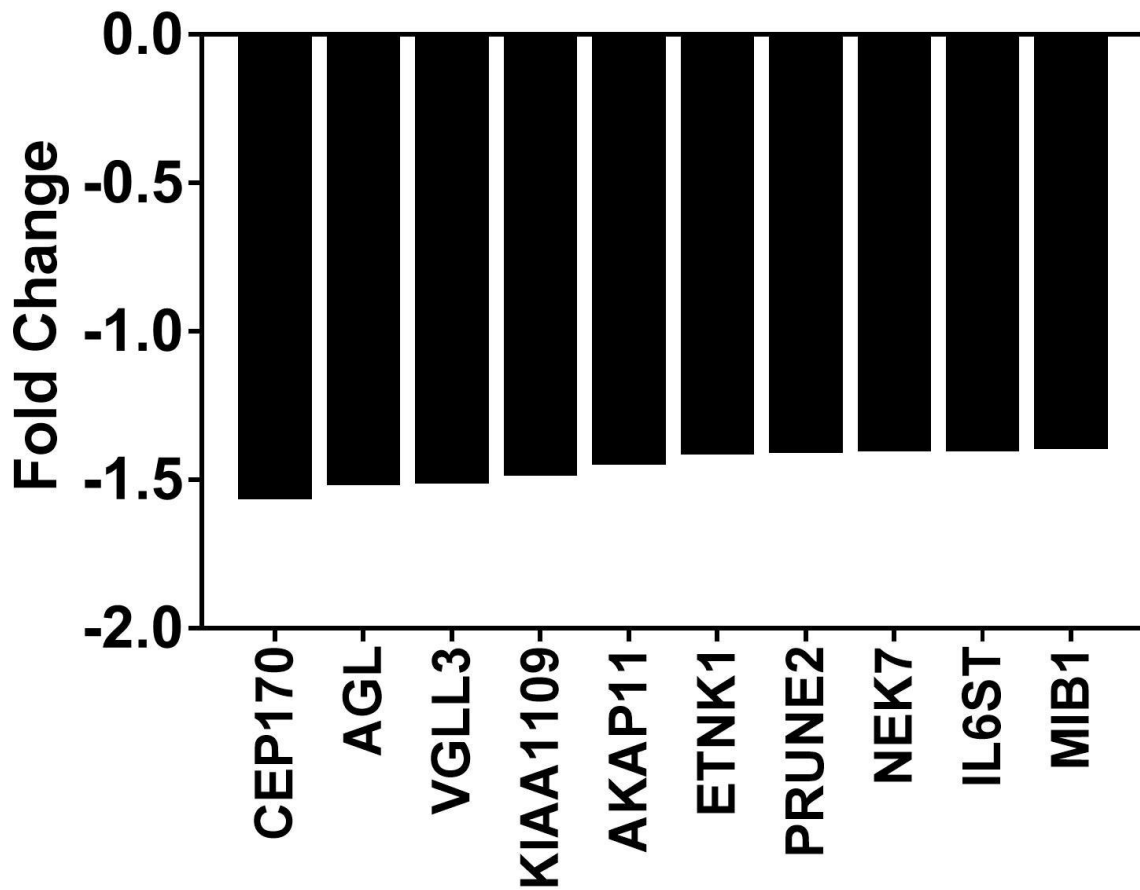


Figure 3- 3: Top 10 genes that are regulated by GLI2 identified by RNA-sequencing.

**Figure 3-3: Top 10 genes that are regulated by GLI2 identified by RNA-sequencing.** Bioinformatic analysis of RNA-seq data in L88 cells identified genes that are differentially expressed in the presence/absence of GLI2. The 10 genes presented have the most fold regulation in the significantly different genes.

## DISCUSSION

Interactions between malignant B cells and stromal cells in the TME play an essential role in regulating malignant B cell biology including cell growth, cell survival and Ig secretion (Burger et al. 2009, Elsawa et al. 2011a, Jalali and Ansell 2016), and cytokines in the TME play important roles in these interactions. Tocilizumab, as an anti-IL-6R antibody, has been investigated in several clinical trial in patients with inflammatory-mediated disease, including Castleman's disease, Rheumatoid arthritis, Systemic juvenile idiopathic arthritis, Crohn's disease, Giant cell arteritis, Systemic sclerosis, Systemic lupus erythematosus and multiple sclerosis (Tanaka et al. 2011). Several studies have also investigated the addition of anti-IL-6 therapy in multiple myeloma (MM) and report enhanced results over chemotherapeutic regimens (Hunsucker et al. 2011, Rossi et al. 2005, Shah et al. 2016). In previous work, we have shown that IL-6 levels are elevated in WM patients and IL-6 secreted from human bone marrow stromal cells can promote WM cell growth and IgM secretion (Elsawa et al. 2011a, Elsawa and Ansell 2009). This finding led us to investigate the efficacy of blocking IL-6 to block the interaction between the TME and WM cells, and evaluate the therapeutic potential of targeting this molecule with the goal of laying the foundation for its evaluation in clinical trials.

Our results show that targeting IL-6 in the WM TME did not affect the survival in two WM cell line models investigated (**Figure 1-1B**). As reported, the role of IL-6 in WM was shown to promote IgM secretion and WM cell growth (Elsawa et al. 2011a, Elsawa et al. 2011b), not inducing apoptosis of WM cells (Elsawa et al. 2011b), so targeting IL-6

did not affect the survival is not surprising. Consistent with this role, we found a reduction in tumor growth rate when tumor bearing mice were treated with Tocilizumab (**Figure 1-2**). These results indicate that targeting IL-6 in the TME in WM may slow the rate of tumor growth. Since WM remains an incurable disease, a therapeutic drug that slows down the rate of tumor growth may be a feasible option for WM patients.

One of the hallmarks of WM is the overproduction of monoclonal IgM protein (Elsawa et al. 2011a, Elsawa et al. 2006, Elsawa et al. 2011b, Han et al. 2017, Jackson et al. 2015). Our results show that tumor-bearing mice treated with Tocilizumab had significantly reduced levels of human IgM secretion in mice serum (**Figure 1-3**). This suggests that targeting IL-6 in the TME in WM may provide a much-needed symptomatic relief for WM patients. Interestingly, RPCI-WM1 tumor-bearing mice treated with Tocilizumab had similar levels of IgM as control mice (**Figure 1-3**). The 2 WM cell lines used in this study are derived from different WM patients (Chitta et al. 2013, Ditzel Santos et al. 2007). Interestingly, RPCI-WM1 cells secrete the highest levels of IgM among the available WM cell lines, raising the possibility that the rate of increase in IgM levels in patients may correlate with the efficacy of Tocilizumab monotherapy. Furthermore, the differences in responses between the 2 WM cell lines investigated may represent the natural diversity that exists between various patients. Therefore, an examination of the efficacy of IL-6 therapy in WM patients is necessary to evaluate its effect on IgM levels and tumor growth.

Current therapies used for the treatment of WM patients mainly focus on targeting cancer cells directly using monotherapy or combination therapies, such treatments including ibrutinib (the only drug approved by FDA for WM patients),

Rituximab (in combination with CHOP; R-CHOP), Bendamustine, Dexamethasone, Fludarabine, Chlorambucil, Everolimus, among others (Kapoor et al. 2015). Our results show that targeting IL-6 in the TME can reduce the rate of WM cell growth and the secretion of IgM, but not enhance survival or induce cell death (**Figure 1-1, 2, and 3**). This is consistent with previous studies that showed IL-6 has no effect on WM cell survival but modulates cell growth and IgM secretion (Elsawa et al. 2011a, Elsawa et al. 2011b). Future studies that investigate the efficiency of combination of targeting the TME and cancer cells together will therefore shed more light on the potential of Tocilizumab in WM. Combined targeting of IL-6, which slows WM cell growth and reduces IgM secretion, and malignant cells, which induces apoptosis of WM cells, may prove to be effective and provide therapeutic benefit to patients.

In bone marrow malignancies such as multiple myeloma (MM) and Waldenström macroglobulinemia (WM), bone marrow stromal cells play an important role in malignant cell biology by secreting a variety of cytokines that are used by malignant cells to survive and proliferate (Azab et al. 2012b, Burger et al. 2009, Burger and Peled 2009, Cheung and Van Ness 2001, Elsawa et al. 2011b, Kurtova et al. 2009a). Previous studies have shown that GLI2 can modulate the expression of TGF- $\beta$  (Furler and Uittenbogaart 2012). Furthermore, we have shown that GLI2 modulates the expression and secretion of IL-6 and IL-6R (Elsawa et al. 2011a, Jackson et al. 2015). When targeting the IL-6 in the TME in WM, we found reduced tumor growth rate and IgM secretion (**Figure 1-1, 2 and 3**). These studies indicate that the interaction between malignant B cells and bone marrow stromal cells are essential for malignant B cell biology (Burger et al. 2009, Podar et al. 2008). More specifically, cytokines that are

regulated by GLI2 play important roles in WM biology. This leads to an important finding and necessitates the identification of novel transcriptional targets of GLI2 in bone marrow stromal cells. This may lead to the selection of new target genes in the TME that are important to WM cells.

Here, we focused our studies on the molecular mechanism of cytokine regulation by the oncogenic transcription factor GLI2. The results from our initial screening of cytokines potentially modulated by GLI2 identified several cytokines that are differentially regulated by GLI2 (**Figure 2-1**). Among those, CD40L was identified as a new novel GLI2 target gene (**Figure 2-1**). CD40L is an important cytokine that has been shown to play an important role during normal B cell responses where it regulates B cell growth, survival, antibody secretion as well as class switch recombination (CSR) (Wykes 2003). In addition to its role in normal B cell functions, it has also been shown to modulate malignant B cells (Burger and Gandhi 2009, Fiumara and Younes 2001, Tournilhac et al. 2006). Therefore, understanding the mechanism of its regulation is important to further our understanding of normal and malignant B cells responses.

We found that the oncogenic transcription factor GLI2 induces the expression of CD40L in stromal cells (**Figure 2-2**). Moreover, we found that GLI2 modulates CD40L expression by directly binding to its promoter (**Figures 2-3 & 4**). Our data identified CD40L as a novel direct target of the GLI2 transcription factor in human bone marrow stromal cells (**Figures 2-1, 2 and 3**). Our group has shown that GLI2 can directly target the IL-6 promoter in bone marrow stromal cells and lead to increased IL-6 expression and secretion (Elsawa et al. 2011a). We also demonstrated a direct role for GLI2 in regulating IgM secretion by WM cells (Jackson et al. 2015). Therefore, this study



identifies CD40L as a novel GLI2 target gene and suggests a novel mechanism by which GLI2 modulates malignant B cell biology via modulation of the TME.

Previous studies have shown that CD40L expression on mast cells in the TME promotes CD40 signaling on primary WM B cells (Tournilhac et al. 2006). Our data support these studies showing GLI2-mediated CD40L increases WM B cell activation (as evidenced by Erk phosphorylation) (**Figure 2-1C**) and ultimately increased malignant B cell proliferation (**Figure 2-1D**) upon co-culture with stromal cells stably expressing CD40L. We also show that GLI2 induces an increase in sCD40L and conditioned media from  $\Delta$ N-GLI2 transfected stromal cells induced an increase in WM cell proliferation (**Figure 2-2F and 2G**). The increase in proliferation was modest (12-13%), which did not allow us to observe a statistical difference in WM cell proliferation in the presence of CD40L blocking antibodies, although the trend was consistent with, at least, a partial role for sCD40L (data not shown). In a previous study we have shown that the CCR3-PI3K-AKT signaling pathway is a regulator of GLI2 (Elsawa et al. 2011a). Here, we found that the CCR3-PI3K-AKT signaling pathway can also modulate CD40L expression and promoter activity and this regulation of CD40L requires an intact GLI2 (**Figure 2-5**).

In summary, our studies demonstrate a novel role for GLI2 in promoting malignant B cell activation and proliferation via regulation of CD40L expression in the TME and signaling through the CCR3-PI3K-AKT pathway can modulate CD40L expression and promoter activity and this regulation requires an intact GLI2 (**Figure 2-6C**). These studies define the molecular mechanism by which GLI2 regulates CD40L expression in human bone marrow stromal cells. Further investigation of GLI2 target

genes will expand our understanding of the role of GLI2 in modulating inflammatory cytokines in the TME.

A role for CD40L has been described in mantle cell lymphoma (MCL) where CD40L promotes activation and proliferation of MCL cells (Andersen et al. 2000). Similarly, a role for CD40L/CD40 signaling has been described in Hodgkin lymphoma, B cell lymphoma, chronic lymphocytic leukemia and other hematological neoplasms (Aldinucci et al. 2012, Burger and Gandhi 2009, Fiumara and Younes 2001, Jin et al. 2004, Schattner 2000, Srahna et al. 2001, Tong et al. 2000, Tournilhac et al. 2006, Tsirakis et al. 2012, Voorzanger-Rousselot et al. 1998, Younes 2001). Despite the importance of CD40L-CD40 signaling in normal and malignant B cells, little is known about the mechanisms that regulate CD40L expression. The identification of GLI2 as a regulator of CD40L promoter advances our understanding of the regulation of this important cytokine.

In human T cells, several transcription factors including AP-1, NF-AT and NF- $\kappa$ B have been shown to modulate CD40L (Srahna et al. 2001). These transcription factors regulate a region closer to the transcription start site. Here we show that GLI2 can directly bind to the promoter of CD40L (binding site 1: -1478, **Figure 2-3A**). This region is located further upstream from the regulatory regions of these other transcription factors. It would be interesting in future studies to address whether GLI2 cooperates with AP-1, NF-AT and NF- $\kappa$ B to regulate CD40L expression.

The role of GLI2 as a transcription factor has been widely studied. Several downstream targets that are regulated by GLI2, such as TGF- $\beta$  (Furler and Uittenbogaart 2012), IL-6 (Elsawa et al. 2011a), IL-6R $\alpha$  (Jackson et al. 2015) and

CD40L (Han et al. 2017) have been characterized. Moreover, GLI2 is directly involved in driving melanoma invasion and metastasis (Mauviel et al. 2010) and plays a critical role in maintaining the tumorigenic properties of prostate cancer cells (Thiyagarajan et al. 2007). We have identified several other genes that are potentially regulated by GLI2 (**Figure 2-1 and Figure 3-3**). Further analysis of the mechanism by which GLI2 modulates these genes and their role in cancer cell biology will be important for our understanding of the role of GLI2 in regulating inflammation in the TME.

In our study, we investigated a GLI2-target gene in the TME, IL-6, as a therapeutic target to study the effect of disrupting the crosstalk between cancer cells and the TME. The outcome of targeting IL-6 reduced human IgM secretion and slowed down tumor growth rate of WM B cells. However, this did not affect mice survival (**Figure 1-1, 2 and 3**). This leads to the question of whether we can switch from targeting the genes regulated by GLI2 to targeting the transcription factor GLI2 itself.

Currently, several studies focus on targeting different transcription factors that play critical roles in cancer biology. Hypoxia-inducible factor 1 (HIF-1) regulates genes that are involved in cancer biology, which including angiogenesis, cell survival, glucose metabolism and invasion, and in preclinical studies, inhibition of HIF-1 activity has marked effects on tumor growth. Therefore, HIF-1 can be used as a potential therapeutic target (Semenza 2003). Forkhead O (FOXO) transcription factors play an essential role in the regulation of cellular functions such as cell cycle arrest, cell death, and protection from stress stimuli, and inactivation of FOXO protein is associated with several neoplasms including breast cancer, prostate cancer, glioblastoma, rhabdomyosarcoma and leukemia. Moreover, clinical studies have shown therapeutic

benefit from using drugs like paclitaxel, imatinib, and doxorubicin that activate FOXO targets (Yang and Hung 2009). The transcription factor NF- $\kappa$ B is frequently encountered in tumor cells and contributes to aggressive tumor growth and resistance to chemotherapy and radiation during cancer treatment. Accumulating evidence shows that induction of chemoresistance and radioresistance are mediated by several genes regulated by NF- $\kappa$ B and inhibition of NF- $\kappa$ B increases sensitivity of cancer cells to chemotherapeutic agents and radiation exposure. Therefore, targeting NF- $\kappa$ B may be a potential therapeutic strategy to overcome chemoresistance and radioresistance in cancer treatment (Li and Sethi 2010). In addition, transcription factors from the STAT family (STAT3 and STAT5), are important players in human cancers and are validated targets for therapeutic intervention (Libermann and Zerbini 2006, Redell and Tweardy 2005).

Inhibition of Smoothed, the signal transduction subunit of the Hedgehog (HH) receptor initially identified GLI2 as a transcriptional activator and has been shown to abrogate leukemia stem cell dormancy and reduce the dormant leukemia stem cells burden using a clinical antagonist (Sadarangani et al. 2015). This suggests that smoothed inhibition can provide therapeutic efficacy in cancer patients. Currently, there are two Smoothed inhibitors (Vismodegib and Sonidegib) that have been approved by the FDA (Rimkus et al. 2016). However, since GLI proteins can also be activated through HH signaling-independent mechanisms, this raises the possibility that targeting GLI may be more therapeutically beneficial than targeting HH.

Direct inhibition of GLI has gained interest and shows promise to target these transcription factors and their targets. In malignant pleural mesothelioma, inhibition of

GLI by siRNA or a novel small molecule GLI inhibitor suppressed tumor cell growth dramatically both *in vitro* and *in vivo*. Furthermore, inhibition of GLI exhibited better cytotoxicity than inhibition of Smoothed, suggesting that inhibition of GLI function could be a strong novel and effective approach to treat malignant pleural mesothelioma (Li et al. 2013). In acute myeloid leukemia, inhibition of GLI by GLI inhibitor GANT61 causes growth arrest and apoptosis in human myeloid leukemia cell lines (Pan et al. 2012). Lauth et al screened for small-molecule antagonists of GLI-mediated transcription and revealed two molecules that can selectively inhibit GLI-mediated gene transactivation by acting in the nucleus to block GLI function, and one of them interferes with GLI1 DNA binding in living cells. More importantly, these compounds efficiently inhibited *in vitro* tumor cell proliferation and successfully blocked cell growth in an *in vivo* xenograft model using human prostate cancer cells (Lauth et al. 2007)

This suggests that directly targeting GLI2 in cancer cells may provide therapeutic efficacy. Future studies in WM and other neoplasms should focus on investigating the therapeutic efficacy of targeting GLI. Furthermore, combined targeting of cancer cells, cells in the TM, and/or molecules such as cytokines, which mediate the interaction between these cells may be beneficial for cancer patients.

## LIST OF REFERENCES

(2005) Advances in Biology and Therapy of Waldenstrom's Macroglobulinemia. *Ann Oncol* 16(suppl\_5), v261-v262.

Abonour, R., Zhang, L.A., Rajkumar, V., Srkalovic, G., Greipp, P.R., Fonseca, R. and Gertz, M. (2007) Phase II Pilot Study of Rituximab + CHOP in Patients with Newly Diagnosed Waldenström's Macroglobulinemia, an Eastren Cooperative Oncology Group Trial (Study E1A02). *Blood* 110(11), 3616-3616.

Abroun, S., Ishikawa, H., Tsuyama, N., Liu, S., Li, F.-J., Otsuyama, K.-i., Zheng, X., Obata, M. and Kawano, M.M. (2004) Receptor synergy of interleukin-6 (IL-6) and insulin-like growth factor-I in myeloma cells that highly express IL-6 receptor  $\alpha$ . *Blood* 103(6), 2291-2298.

Aldinucci, D., Gloghini, A., Pinto, A., Colombatti, A. and Carbone, A. (2012) The role of CD40/CD40L and interferon regulatory factor 4 in Hodgkin lymphoma microenvironment. *Leukemia & Lymphoma* 53(2), 195-201.

Aldinucci, D., Lorenzon, D., Olivo, K., Rapanà, B. and Gattei, V. (2004) Interactions Between Tissue Fibroblasts in Lymph Nodes and Hodgkin/Reed-Sternberg Cells. *Leukemia & Lymphoma* 45(9), 1731-1739.

Amakye, D., Jagani, Z. and Dorsch, M. (2013) Unraveling the therapeutic potential of the Hedgehog pathway in cancer. *Nature medicine* 19, 1410.

Amarsaikhan, N. and Elsawa, S.F. (2013) GLI2 (GLI family zinc finger 2). *Atlas of Genetic and Cytogenetics in Oncology and Haematology* 17(8), 516-522.

Ambs, S., Merriam, W.G., Bennett, W.P., Felley-Bosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R. and Harris, C.C. (1998) Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. *Cancer research* 58(2), 334-341.

Andersen, N.S., Larsen, J.K., Christiansen, J., Pedersen, L.B., Christophersen, N.S., Geisler, C.H. and Jurlander, J. (2000) Soluble CD40 ligand induces selective proliferation of lymphoma cells in primary mantle cell lymphoma cell cultures. *Blood* 96(6), 2219-2225.

Ansell, S.M., Kyle, R.A., Reeder, C.B., Fonseca, R., Mikhael, J.R., Morice, W.G., Bergsagel, P.L., Buadi, F.K., Colgan, J.P., Dingli, D., Dispenzieri, A., Greipp, P.R., Habermann, T.M., Hayman, S.R., Inwards, D.J., Johnston, P.B., Kumar, S.K., Lacy, M.Q., Lust, J.A., Markovic, S.N., Micallef, I.N., Nowakowski, G.S., Porrata, L.F., Roy, V.,

Russell, S.J., Short, K.E., Stewart, A.K., Thompson, C.A., Witzig, T.E., Zeldenrust, S.R., Dalton, R.J., Rajkumar, S.V. and Gertz, M.A. (2010) Diagnosis and management of Waldenstrom macroglobulinemia: Mayo stratification of macroglobulinemia and risk-adapted therapy (mSMART) guidelines. *Mayo Clin Proc* 85(9), 824-833.

Arendt, B.K., Velazquez-Dones, A., Tschumper, R.C., Howell, K.G., Ansell, S.M., Witzig, T.E. and Jelinek, D.F. (2002) Interleukin 6 induces monocyte chemoattractant protein-1 expression in myeloma cells. *Leukemia* 16, 2142.

Armulik, A., Abramsson, A. and Betsholtz, C. (2005) Endothelial/Pericyte Interactions. *Circulation research* 97(6), 512-523.

Arnott, C.H., Scott, K.A., Moore, R.J., Robinson, S.C., Thompson, R.G. and Balkwill, F.R. (2003) Expression of both TNF- $\alpha$  receptor subtypes is essential for optimal skin tumour development. *Oncogene* 23, 1902.

Azab, A.K., Quang, P., Azab, F., Pitsillides, C., Thompson, B., Chonghaile, T., Patton, J.T., Maiso, P., Monrose, V., Sacco, A., Ngo, H.T., Flores, L.M., Lin, C.P., Magnani, J.L., Kung, A.L., Letai, A., Carrasco, R., Roccaro, A.M. and Ghobrial, I.M. (2012a) P-selectin glycoprotein ligand regulates the interaction of multiple myeloma cells with the bone marrow microenvironment. *Blood* 119(6), 1468-1478.

Azab, F., Azab, A.K., Maiso, P., Calimeri, T., Flores, L., Liu, Y., Quang, P., Roccaro, A.M., Sacco, A., Ngo, H.T., Zhang, Y., Morgan, B.L., Carrasco, R.D. and Ghobrial, I.M. (2012b) Eph-B2/Ephrin-B2 Interaction Plays a Major Role in the Adhesion and Proliferation of Waldenstrom's Macroglobulinemia. *Clinical Cancer Research* 18(1), 91-104.

Balkwill, F., Charles, K.A. and Mantovani, A. (2005) Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer cell* 7(3), 211-217.

Balkwill, F.R., Capasso, M., and Hagemann, T. (2012). The tumor microenvironment at a glance. 5591-5596.

Bar, E.E., Chaudhry, A., Lin, A., Fan, X., Schreck, K., Matsui, W., Piccirillo, S., Vescovi, A.L., DiMeco, F., Olivi, A. and Eberhart, C.G. (2007) Cyclopamine-Mediated Hedgehog Pathway Inhibition Depletes Stem-Like Cancer Cells in Glioblastoma. *Stem Cells* 25(10), 2524-2533.

Becker, C., Fantini, M.C. and Neurath, M.F. (2006) TGF-beta as a T cell regulator in colitis and colon cancer. *Cytokine & growth factor reviews* 17(1), 97-106.

Becker, C., Fantini, M.C., Schramm, C., Lehr, H.A., Wirtz, S., Nikolaev, A., Burg, J., Strand, S., Kiesslich, R. and Huber, S. (2004) TGF- $\beta$  suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity* 21(4), 491-501.



Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z. and Hanahan, D. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nature Cell Biology* 2, 737.

Bergers, G., Javaherian, K., Lo, K.-M., Folkman, J. and Hanahan, D. (1999) Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science* 284(5415), 808-812.

Berndt, J.D., Aoyagi, A., Yang, P., Anastas, J.N., Tang, L. and Moon, R.T. (2011) Mindbomb 1, an E3 ubiquitin ligase, forms a complex with RYK to activate Wnt/ $\beta$ -catenin signaling. *The Journal of cell biology* 194(5), 737-750.

Bingle, L., Brown, N. and Lewis, C. (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland* 196(3), 254-265.

Bissell, M.J. and Hines, W.C. (2011) Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature medicine* 17, 320.

Bochet, L., Meulle, A., Imbert, S., Salles, B., Valet, P. and Muller, C. (2011) Cancer-associated adipocytes promotes breast tumor radioresistance. *Biochemical and Biophysical Research Communications* 411(1), 102-106.

Bommert, K., Bargou, R.C. and Stühmer, T. (2006) Signalling and survival pathways in multiple myeloma. *European Journal of Cancer* 42(11), 1574-1580.

Bouزيد, D., Fourati, H., Amouri, A., Marques, I., Abida, O., Tahri, N., Penha-Gonçalves, C. and Masmoudi, H. (2014) Autoimmune diseases association study with the KIAA1109–IL2–IL21 region in a Tunisian population. *Molecular Biology Reports* 41(11), 7133-7139.

Branco-Price, C., Zhang, N., Schnelle, M., Evans, C., Katschinski, D.M., Liao, D., Ellies, L. and Johnson, R.S. (2012) Endothelial cell HIF-1 $\alpha$  and HIF-2 $\alpha$  differentially regulate metastatic success. *Cancer cell* 21(1), 52-65.

Briones, J., Timmerman, J.M., Hilbert, D.M. and Levy, R. (2002) BLYS and BLYS receptor expression in non-Hodgkin's lymphoma. *Experimental Hematology* 30(2), 135-141.

Burger, J.A. and Gandhi, V. (2009) The lymphatic tissue microenvironments in chronic lymphocytic leukemia: in vitro models and the significance of CD40-CD154 interactions. *Blood* 114(12), 2560-2561; author reply 2561-2562.



Burger, J.A., Ghia, P., Rosenwald, A. and Caligaris-Cappio, F. (2009) The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 114(16), 3367-3375.

Burger, J.A. and Peled, A. (2009) CXCR4 antagonists: targeting the microenvironment in leukemia and other cancers. *Leukemia* 23(1), 43-52.

Burger, J.A. and Stewart, D.J. (2009) CXCR4 chemokine receptor antagonists: perspectives in SCLC. *Expert Opin Investig Drugs* 18(4), 481-490.

Cao, Y., Zhang, H., Liu, H., Lin, C., Li, R., Wu, S., He, H., Li, H. and Xu, J. (2016) Glycoprotein 130 is associated with adverse postoperative clinical outcomes of patients with late-stage non-metastatic gastric cancer. *Scientific Reports* 6, 38364.

Carmeliet, P. and Jain, R.K. (2011) Molecular mechanisms and clinical applications of angiogenesis. *Nature* 473(7347), 298.

Chaffer, C.L. and Weinberg, R.A. (2011) A Perspective on Cancer Cell Metastasis. *Science* 331(6024), 1559-1564.

Chakraborty, R., Kapoor, P., Ansell, S.M. and Gertz, M.A. (2015) Ibrutinib for the treatment of Waldenström macroglobulinemia. *Expert Review of Hematology* 8(5), 569-579.

Chambers, A.F., Groom, A.C. and MacDonald, I.C. (2002) Metastasis: dissemination and growth of cancer cells in metastatic sites. *Nature Reviews Cancer* 2(8), 563.

Chatterjee, M., Stühmer, T., Herrmann, P., Bommert, K., Dörken, B. and Bargou, R.C. (2004) Combined disruption of both the MEK/ERK and the IL-6R/STAT3 pathways is required to induce apoptosis of multiple myeloma cells in the presence of bone marrow stromal cells. *Blood* 104(12), 3712-3721.

Chauhan, D., Uchiyama, H., Akbarali, Y., Urashima, M., Yamamoto, K., Libermann, T. and Anderson, K. (1996) Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. *Blood* 87(3), 1104-1112.

Chen, M.-L., Pittet, M.J., Gorelik, L., Flavell, R.A., Weissleder, R., von Boehmer, H. and Khazaie, K. (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF- $\beta$  signals in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 102(2), 419-424.

Chen, Q., Zhang, Xiang H.F. and Massagué, J. (2011) Macrophage Binding to Receptor VCAM-1 Transmits Survival Signals in Breast Cancer Cells that Invade the Lungs. *Cancer cell* 20(4), 538-549.

Chen, X., Du, Y., Lin, X., Qian, Y., Zhou, T. and Huang, Z. (2016) CD4+CD25+ regulatory T cells in tumor immunity. *International immunopharmacology* 34, 244-249.

Cheng, A., Zhang, M., Saltiel, A.R., Okubo, M. and Omichi, K. (2009) Distinct mutations in the glycogen debranching enzyme found in glycogen storage disease type III lead to impairment in diverse cellular functions. *Human Molecular Genetics* 18(11), 2045-2052.

Cheung, W.C. and Van Ness, B. (2001) The bone marrow stromal microenvironment influences myeloma therapeutic response in vitro. *Leukemia* 15(2), 264-271.

Chitta, K.S., Paulus, A., Ailawadhi, S., Foster, B.A., Moser, M.T., Starostik, P., Masood, A., Sher, T., Miller, K.C., Iancu, D.M., Conroy, J., Nowak, N.J., Sait, S.N., Personett, D.A., Coleman, M., Furman, R.R., Martin, P., Ansell, S.M., Lee, K. and Chanan-Khan, A.A. (2013) Development and characterization of a novel human Waldenström macroglobulinemia cell line: RPCI-WM1, Roswell Park Cancer Institute – Waldenström Macroglobulinemia 1. *Leukemia & Lymphoma* 54(2), 387-396.

Chu, Y. and Corey, D.R. (2012) RNA Sequencing: Platform Selection, Experimental Design, and Data Interpretation. *Nucleic Acid Therapeutics* 22(4), 271-274.

Cirri, P. and Chiarugi, P. (2012) Cancer-associated-fibroblasts and tumour cells: a diabolic liaison driving cancer progression. *Cancer and metastasis reviews* 31(1), 195-208.

Clodi, K., Asgary, Z., Zhao, S., Kliche, K.O., Cabanillas, F., Andreeff, M. and Younes, A. (1998) Coexpression of CD40 and CD40 ligand in B-cell lymphoma cells. *Br J Haematol* 103(1), 270-275.

Colledge, M. and Scott, J.D. (1999) AKAPs: from structure to function. *Trends in Cell Biology* 9(6), 216-221.

Correa-Rodríguez, M., Schmidt Rio-Valle, J. and Rueda-Medina, B. (2018) AKAP11 gene polymorphism is associated with bone mass measured by quantitative ultrasound in young adults. *International journal of medical sciences* 15(10), 999-1004.

Cozen, W., Gill, P.S., Ingles, S.A., Masood, R., Martínez-Maza, O., Cockburn, M.G., Gauderman, W.J., Pike, M.C., Bernstein, L., Nathwani, B.N., Salam, M.T., Danley, K.L., Wang, W., Gage, J., Gundell-Miller, S. and Mack, T.M. (2004) IL-6 levels and genotype are associated with risk of young adult Hodgkin lymphoma. *Blood* 103(8), 3216-3221.

Crawford, Y., Kasman, I., Yu, L., Zhong, C., Wu, X., Modrusan, Z., Kaminker, J. and Ferrara, N. (2009) PDGF-C Mediates the Angiogenic and Tumorigenic Properties of Fibroblasts Associated with Tumors Refractory to Anti-VEGF Treatment. *Cancer cell* 15(1), 21-34.

Curiel, T.J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J.R., Zhang, L. and Burow, M. (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine* 10(9), 942.

Daenen, L.G., Shaked, Y., Man, S., Xu, P., Voest, E.E., Hoffman, R.M., Chaplin, D.J. and Kerbel, R.S. (2009) Low-dose metronomic cyclophosphamide combined with vascular disrupting therapy induces potent antitumor activity in preclinical human tumor xenograft models. *Molecular Cancer Therapeutics* 8(10), 2872-2881.

Dahmane, N., Lee, J., Robins, P., Heller, P. and Altaba, A.R.i. (1997) Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* 389(6653), 876-881.

Dalton, W.S. (1999) The tumor microenvironment as a determinant of drug response and resistance. *Drug Resistance Updates* 2(5), 285-288.

Dang, C.V. and Semenza, G.L. (1999) Oncogenic alterations of metabolism. *Trends in biochemical sciences* 24(2), 68-72.

De Jaeger, K., Merlo, F.M., Kavanagh, M.-C., Fyles, A.W., Hedley, D. and Hill, R.P. (1998) Heterogeneity of tumor oxygenation: relationship to tumor necrosis, tumor size, and metastasis. *International Journal of Radiation Oncology\* Biology\* Physics* 42(4), 717-721.

DeNardo, D.G., Brennan, D.J., Rexhepaj, E., Ruffell, B., Shiao, S.L., Madden, S.F., Gallagher, W.M., Wadhvani, N., Keil, S.D., Junaid, S.A., Rugo, H.S., Hwang, E.S., Jirström, K., West, B.L. and Coussens, L.M. (2011) Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer discovery* 1(1), 54-67.

Dennler, S., André, J., Alexaki, I., Li, A., Magnaldo, T., ten Dijke, P., Wang, X.-J., Verrecchia, F. and Mauviel, A. (2007) Induction of Sonic Hedgehog Mediators by Transforming Growth Factor- $\beta$ : Smad3-Dependent Activation of Gli2 and Gli1 Expression In vitro and In vivo. *Cancer research* 67(14), 6981-6986.

Derynck, R., Akhurst, R.J. and Balmain, A. (2001) TGF- $\beta$  signaling in tumor suppression and cancer progression. *Nature Genetics* 29, 117.

di Magliano, M.P. and Hebrok, M. (2003) Hedgehog signalling in cancer formation and maintenance. *Nature Reviews Cancer* 3, 903.

Dierks, C., Beigi, R., Guo, G.R., Zirlik, K., Stegert, M.R. and Manley, P. (2008) Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer cell* 14.

Dimopoulos, M.A. and Alexanian, R. (1994) Waldenstrom's macroglobulinemia. *Blood* 83(6), 1452-1459.

Dimopoulos, M.A. and Anagnostopoulos, A. (2005) Waldenstrom's macroglobulinemia. *Best Pract Res Clin Haematol* 18(4), 747-765.

Dimopoulos, M.A., Anagnostopoulos, A., Kyrtsolis, M.-C., Zervas, K., Tsatalas, C., Kokkinis, G., Repoussis, P., Symeonidis, A., Delimpasi, S., Katodritou, E., Vervessou, E., Michali, E., Pouli, A., Gika, D., Vassou, A., Terpos, E., Anagnostopoulos, N., Economopoulos, T. and Pangalis, G. (2007) Primary Treatment of Waldenström Macroglobulinemia With Dexamethasone, Rituximab, and Cyclophosphamide. *Journal of Clinical Oncology* 25(22), 3344-3349.

Dimopoulos, M.A., Gertz, M.A., Kastiris, E., Garcia-Sanz, R., Kimby, E.K., Leblond, V., Femand, J.-P., Merlini, G., Morel, P., Morra, E., Ocio, E.M., Owen, R., Ghobrial, I.M., Seymour, J., Kyle, R.A. and Treon, S.P. (2009) Update on treatment recommendations from the Fourth International Workshop on Waldenstrom's Macroglobulinemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 27(1), 120-126.

Dimopoulos, M.A., Kyle, R.A., Anagnostopoulos, A. and Treon, S.P. (2005) Diagnosis and Management of Waldenstrom's Macroglobulinemia. *Journal of Clinical Oncology* 23(7), 1564-1577.

Dimopoulos, M.A., Panayiotidis, P., Mouloupoulos, L.A., Sfrikakis, P. and Dalakas, M. (2000) Waldenström's macroglobulinemia: clinical features, complications, and management. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 18(1), 214-226.

Ditzel Santos, D., Ho, A.W., Tournilhac, O., Hatjiharissi, E., Leleu, X., Xu, L., Tassone, P., Neri, P., Hunter, Z.R., Chemaly, M.A., Branagan, A.R., Manning, R.J., Patterson, C.J., Moreau, A.S., Ciccarelli, B., Adamia, S., Kriangkum, J., Kutok, J.L., Tai, Y.T., Zhang, J., Pilarski, L.M., Anderson, K.C., Munshi, N. and Treon, S.P. (2007) Establishment of BCWM.1 cell line for Waldenstrom's macroglobulinemia with productive in vivo engraftment in SCID-hu mice. *Exp Hematol* 35(9), 1366-1375.

Doedens, A.L., Stockmann, C., Rubinstein, M.P., Liao, D., Zhang, N., DeNardo, D.G., Coussens, L.M., Karin, M., Goldrath, A.W. and Johnson, R.S. (2010) Macrophage expression of hypoxia-inducible factor-1 $\alpha$  suppresses T-cell function and promotes tumor progression. *Cancer research* 70(19), 7465-7475.

Drexler, H.G., Chen, S. and Macleod, R.A.F. (2013) Would the real Waldenström cell line please stand up? *Leukemia & Lymphoma* 54(2), 224-226.

Du, R., Lu, K.V., Petritsch, C., Liu, P., Ganss, R., Passequé, E., Song, H., VandenBerg, S., Johnson, R.S., Werb, Z. and Bergers, G. (2008) HIF1- $\alpha$  Induces the Recruitment of

Bone Marrow-Derived Vascular Modulatory Cells to Regulate Tumor Angiogenesis and Invasion. *Cancer cell* 13(3), 206-220.

DuVillard, L., Guiguet, M., Casasnovas, R.-O., Caillot, D., Monnier-Zeller, V., Bernard, A., Guy, H. and Solary, E. (1995a) Diagnostic value of serum IL-6 level in monoclonal gammopathies. *British Journal of Haematology* 89(2), 243-249.

Elsawa, S.F., Almada, L.L., Ziesmer, S.C., Novak, A.J., Witzig, T.E., Ansell, S.M. and Fernandez-Zapico, M.E. (2011a) GLI2 Transcription Factor Mediates Cytokine Cross-talk in the Tumor Microenvironment. *Journal of Biological Chemistry* 286(24), 21524-21534.

Elsawa, S.F. and Ansell, S.M. (2009) Cytokines in the Microenvironment of Waldenström's Macroglobulinemia. *Clinical Lymphoma and Myeloma* 9(1), 43-45.

Elsawa, S.F., Novak, A.J., Grote, D., Konopleva, M., Andreeff, M., Witzig, T.E. and Ansell, S.M. (2008) CDDO-imidazole mediated inhibition of malignant cell growth in Waldenström macroglobulinemia. *Leuk Res* 32(12), 1895-1902.

Elsawa, S.F., Novak, A.J., Grote, D.M., Ziesmer, S.C., Witzig, T.E., Kyle, R.A., Dillon, S.R., Harder, B., Gross, J.A. and Ansell, S.M. (2006) B-lymphocyte stimulator (BLyS) stimulates immunoglobulin production and malignant B-cell growth in Waldenström macroglobulinemia. *Blood* 107(7), 2882-2888.

Elsawa, S.F., Novak, A.J., Ziesmer, S.C., Almada, L.L., Hodge, L.S., Grote, D.M., Witzig, T.E., Fernandez-Zapico, M.E. and Ansell, S.M. (2011b) Comprehensive analysis of tumor microenvironment cytokines in Waldenström macroglobulinemia identifies CCL5 as a novel modulator of IL-6 activity. *Blood* 118(20), 5540-5549.

Engle, S.J., Ormsby, I., Pawlowski, S., Boivin, G.P., Croft, J., Balish, E. and Doetschman, T. (2002) Elimination of Colon Cancer in Germ-free Transforming Growth Factor Beta 1-deficient Mice. *Cancer research* 62(22), 6362-6366.

Fernandes, M.S., Gomes, E.M., Butcher, L.D., Hernandez-Alcoceba, R., Chang, D., Kansopon, J., Newman, J., Stone, M.J. and Tong, A.W. (2009) Growth inhibition of human multiple myeloma cells by an oncolytic adenovirus carrying the CD40 ligand transgene. *Clin Cancer Res* 15(15), 4847-4856.

Ferrara, N. and Alitalo, K. (1999) Clinical applications of angiogenic growth factors and their inhibitors. *Nature medicine* 5(12), 1359.

Finger, E.C. and Giaccia, A.J. (2010) Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer metastasis reviews* 29(2), 285-293.

Fiumara, P. and Younes, A. (2001) CD40 ligand (CD154) and tumour necrosis factor-related apoptosis inducing ligand (Apo-2L) in haematological malignancies. *British Journal of Haematology* 113(2), 265-274.

Flaberg, E., Markasz, L., Petranyi, G., Stuber, G., Dicso, F., Alchihabi, N., Oláh, È., Csízy, I., Józsa, T., Andrén, O., Johansson, J.-E., Andersson, S.-O., Klein, G. and Szekely, L. (2011) High-throughput live-cell imaging reveals differential inhibition of tumor cell proliferation by human fibroblasts. *International Journal of Cancer* 128(12), 2793-2802.

Fonseca, R. and Hayman, S. (2007) Waldenstrom macroglobulinaemia. *Br J Haematol* 138(6), 700-720.

French, J.D., Tschumper, R.C. and Jelinek, D.F. (2002) Analysis of IL-6-mediated growth control of myeloma cells using a gp130 chimeric receptor approach. *Leukemia* 16(6), 1189-1196.

French, J.D., Walters, D.K. and Jelinek, D.F. (2003) Transactivation of gp130 in Myeloma Cells. *The Journal of Immunology* 170(7), 3717-3723.

Furler, R.L. and Uittenbogaart, C.H. (2012) GLI2 Regulates TGF- $\beta$ 1 in Human CD4(+) T Cells: Implications in Cancer and HIV Pathogenesis. *PloS one* 7(7), e40874.

Galmarini, C.M., Tannock, I.F., Patel, K. and Trédan, O. (2007) Drug Resistance and the Solid Tumor Microenvironment. *JNCI: Journal of the National Cancer Institute* 99(19), 1441-1454.

Gambacorti-Passerini, C.B., Donadoni, C., Parmiani, A., Pirola, A., Redaelli, S., Signore, G., Piazza, V., Malcovati, L., Fontana, D., Spinelli, R., Magistroni, V., Gaipa, G., Peronaci, M., Morotti, A., Panuzzo, C., Saglio, G., Usala, E., Kim, D.-W., Rea, D., Zervakis, K., Viniou, N., Symeonidis, A., Becker, H., Boulwood, J., Campiotti, L., Carrabba, M., Elli, E., Bignell, G.R., Papaemmanuil, E., Campbell, P.J., Cazzola, M. and Piazza, R. (2015) Recurrent ETNK1 mutations in atypical chronic myeloid leukemia. *Blood* 125(3), 499-503.

Gambaro, K., Quinn, M.C.J., Wojnarowicz, P.M., Arcand, S.L., de Ladurantaye, M., Barrès, V., Ripeau, J.-S., Killary, A.M., Davis, E.C., Lavoie, J., Provencher, D.M., Mes-Masson, A.-M., Chevrette, M. and Tonin, P.N. (2013) VGLL3 expression is associated with a tumor suppressor phenotype in epithelial ovarian cancer. *Molecular Oncology* 7(3), 513-530.

Gan, A., Ong, C.K., Huang, D., Heng, H.L., Poon, S.L., Yu, W., Teh, B.T., McPherson, J.R., Rozen, S.G., Tan, P., Stevenson, M., Newey, P., Thakker, R.V., Ruano, D., van Eijk, R., van Wezel, T., Morreau, H. and Cavaco, B. (2015) Whole-Exome Sequencing Studies of Parathyroid Carcinomas Reveal Novel PRUNE2 Mutations, Distinctive Mutational Spectra Related to APOBEC-Catalyzed DNA Mutagenesis and Mutational



Enrichment in Kinases Associated With Cell Migration and Invasion. *The Journal of Clinical Endocrinology & Metabolism* 100(2), E360-E364.

García-Sanz, R., Montoto, S., Torrequebrada, A., De Coca, A.G., Petit, J., Sureda, A., Rodríguez-García, J.A., Massó, P., Pérez-Aliaga, A., Monteagudo, M.D., Navarro, I., Moreno, G., Toledo, C., Alonso, A., Besses, C., Besalduch, J., Jarque, I., Salama, P., Rivas, J.A.H., Navarro, B., Bladé, J. and Miguel, J.F.S. (2001) Waldenström macroglobulinaemia: presenting features and outcome in a series with 217 cases. *British Journal of Haematology* 115(3), 575-582.

Gertz, M.A. (2018a) Selecting Initial Therapy for Newly Diagnosed Waldenström Macroglobulinemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 36(27), 2749-2751.

Gertz, M.A. (2018b) Waldenström macroglobulinemia treatment algorithm 2018. *Blood Cancer Journal* 8(4).

Gertz, M.A., Kyle, R.A. and Noel, P. (1993) Primary systemic amyloidosis: a rare complication of immunoglobulin M monoclonal gammopathies and Waldenström's macroglobulinemia. *Journal of Clinical Oncology* 11(5), 914-920.

Ghiringhelli, F., Ménard, C., Martin, F. and Zitvogel, L. (2006) The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunological reviews* 214, 229-238.

Ghobrial, I.M. (2012) Are you sure this is Waldenstrom macroglobulinemia? Hematology. *American Society of Hematology*. Education Program 2012, 586-594.

Ghobrial, I.M., Gertz, M.A. and Fonseca, R. (2003) Waldenström macroglobulinaemia. *The Lancet Oncology* 4(11), 679-685.

Ghobrial, I.M., Maiso, P., Azab, A., Liu, Y., Zhang, Y., Issa, G., Azab, F., Sacco, A., Quang, P., Ngo, H. and Roccaro, A. (2011) The bone marrow microenvironment in Waldenstrom macroglobulinemia. *Therapeutic Advances in Hematology* 2(4), 267-272.

Ghobrial, I.M. and Witzig, T.E. (2004) Waldenström macroglobulinemia. *Current treatment options in oncology* 5(3), 239-247.

Giraud, E., Inoue, M. and Hanahan, D. (2004) An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *The Journal of Clinical Investigation* 114(5), 623-633.

Goldstein, J.L., Austin, S.L., Boyette, K., Kanaly, A., Veerapandiyan, A., Rehder, C., Kishnani, P.S. and Bali, D.S. (2010) Molecular analysis of the AGL gene: Identification of 25 novel mutations and evidence of genetic heterogeneity in patients with Glycogen Storage Disease Type III. *Genetics In Medicine* 12, 424.

Grachtchouk, M., Mo, R., Yu, S., Zhang, X., Sasaki, H., Hui, C.-c. and Dlugosz, A.A. (2000) Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nature Genetics* 24, 216.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W. and Giaccia, A.J. (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 379(6560), 88.

Grivennikov, S.I., Greten, F.R. and Karin, M. (2010) Immunity, Inflammation, and Cancer. *Cell* 140(6), 883-899.

Gruber Filbin, M., Dabral, S.K., Pazyra-Murphy, M.F., Ramkissoon, S., Kung, A.L., Pak, E., Chung, J., Theisen, M.A., Sun, Y., Franchetti, Y., Sun, Y., Shulman, D.S., Redjal, N., Tabak, B., Beroukhim, R., Wang, Q., Zhao, J., Dorsch, M., Buonamici, S., Ligon, K.L., Kelleher, J.F. and Segal, R.A. (2013) Coordinate activation of Shh and PI3K signaling in PTEN-deficient glioblastoma: new therapeutic opportunities. *Nat Med* 19(11), 1518-1523.

Guarguaglini, G., Duncan, P.I., Stierhof, Y.D., Holmström, T., Duensing, S. and Nigg, E.A. (2005) The Forkhead-associated Domain Protein Cep170 Interacts with Polo-like Kinase 1 and Serves as a Marker for Mature Centrioles. *Molecular Biology of the Cell* 16(3), 1095-1107.

Guasch, G., Schober, M., Pasolli, H.A., Conn, E.B., Polak, L. and Fuchs, E. (2007) Loss of TGF $\beta$  Signaling Destabilizes Homeostasis and Promotes Squamous Cell Carcinomas in Stratified Epithelia. *Cancer cell* 12(4), 313-327.

Gupta, A., Tsuchiya, Y., Ohta, M., Shiratsuchi, G. and Kitagawa, D. (2017) NEK7 is required for G1 progression and procentriole formation. *Molecular Biology of the Cell* 28(15), 2123-2134.

Gupta, G.P. and Massagué, J. (2006) Cancer metastasis: building a framework. *Cell* 127(4), 679-695.

Haase, V.H. (2009) Oxygen regulates epithelial-to-mesenchymal transition: insights into molecular mechanisms and relevance to disease. *Kidney international* 76(5), 492-499.

Han, W., Jackson, D.A., Matissek, S.J., Misurelli, J.A., Neil, M.S., Sklavanitis, B., Amarsaikhan, N. and ElSawa, S.F. (2017) Novel Molecular Mechanism of Regulation of CD40 Ligand by the Transcription Factor GLI2. *The Journal of Immunology* 198(11), 4481-4489.

Hanahan, D. and Coussens, L.M. (2012) Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell* 21(3), 309-322.



Hanahan, D. and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86(3), 353-364.

Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144(5), 646-674.

Hao, J.-S. and Shan, B.-E. (2006) Immune enhancement and anti-tumour activity of IL-23. *Cancer Immunology, Immunotherapy* 55(11), 1426-1431.

Hatzimichael, E.C., Christou, L., Bai, M., Kolios, G., Kefala, L. and Bourantas, K.L. (2001) Serum levels of IL-6 and its soluble receptor (sIL-6R) in Waldenström's macroglobulinemia. *European Journal of Haematology* 66(1), 1-6.

Haura, E.B., Turkson, J. and Jove, R. (2005) Mechanisms of Disease: insights into the emerging role of signal transducers and activators of transcription in cancer. *Nature Clinical Practice Oncology* 2, 315.

Hazlehurst, L.A., Landowski, T.H. and Dalton, W.S. (2002) Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. *Oncogene* 22, 7396.

He, B., Chadburn, A., Jou, E., Schattner, E.J., Knowles, D.M. and Cerutti, A. (2004) Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. *J Immunol* 172(5), 3268-3279.

He, Y., Zeng, M.Y., Yang, D., Motro, B. and Núñez, G. (2016) NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux. *Nature* 530, 354.

Heinrich, P.C., Behrmann, I., Müller-newen, G., Schaper, F. and Graeve, L. (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochemical Journal* 334(2), 297-314.

Hilbert, D.M., Kopf, M., Mock, B.A., Köhler, G. and Rudikoff, S. (1995) Interleukin 6 is essential for in vivo development of B lineage neoplasms. *The Journal of Experimental Medicine* 182(1), 243-248.

Hirano, T. (1991) Interleukin 6 (IL-6) and its receptor: their role in plasma cell neoplasias. *Int J Cell Cloning* 9(3), 166-184.

Hodge, D.R., Hurt, E.M. and Farrar, W.L. (2005) The role of IL-6 and STAT3 in inflammation and cancer. *European Journal of Cancer* 41(16), 2502-2512.

Hodge, L.S., Novak, A.J., Grote, D.M., Braggio, E., Ketterling, R.P., Manske, M.K., Price Troska, T.L., Ziesmer, S.C., Fonseca, R., Witzig, T.E., Morice, W.G., Gertz, M.A. and Ansell, S.M. (2011) Establishment and characterization of a novel Waldenström macroglobulinemia cell line, MWCL-1. *Blood* 117(19), e190-197.

Hollis-Moffatt, J.E., Chen-Xu, M., Topless, R., Dalbeth, N., Gow, P.J., Harrison, A.A., Highton, J., Jones, P.B., Nissen, M., Smith, M.D., van Rij, A., Jones, G.T., Stamp, L.K. and Merriman, T.R. (2010) Only one independent genetic association with rheumatoid arthritis within the KIAA1109-TENR-IL2-IL21 locus in Caucasian sample sets: confirmation of association of rs6822844 with rheumatoid arthritis at a genome-wide level of significance. *Arthritis Research & Therapy* 12(3), R116.

Holohan, C., Van Schaeybroeck, S., Longley, D.B. and Johnston, P.G. (2013) Cancer drug resistance: an evolving paradigm. *Nature Reviews Cancer* 13, 714.

Horn, S., Kobberup, S., Jørgensen, M.C., Kalisz, M., Klein, T., Kageyama, R., Gegg, M., Lickert, H., Lindner, J., Magnuson, M.A., Kong, Y.-Y., Serup, P., Ahnfelt-Rønne, J. and Jensen, J.N. (2012) Mind bomb 1 is required for pancreatic  $\beta$ -cell formation. *Proceedings of the National Academy of Sciences* 109(19), 7356-7361.

Hunsucker, S.A., Magarotto, V., Kuhn, D.J., Kornblau, S.M., Wang, M., Weber, D.M., Thomas, S.K., Shah, J.J., Voorhees, P.M., Xie, H., Cornfeld, M., Nemeth, J.A. and Orłowski, R.Z. (2011) Blockade of interleukin-6 signalling with siltuximab enhances melphalan cytotoxicity in preclinical models of multiple myeloma. *British Journal of Haematology* 152(5), 579-592.

Hussain, S.P., Hofseth, L.J. and Harris, C.C. (2003) Radical causes of cancer. *Nature Reviews Cancer* 3, 276.

Inácio Pinto, N., Carnier, J., Oyama, L.M., Otoch, J.P., Alcântara, P.S., Tokeshi, F. and Nascimento, C.M. (2015) Cancer as a Proinflammatory Environment: Metastasis and Cachexia. *Mediators of Inflammation* 2015, 13.

Ingham, P.W. and McMahon, A.P. (2001) Hedgehog signaling in animal development: paradigms and principles. *Genes & development* 15(23), 3059-3087.

Ingolia, N.T., Brar, G.A., Rouskin, S., McGeachy, A.M. and Weissman, J.S. (2012) The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. *Nature Protocols* 7, 1534.

Iwama, E., Tsuchimoto, D., Iyama, T., Sakumi, K., Nakagawara, A., Takayama, K., Nakanishi, Y. and Nakabeppu, Y. (2011) Cancer-Related PRUNE2 Protein Is Associated with Nucleotides and Is Highly Expressed in Mature Nerve Tissues. *Journal of Molecular Neuroscience* 44(2), 103-114.

Jackson, D.A., Smith, T.D., Amarsaikhan, N., Han, W., Neil, M.S., Boi, S.K., Vrabel, A.M., Tolosa, E.J., Almada, L.L., Fernandez-Zapico, M.E. and Elsawa, S.F. (2015) Modulation of the IL-6 Receptor  $\alpha$  Underlies GLI2-Mediated Regulation of Ig Secretion in Waldenström Macroglobulinemia Cells. *The Journal of Immunology* 195(6), 2908-2916.

Jalali, S. and Ansell, S.M. (2016) Bone marrow microenvironment in Waldenstrom's Macroglobulinemia. *Best Practice & Research Clinical Haematology* 29(2), 148-155.

Javelaud, D., Alexaki, V.I., Dennler, S., Mohammad, K.S., Guise, T.A. and Mauviel, A. (2011) The TGF- $\beta$ /SMAD/GLI2 signaling axis in cancer progression and metastasis. *Cancer research* 71(17), 5606-5610.

Jin, Z., Teramoto, N., Hayashi, K., Liu, Y.X., Jin, G., Oka, T., Takahashi, K., Yoshino, T. and Akagi, T. (2004) CD40 ligand stimulation inhibits the proliferation of mantle cell lymphoma lines. *Anticancer Res* 24(2B), 691-697.

Joyce, J.A. and Fearon, D.T. (2015) T cell exclusion, immune privilege, and the tumor microenvironment. *Science* 348(6230), 74-80.

Kalluri, R. and Zeisberg, M. (2006) Fibroblasts in cancer. *Nature Reviews Cancer* 6, 392.

Kamimura, D., Ishihara, K. and Hirano, T. (2003). IL-6 signal transduction and its physiological roles: the signal orchestration model. In *Reviews of Physiology, Biochemistry and Pharmacology* (pp. 1-38). Springer, Berlin, Heidelberg.

Kapoor, P., Paludo, J., Vallumsetla, N. and Greipp, P.R. (2015) Waldenstrom macroglobulinemia: What a hematologist needs to know. *Blood Rev* 29(5), 301-319.

Karin, M. (2006) Nuclear factor- $\kappa$ B in cancer development and progression. *Nature* 441(7092), 431-436.

Karin, M. and Greten, F.R. (2005) NF- $\kappa$ B: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology* 5, 749.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R. and Weinberg, R.A. (2007) Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 449, 557.

Kim, S.-Y., Kang, J.W., Song, X., Kim, B.K., Yoo, Y.D., Kwon, Y.T. and Lee, Y.J. (2013) Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cellular Signalling* 25(4), 961-969.

Kim, S., Lee, K. and Rhee, K. (2007) NEK7 is a centrosomal kinase critical for microtubule nucleation. *Biochemical and Biophysical Research Communications* 360(1), 56-62.

Kinzler, K., Bigner, S., Bigner, D., Trent, J., Law, M., O'Brien, S., Wong, A. and Vogelstein, B. (1987) Identification of an amplified, highly expressed gene in a human glioma. *Science* 236(4797), 70-73.

- Kishimoto, T. (2005) Interleukin-6: from basic science to medicine--40 years in immunology. *Annu Rev Immunol* 23, 1-21.
- Kishimoto, T., Akira, S., Narazaki, M. and Taga, T. (1995) Interleukin-6 family of cytokines and gp130. *Blood* 86(4), 1243-1254.
- Kishimoto, T. and Tanaka, T. (2015) Interleukin6. *Encyclopedia of Inflammatory Diseases*, 1-8.
- Kondo, A., Safaei, R., Mishima, M., Niedner, H., Lin, X. and Howell, S.B. (2001) Hypoxia-induced enrichment and mutagenesis of cells that have lost DNA mismatch repair. *Cancer research* 61(20), 7603-7607.
- Konforte, D., Simard, N. and Paige, C.J. (2009) IL-21: An Executor of B Cell Fate. *The Journal of Immunology* 182(4), 1781-1787.
- Kortylewski, M., Xin, H., Kujawski, M., Lee, H., Liu, Y., Harris, T., Drake, C., Pardoll, D. and Yu, H. (2009) Regulation of the IL-23 and IL-12 Balance by Stat3 Signaling in the Tumor Microenvironment. *Cancer cell* 15(2), 114-123.
- Kosmider, O. (2015) Mutations of ETNK1 in aCML and CMML. *Blood* 125(3), 422-423.
- Kryczek, I., Zou, L., Rodriguez, P., Zhu, G., Wei, S., Mottram, P., Brumlik, M., Cheng, P., Curiel, T. and Myers, L. (2006) B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *Journal of Experimental Medicine* 203(4), 871-881.
- Kukurba, K.R. and Montgomery, S.B. (2015). RNA Sequencing and Analysis. *Cold Spring Harbor Protocols*, 2015(11), 951-969.
- Kumar, K., Raza, S.S., Knab, L.M., Chow, C.R., Kwok, B., Bentrem, D.J., Popovic, R., Ebine, K., Licht, J.D. and Munshi, H.G. (2015) GLI2-dependent c-MYC upregulation mediates resistance of pancreatic cancer cells to the BET bromodomain inhibitor JQ1. *Scientific Reports* 5, 9489.
- Kurtova, A.V., Balakrishnan, K., Chen, R., Ding, W., Schnabl, S., Quiroga, M.P., Sivina, M., Wierda, W.G., Estrov, Z., Keating, M.J., Shehata, M., Jager, U., Gandhi, V., Kay, N.E., Plunkett, W. and Burger, J.A. (2009a) Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. *Blood* 114(20), 4441-4450.
- Kurtova, A.V., Tamayo, A.T., Ford, R.J. and Burger, J.A. (2009b) Mantle cell lymphoma cells express high levels of CXCR4, CXCR5, and VLA-4 (CD49d): importance for

interactions with the stromal microenvironment and specific targeting. *Blood* 113(19), 4604-4613.

Landskron, G., De la Fuente, M., Thuwajit, P., Thuwajit, C. and Hermoso, M.A. (2014) Chronic inflammation and cytokines in the tumor microenvironment. *Journal of immunology research* 2014, 149185.

Langowski, J.L., Zhang, X., Wu, L., Mattson, J.D., Chen, T., Smith, K., Basham, B., McClanahan, T., Kastelein, R.A. and Oft, M. (2006) IL-23 promotes tumour incidence and growth. *Nature* 442(7101), 461-465.

Lasho, T.L., Finke, C.M., Zblewski, D., Patnaik, M., Ketterling, R.P., Chen, D., Hanson, C.A., Tefferi, A. and Pardanani, A. (2015) Novel recurrent mutations in ethanolamine kinase 1 (ETNK1) gene in systemic mastocytosis with eosinophilia and chronic myelomonocytic leukemia. *Blood Cancer Journal* 5, e275.

Lauth, M., Bergström, Å., Shimokawa, T. and Toftgård, R. (2007) Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *Proceedings of the National Academy of Sciences* 104(20), 8455-8460.

Lee, E., Trepicchio, W.L., Oestreicher, J.L., Pittman, D., Wang, F., Chamian, F., Dhodapkar, M. and Krueger, J.G. (2004) Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *Journal of Experimental Medicine* 199(1), 125-130.

Leleu, X., Jia, X., Runnels, J., Ngo, H.T., Moreau, A.-S., Farag, M., Spencer, J.A., Pitsillides, C.M., Hatjiharissi, E., Roccaro, A., O'Sullivan, G., McMillin, D.W., Moreno, D., Kiziltepe, T., Carrasco, R., Treon, S.P., Hideshima, T., Anderson, K.C., Lin, C.P. and Ghobrial, I.M. (2007) The Akt pathway regulates survival and homing in Waldenstrom macroglobulinemia. *Blood* 110(13), 4417-4426.

Li, F. and Sethi, G. (2010) Targeting transcription factor NF- $\kappa$ B to overcome chemoresistance and radioresistance in cancer therapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* 1805(2), 167-180.

Li, H., Lui, N., Cheng, T., Tseng, H.-H.K., Yue, D., Giroux-Leprieur, E., Do, H.T., Sheng, Q., Jin, J.Q. and Luh, T.W. (2013) Gli as a novel therapeutic target in malignant pleural mesothelioma. *PloS one* 8(3), e57346.

Li, Z.-W. and Dalton, W.S. (2006) Tumor microenvironment and drug resistance in hematologic malignancies. *Blood Reviews* 20(6), 333-342.

Liang, Y., Tsoi, L.C., Xing, X., Beamer, M.A., Swindell, W.R., Sarkar, M.K., Berthier, C.C., Stuart, P.E., Harms, P.W., Nair, R.P., Elder, J.T., Voorhees, J.J., Kahlenberg, J.M. and Gudjonsson, J.E. (2017) VGLL3-regulated gene network as a promoter of sex-biased autoimmune diseases. *Nature immunology* 18(2), 152-160.

Liao, D., Corle, C., Seagroves, T.N. and Johnson, R.S. (2007) Hypoxia-Inducible Factor-1A Is a Key Regulator of Metastasis in a Transgenic Model of Cancer Initiation and Progression. *Cancer Research*, 67(2), 563-572.

Libermann, T.A. and Zerbini, L.F. (2006) Targeting transcription factors for cancer gene therapy. *Current gene therapy* 6(1), 17-33.

Lin, E.Y., Li, J.-f., Bricard, G., Wang, W., Deng, Y., Sellers, R., Porcelli, S.A. and Pollard, J.W. (2007) Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages. *Molecular Oncology* 1(3), 288-302.

Lin, W.-W. and Karin, M. (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. *The Journal of Clinical Investigation* 117(5), 1175-1183.

Lindau, D., Gielen, P., Kroesen, M., Wesseling, P. and Adema, G.J. (2013) The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. *Immunology* 138(2), 105-115.

Liu, S., Dontu, G., Mantle, I.D., Patel, S., Ahn, N.-s., Jackson, K.W., Suri, P. and Wicha, M.S. (2006) Hedgehog Signaling and Bmi-1 Regulate Self-renewal of Normal and Malignant Human Mammary Stem Cells. *Cancer research* 66(12), 6063-6071.

Ljungkvist, A.S., Bussink, J., Rijken, P.F., Kaanders, J.H., van der Kogel, A.J. and Denekamp, J. (2002) Vascular architecture, hypoxia, and proliferation in first-generation xenografts of human head-and-neck squamous cell carcinomas. *International Journal of Radiation Oncology\* Biology\* Physics* 54(1), 215-228.

Lo Re, A.E., Fernandez-Barrena, M.G., Almada, L.L., Mills, L.D., ElSawa, S.F., Lund, G., Ropolo, A., Molejon, M.I., Vaccaro, M.I. and Fernandez-Zapico, M.E. (2012) Novel AKT1-GLI3-VMP1 pathway mediates KRAS oncogene-induced autophagy in cancer cells. *J Biol Chem* 287(30), 25325-25334.

Loeffler, M., Krüger, J.A., Niethammer, A.G. and Reisfeld, R.A. (2006) Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. *The Journal of Clinical Investigation* 116(7), 1955-1962.

Lu, P., Takai, K., Weaver, V.M. and Werb, Z. (2011) Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor perspectives in biology* 3(12), a005058.

Luo, J.-L., Maeda, S., Hsu, L.-C., Yagita, H. and Karin, M. (2004) Inhibition of NF- $\kappa$ B in cancer cells converts inflammation-induced tumor growth mediated by TNF $\alpha$  to TRAIL-mediated tumor regression. *Cancer cell* 6(3), 297-305.



Lykidis, A., Wang, J., Karim, M.A. and Jackowski, S. (2001) Overexpression of a mammalian ethanolamine-specific kinase accelerates the CDP-ethanolamine pathway. *The Journal of biological chemistry* 276(3), 2174-2179.

Mach, F., Schönbeck, U., Sukhova, G.K., Bourcier, T., Bonnefoy, J.-Y., Pober, J.S. and Libby, P. (1997) Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: Implications for CD40–CD40 ligand signaling in atherosclerosis. *Proceedings of the National Academy of Sciences of the United States of America* 94(5), 1931-1936.

Maggio-Price, L., Treuting, P., Zeng, W., Tsang, M., Bielefeldt-Ohmann, H. and Iritani, B.M. (2006) Helicobacter Infection Is Required for Inflammation and Colon Cancer in Smad3-Deficient Mice. *Cancer research* 66(2), 828-838.

Maher, C.A., Kumar-Sinha, C., Cao, X., Kalyana-Sundaram, S., Han, B., Jing, X., Sam, L., Barrette, T., Palanisamy, N. and Chinnaiyan, A.M. (2009) Transcriptome sequencing to detect gene fusions in cancer. *Nature* 458, 97.

Mantovani, A., Allavena, P., Sica, A. and Balkwill, F. (2008) Cancer-related inflammation. *Nature* 454, 436.

Massagué, J. (2008) TGF $\beta$  in Cancer. *Cell* 134(2), 215-230.

Matsuzaki, K. (2013) Smad phospho-isoforms direct context-dependent TGF- $\beta$  signaling. *Cytokine & growth factor reviews* 24(4), 385-399.

Mauviel, A., Sibon, C., Bertolotto, C., Javelaud, D., Luciani, F., Verrecchia, F., Goydos, J.S., Hoek, K.S., Mohammad, K.S., Van Kempen, L.C.L., Larue, L., Juárez, P., Saiag, P., Fournier, P.J., Ballotti, R., Saule, S., Dennler, S., Guise, T.A., Alexaki, V.-I. and Delmas, V. (2010) GLI2-Mediated Melanoma Invasion and Metastasis. *JNCI: Journal of the National Cancer Institute* 102(15), 1148-1159.

Meads, M.B., Hazlehurst, L.A. and Dalton, W.S. (2008) The Bone Marrow Microenvironment as a Tumor Sanctuary and Contributor to Drug Resistance. *Clinical Cancer Research* 14(9), 2519-2526.

Mendelson, A. and Frenette, P.S. (2014) Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nature medicine* 20, 833.

Mohamed, M.M. and Sloane, B.F. (2006) Cysteine cathepsins: multifunctional enzymes in cancer. *Nature Reviews Cancer* 6(10), 764.

Moore, R.J., Owens, D.M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., Kollias, G. and Balkwill, F. (1999) Mice deficient in tumor necrosis factor- $\alpha$  are resistant to skin carcinogenesis. *Nature medicine* 5, 828.

Moreaux, J., Legouffe, E., Jourdan, E., Quittet, P., Reme, T., Lugagne, C., Moine, P., Rossi, J.F., Klein, B. and Tarte, K. (2004) BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood* 103(8), 3148-3157.

Morin, P.J. (2003) Drug resistance and the microenvironment: nature and nurture. *Drug Resistance Updates* 6(4), 169-172.

Morrison, C.D., Parvani, J.G. and Schiemann, W.P. (2013) The relevance of the TGF- $\beta$  Paradox to EMT-MET programs. *Cancer Letters* 341(1), 30-40.

Movahedi, K., Laoui, D., Gysemans, C., Baeten, M., Stangé, G., Van den Bossche, J., Mack, M., Pipeleers, D., In't Veld, P. and De Baetselier, P. (2010) Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C (high) monocytes. *Cancer research* 70(14), 5728-5739.

Nagasawa, T. (2006) Microenvironmental niches in the bone marrow required for B-cell development. *Nature Reviews Immunology* 6, 107.

Ngo, H.T., Azab, A.K., Farag, M., Jia, X., Melhem, M.M., Runnels, J., Roccaro, A.M., Azab, F., Sacco, A., Leleu, X., Anderson, K.C. and Ghobrial, I.M. (2009) Src Tyrosine Kinase Regulates Adhesion and Chemotaxis in Waldenstrom Macroglobulinemia. *Clinical Cancer Research* 15(19), 6035-6041.

Nilsson, M., Undèn, A.B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P.G. and Toftgård, R. (2000) Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. *Proceedings of the National Academy of Sciences* 97(7), 3438-3443.

O'Shea, J.J., Gadina, M. and Schreiber, R.D. (2002) Cytokine Signaling in 2002: New Surprises in the Jak/Stat Pathway. *Cell* 109(2, Supplement 1), S121-S131.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J. and Singh, K. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13(5), 715-725.

Oro, A.E., Higgins, K.M., Hu, Z., Bonifas, J.M., Epstein, E.H. and Scott, M.P. (1997) Basal Cell Carcinomas in Mice Overexpressing Sonic Hedgehog. *Science* 276(5313), 817-821.

Owen, R.G., Treon, S.P., Al-Katib, A., Fonseca, R., Greipp, P.R., McMaster, M.L., Morra, E., Pangalis, G.A., San Miguel, J.F., Branagan, A.R. and Dimopoulos, M.A. (2003) Clinicopathological definition of Waldenstrom's macroglobulinemia: consensus panel recommendations from the Second International Workshop on Waldenstrom's Macroglobulinemia. *Semin Oncol* 30(2), 110-115.



Pan, D., Li, Y., Li, Z., Wang, Y., Wang, P. and Liang, Y. (2012) Gli inhibitor GANT61 causes apoptosis in myeloid leukemia cells and acts in synergy with rapamycin. *Leukemia research* 36(6), 742-748.

Parangi, S., O'Reilly, M., Christofori, G., Holmgren, L., Grosfeld, J., Folkman, J. and Hanahan, D. (1996) Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. *Proceedings of the National Academy of Sciences* 93(5), 2002-2007.

Park, E.J., Lee, J.H., Yu, G.-Y., He, G., Ali, S.R., Holzer, R.G., Österreicher, C.H., Takahashi, H. and Karin, M. (2010) Dietary and Genetic Obesity Promote Liver Inflammation and Tumorigenesis by Enhancing IL-6 and TNF Expression. *Cell* 140(2), 197-208.

Pasca di Magliano, M., Sekine, S., Ermilov, A., Ferris, J., Dlugosz, A.A. and Hebrok, M. (2006) Hedgehog/Ras interactions regulate early stages of pancreatic cancer. *Genes & development* 20(22), 3161-3173.

Pedersen, L.M., Jürgensen, G.W. and Johnsen, H.E. (2005) Serum levels of inflammatory cytokines at diagnosis correlate to the bcl-6 and CD10 defined germinal centre (GC) phenotype and bcl-2 expression in patients with diffuse large B-cell lymphoma. *British Journal of Haematology* 128(6), 813-819.

Pellegrino, A., Ria, R., Di Pietro, G., Cirulli, T., Surico, G., Pennisi, A., Morabito, F., Ribatti, D. and Vacca, A. (2005) Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *Br J Haematol* 129(2), 248-256.

Pikarsky, E., Porat, R.M., Stein, I., Abramovitch, R., Amit, S., Kasem, S., Galkovitch, P., Pyest, E., Urieli-Shoval, S., Galun, E. and Ben-Neriah, Y. (2004) NF- $\kappa$ B functions as a tumour promoter in inflammation-associated cancer. *Nature* 431(7007), 461-466.

Pirhonen, J., Matikainen, S. and Julkunen, I. (2002) Regulation of virus-induced IL-12 and IL-23 expression in human macrophages. *The Journal of Immunology* 169(10), 5673-5678.

Podar, K., Chauhan, D. and Anderson, K.C. (2008) Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia* 23, 10.

Pollard, J.W. (2004) Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews Cancer* 4(1), 71.

Pontiggia, O., Sampayo, R., Raffo, D., Motter, A., Xu, R., Bissell, M.J., de Kier Joffé, E.B. and Simian, M. (2012) The tumor microenvironment modulates tamoxifen resistance in breast cancer: a role for soluble stromal factors and fibronectin through  $\beta$ 1 integrin. *Breast cancer research and treatment* 133(2), 459-471.

Pouysségur, J., Dayan, F. and Mazure, N.M. (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441(7092), 437.

Qian, B.-Z. and Pollard, J.W. (2010) Macrophage diversity enhances tumor progression and metastasis. *Cell* 141(1), 39-51.

Rajkumar, S.V., Dispenzieri, A. and Kyle, R.A. (2006) Monoclonal Gammopathy of Undetermined Significance, Waldenström Macroglobulinemia, AL Amyloidosis, and Related Plasma Cell Disorders: Diagnosis and Treatment. *Mayo Clinic Proceedings* 81(5), 693-703.

Räsänen, K. and Vaheri, A. (2010) Activation of fibroblasts in cancer stroma. *Experimental Cell Research* 316(17), 2713-2722.

Redell, M.S. and Tweardy, D.J. (2005) Targeting transcription factors for cancer therapy. *Current pharmaceutical design* 11(22), 2873-2887.

Rimkus, T.K., Carpenter, R.L., Qasem, S., Chan, M. and Lo, H.-W. (2016) Targeting the sonic hedgehog signaling pathway: review of smoothed and GLI inhibitors. *Cancers* 8(2), 22.

Roccaro, A.M., Sacco, A., Husu, E.N., Pitsillides, C., Vesole, S., Azab, A.K., Azab, F., Melhem, M., Ngo, H.T., Quang, P., Maiso, P., Runnels, J., Liang, M.-C., Wong, K.-K., Lin, C. and Ghobrial, I.M. (2010) Dual targeting of the PI3K/Akt/mTOR pathway as an antitumor strategy in Waldenstrom macroglobulinemia. *Blood* 115(3), 559-569.

Roessler, E., Ermilov, A.N., Grange, D.K., Wang, A., Grachtchouk, M. and Dlugosz, A.A. (2005) A previously unidentified amino-terminal domain regulates transcriptional activity of wild-type and disease-associated human GLI2. *Hum Mol Genet* 14.

Rohatgi, R. and Scott, M.P. (2007) Patching the gaps in Hedgehog signalling. *Nature Cell Biology* 9, 1005.

Rolny, C., Mazzone, M., Tugues, S., Laoui, D., Johansson, I., Coulon, C., Squadrito, M.L., Segura, I., Li, X., Knevels, E., Costa, S., Vinckier, S., Dresselaer, T., Åkerud, P., De Mol, M., Salomäki, H., Phillipson, M., Wyns, S., Larsson, E., Buysschaert, I., Botling, J., Himmelreich, U., Van Ginderachter, J.A., De Palma, M., Dewerchin, M., Claesson-Welsh, L. and Carmeliet, P. (2011) HRG Inhibits Tumor Growth and Metastasis by Inducing Macrophage Polarization and Vessel Normalization through Downregulation of PlGF. *Cancer cell* 19(1), 31-44.

Rose-John, S., Scheller, J., Elson, G. and Jones, S.A. (2006) Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *Journal of leukocyte biology* 80(2), 227-236.

Rossi, J.F., Fegueux, N., Lu, Z.Y., Legouffe, E., Exbrayat, C., Bozonnat, M.C., Navarro, R., Lopez, E., Quittet, P., Daures, J.P., Rouillé, V., Kanouni, T., Widjenes, J. and Klein, B. (2005) Optimizing the use of anti-interleukin-6 monoclonal antibody with dexamethasone and 140 mg/m<sup>2</sup> of melphalan in multiple myeloma: results of a pilot study including biological aspects. *Bone Marrow Transplantation* 36, 771.

Ruffell, B., DeNardo, D.G., Affara, N.I. and Coussens, L.M. (2010) Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine & growth factor reviews* 21(1), 3-10.

Ruiz i Altaba, A., Mas, C. and Stecca, B. (2007) The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends in Cell Biology* 17(9), 438-447.

Sadarangani, A., Pineda, G., Lennon, K.M., Chun, H.-J., Shih, A., Schairer, A.E., Goff, D.J., Prashad, S.L., Geron, I. and Wall, R. (2015) GLI2 inhibition abrogates human leukemia stem cell dormancy. *Journal of translational medicine* 13(1), 98.

Sahin, I., Leblebjian, H., Treon, S.P. and Ghobrial, I.M. (2014) Waldenström macroglobulinemia: from biology to treatment. *Expert Review of Hematology* 7(1), 157-168.

Salameh, A., Lee, A.K., Cardó-Vila, M., Nunes, D.N., Efstathiou, E., Staquicini, F.I., Dobroff, A.S., Marchiò, S., Navone, N.M., Hosoya, H., Lauer, R.C., Wen, S., Salmeron, C.C., Hoang, A., Newsham, I., Lima, L.A., Carraro, D.M., Oliviero, S., Kolonin, M.G., Sidman, R.L., Do, K.-A., Troncoso, P., Logothetis, C.J., Brentani, R.R., Calin, G.A., Cavenee, W.K., Dias-Neto, E., Pasqualini, R. and Arap, W. (2015) PRUNE2 is a human prostate cancer suppressor regulated by the intronic long noncoding RNA PCA3. *Proceedings of the National Academy of Sciences* 112(27), 8403-8408.

Santos, D.D., Hatjiharissi, E., Tournilhac, O., Chemaly, M.Z.A., Leleu, X., Xu, L., Patterson, C., Branagan, A.R., Manning, R.J., Ho, A.W., Hunter, Z.R., Dimmock, E.A., Kutok, J.L., Churchill, W.H., Castells, M.C., Tai, Y.-T., Anderson, K.C. and Treon, S.P. (2006) CD52 Is Expressed on Human Mast Cells and Is a Potential Therapeutic Target in Waldenström's Macroglobulinemia and Mast Cell Disorders. *Clinical Lymphoma and Myeloma* 6(6), 478-483.

Schattner, E.J. (2000) CD40 ligand in CLL pathogenesis and therapy. *Leuk Lymphoma* 37(5-6), 461-472.

Schönbeck, U., Mach, F. and Libby, P. (2000) CD154 (CD40 ligand). *The International Journal of Biochemistry & Cell Biology* 32(7), 687-693.

Schwerd, T., Twigg, S.R.F., Aschenbrenner, D., Manrique, S., Miller, K.A., Taylor, I.B., Capitani, M., McGowan, S.J., Sweeney, E., Weber, A., Chen, L., Bowness, P., Riordan, A., Cant, A., Freeman, A.F., Milner, J.D., Holland, S.M., Frede, N., Müller, M., Schmidt-Arras, D., Grimbacher, B., Wall, S.A., Jones, E.Y., Wilkie, A.O.M. and Uhlig, H.H. (2017)

A biallelic mutation in *IL6ST* encoding the GP130 co-receptor causes immunodeficiency and craniosynostosis. *The Journal of Experimental Medicine* 214(9), 2547-2562.

Semenza, G.L. (2003) Targeting HIF-1 for cancer therapy. *Nature Reviews Cancer* 3, 721.

Shah, J.J., Feng, L., Thomas, S.K., Berkova, Z., Weber, D.M., Wang, M., Qazilbash, M.H., Champlin, R.E., Mendoza, T.R., Cleeland, C. and Orlowski, R.Z. (2016) Siltuximab (CNTO 328) with lenalidomide, bortezomib and dexamethasone in newly-diagnosed, previously untreated multiple myeloma: an open-label phase I trial. *Blood Cancer Journal* 6, e396.

Shaheen, R.M., Davis, D.W., Liu, W., Zebrowski, B.K., Wilson, M.R., Bucana, C.D., McConkey, D.J., McMahon, G. and Ellis, L.M. (1999) Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. *Cancer research* 59(21), 5412-5416.

Shields, J.D., Kourtis, I.C., Tomei, A.A., Roberts, J.M. and Swartz, M.A. (2010) Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* 328(5979), 749-752.

Shree, T., Olson, O.C., Elie, B.T., Kester, J.C., Garfall, A.L., Simpson, K., Bell-McGuinn, K.M., Zabor, E.C., Brogi, E. and Joyce, J.A. (2011) Macrophages and cathepsin proteases blunt chemotherapeutic response in breast cancer. *Genes & development* 25(23), 2465-2479.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Côté, D., Means, T.K., Luster, A.D., Scadden, D.T. and Lin, C.P. (2005) In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* 435(7044), 969-973.

Song, R., Kim, Y.-W., Koo, B.-K., Jeong, H.-W., Yoon, M.-J., Yoon, K.-J., Jun, D.-J., Im, S.-K., Shin, J., Kong, M.-P., Kim, K.-T., Yoon, K. and Kong, Y.-Y. (2008) Mind bomb 1 in the lymphopoietic niches is essential for T and marginal zone B cell development. *The Journal of Experimental Medicine* 205(11), 2525-2536.

Spill, F., Reynolds, D.S., Kamm, R.D. and Zaman, M.H. (2016) Impact of the physical microenvironment on tumor progression and metastasis. *Current opinion in biotechnology* 40, 41-48.

Srahna, M., Remacle, J.E., Annamalai, K., Pype, S., Huylebroeck, D., Boogaerts, M.A. and Vandenberghe, P. (2001) NF-kappaB is involved in the regulation of CD154 (CD40 ligand) expression in primary human T cells. *Clin Exp Immunol* 125(2), 229-236.

Stover, D.G., Bierie, B. and Moses, H.L. (2007) A delicate balance: TGF- $\beta$  and the tumor microenvironment. *Journal of cellular biochemistry* 101(4), 851-861.

Sugiyama, T. and Nagasawa, T. (2012) Bone Marrow Niches for Hematopoietic Stem Cells and Immune Cells. *Inflammation & Allergy - Drug Targets (Formerly Current Drug Targets - Inflammation & Allergy)* 11(3), 201-206.

Takahashi, S., Reddy, S.V., Dallas, M., Devlin, R., Chou, J.Y. and Roodman, G.D. (1995) Development and characterization of a human marrow stromal cell line that enhances osteoclast-like cell formation. *Endocrinology* 136(4), 1441-1449.

Takeda, N., O'Dea, E.L., Doedens, A., Kim, J.-w., Weidemann, A., Stockmann, C., Asagiri, M., Simon, M.C., Hoffmann, A. and Johnson, R.S. (2010) Differential activation and antagonistic function of HIF- $\alpha$  isoforms in macrophages are essential for NO homeostasis. *Genes & development* 24(5), 491-501.

Tanaka, T., Narazaki, M. and Kishimoto, T. (2011) Anti-interleukin-6 receptor antibody, tocilizumab, for the treatment of autoimmune diseases. *FEBS letters* 585(23), 3699-3709.

Tassone, P., Neri, P., Kutok, J.L., Tournilhac, O., Santos, D.D., Hatjiharissi, E., Munshi, V., Venuta, S., Anderson, K.C., Treon, S.P. and Munshi, N.C. (2005) A SCID-hu in vivo model of human Waldenström macroglobulinemia. *Blood* 106(4), 1341-1345.

Teicher, B.A., Herman, T.S., Holden, S.A., Wang, Y., Pfeffer, M.R., Crawford, J.W. and Frei, E. (1990) Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science* 247(4949), 1457-1461.

Teixeira, V.H., Pierlot, C., Migliorini, P., Balsa, A., Westhovens, R., Barrera, P., Alves, H., Vaz, C., Fernandes, M., Pascual-Salcedo, D., Bombardieri, S., Dequeker, J., Radstake, T.R., Van Riel, P., van de Putte, L., Lopes-Vaz, A., Bardin, T., Prum, B., Cornélis, F., Petit-Teixeira, E. and Families, t.E.C.o.R.A. (2009) Testing for the association of the KIAA1109/Tenr/IL2/IL21 gene region with rheumatoid arthritis in a European family-based study. *Arthritis Research & Therapy* 11(2), R45.

Terpos, E., Tasidou, A., Kastiris, E., Eleftherakis-Papaiakovou, E., Gavriatopoulou, M., Migkou, M. and Dimopoulos, M.-A. (2009) Angiogenesis in Waldenström's Macroglobulinemia. *Clinical Lymphoma and Myeloma* 9(1), 46-49.

Thiyagarajan, S., Bhatia, N., Reagan-Shaw, S., Cozma, D., Thomas-Tikhonenko, A., Ahmad, N. and Spiegelman, V.S. (2007) Role of GLI2 Transcription Factor in Growth and Tumorigenicity of Prostate Cells. *Cancer research* 67(22), 10642-10646.

Tong, A.W., Seamour, B., Chen, J., Su, D., Ordonez, G., Frase, L., Netto, G. and Stone, M.J. (2000) CD40 ligand-induced apoptosis is Fas-independent in human multiple myeloma cells. *Leuk Lymphoma* 36(5-6), 543-558.



Tournilhac, O., Santos, D.D., Xu, L., Kutok, J., Tai, Y.T., Le Gouill, S., Catley, L., Hunter, Z., Branagan, A.R., Boyce, J.A., Munshi, N., Anderson, K.C. and Treon, S.P. (2006) Mast cells in Waldenstrom's macroglobulinemia support lymphoplasmacytic cell growth through CD154/CD40 signaling. *Ann Oncol* 17(8), 1275-1282.

Treon, S.P., Patterson, C.J., Kimby, E. and Stone, M.J. (2009) Advances in the Biology and Treatment of Waldenström's Macroglobulinemia: A Report from the 5th International Workshop on Waldenström's Macroglobulinemia, Stockholm, Sweden. *Clinical Lymphoma and Myeloma* 9(1), 10-15.

Treon, S.P., Tripsas, C.K., Meid, K., Warren, D., Varma, G., Green, R., Argyropoulos, K.V., Yang, G., Cao, Y., Xu, L., Patterson, C.J., Rodig, S., Zehnder, J.L., Aster, J.C., Harris, N.L., Kanan, S., Ghobrial, I., Castillo, J.J., Laubach, J.P., Hunter, Z.R., Salman, Z., Li, J., Cheng, M., Clow, F., Graef, T., Palomba, M.L. and Advani, R.H. (2015a) Ibrutinib in previously treated Waldenström's macroglobulinemia. *The New England journal of medicine* 372(15), 1430-1440.

Treon, S.P., Xu, L. and Hunter, Z. (2015b) MYD88 Mutations and Response to Ibrutinib in Waldenström's Macroglobulinemia. *The New England journal of medicine* 373(6), 584-586.

Trinchieri, G. (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology* 3, 133.

Tsirakis, G., Pappa, C.A., Psarakis, F.E., Fragioudaki, M., Tsioutis, C., Stavroulaki, E., Boula, A. and Alexandrakis, M.G. (2012) Serum concentrations and clinical significance of soluble CD40 ligand in patients with multiple myeloma. *Med Oncol* 29(4), 2396-2401.

Vacca, A. and Ribatti, D. (2005) Bone marrow angiogenesis in multiple myeloma. *Leukemia* 20, 193.

van Seventer, J.M., Nagai, T. and van Seventer, G.A. (2002) Interferon- $\beta$  differentially regulates expression of the IL-12 family members p35, p40, p19 and EB13 in activated human dendritic cells. *Journal of Neuroimmunology* 133(1), 60-71.

Varjosalo, M. and Taipale, J. (2007) Hedgehog signaling. *J Cell Sci* 120.

Varjosalo, M. and Taipale, J. (2008) Hedgehog: functions and mechanisms. *Genes & development* 22(18), 2454-2472.

Varnat, F., Duquet, A., Malerba, M., Zbinden, M., Mas, C., Gervaz, P. and Ruiz i Altaba, A. (2009) Human colon cancer epithelial cells harbour active HEDGEHOG-GLI signalling that is essential for tumour growth, recurrence, metastasis and stem cell survival and expansion. *EMBO Molecular Medicine* 1(6-7), 338-351.

Vitale, M., Cantoni, C., Pietra, G., Mingari, M.C. and Moretta, L. (2014) Effect of tumor cells and tumor microenvironment on NK-cell function. *European journal of immunology* 44(6), 1582-1592.

Vong, S. and Kalluri, R. (2011) The Role of Stromal Myofibroblast and Extracellular Matrix in Tumor Angiogenesis. *Genes & Cancer* 2(12), 1139-1145.

Voorzanger-Rousselot, N., Favrot, M. and Blay, J.Y. (1998) Resistance to cytotoxic chemotherapy induced by CD40 ligand in lymphoma cells. *Blood* 92(9), 3381-3387.

Wang, Z., Gerstein, M. and Snyder, M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* 10, 57.

Wasiuk, A., De Vries, V., Hartmann, K., Roers, A. and Noelle, R. (2009) Mast cells as regulators of adaptive immunity to tumours. *Clinical & Experimental Immunology* 155(2), 140-146.

Watford, W.T., Hissong, B.D., Bream, J.H., Kanno, Y., Muul, L. and O'shea, J.J. (2004) Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. *Immunological reviews* 202(1), 139-156.

Wykes, M. (2003) Why do B cells produce CD40 ligand? *Immunol Cell Biol* 81(4), 328-331.

Xu, J., Lu, L. and Li, L. (2016) NEK7: a novel promising therapy target for NLRP3-related inflammatory diseases. *Acta Biochimica et Biophysica Sinica* 48(10), 966-968.

Xu, R., Boudreau, A. and Bissell, M.J. (2009) Tissue architecture and function: dynamic reciprocity via extra-and intra-cellular matrices. *Cancer and metastasis reviews* 28(1-2), 167-176.

Yang, J.-Y. and Hung, M.-C. (2009) A New Fork for Clinical Application: Targeting Forkhead Transcription Factors in Cancer. *Clinical Cancer Research* 15(3), 752-757.

Yang, L., Pang, Y. and Moses, H.L. (2010) TGF- $\beta$  and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends in Immunology* 31(6), 220-227.

Yang, M.-H., Wu, M.-Z., Chiou, S.-H., Chen, P.-M., Chang, S.-Y., Liu, C.-J., Teng, S.-C. and Wu, K.-J. (2008) Direct regulation of TWIST by HIF-1 $\alpha$  promotes metastasis. *Nature Cell Biology* 10, 295.

Yin, T. and Li, L. (2006) The stem cell niches in bone. *The Journal of Clinical Investigation* 116(5), 1195-1201.

Younes, A. (2001) CD40 ligand therapy of lymphoma patients. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 19(23), 4351-4353.

Zhao, M., Qiao, M., Harris, S.E., Chen, D., Oyajobi, B.O. and Mundy, G.R. (2006) The Zinc Finger Transcription Factor Gli2 Mediates Bone Morphogenetic Protein 2 Expression in Osteoblasts in Response to Hedgehog Signaling. *Molecular and Cellular Biology* 26(16), 6197-6208.

Zhou, L., Wang, Z., Xu, X., Wan, Y., Qu, K., Fan, H., Chen, Q., Sun, X. and Liu, C. (2016) Nek7 is overexpressed in hepatocellular carcinoma and promotes hepatocellular carcinoma cell proliferation in vitro and in vivo. *Oncotarget* 7(14), 18620-18630.

Ziche, M. and Morbidelli, L. (2009) Molecular regulation of tumour angiogenesis by nitric oxide. *European cytokine network* 20(4), 164-170.



## APPENDIX



NORTHERN ILLINOIS UNIVERSITY

**Office of Research Compliance,  
Integrity & Safety**

*Division of Research & Innovation Partnerships*

## Notice of Continuing Approval

19-Jul-2016

Sherine Elsawa

Biological Sciences

RE: Review of Application for Continuing Approval

The following application for continuation of approval of the use of live vertebrate animals in research was reviewed and approved by the Institutional Animal Care and Use Committee by designated member review on 15-Jul-2016:

Title: ***Targeting the tumor microenvironment in cancer***

ORC #: **LA12-0007**

This approval will be effective for one year from **16-Jul-2016** until **15-Jul-2017**. It is your responsibility as principal investigator to implement the research/course as specified in your IACUC protocol. If there is a change in the project, a need for additional animals, or if the project will last beyond that date, you will need additional approval and should contact the Office of Research Compliance, Integrity and Safety at 753-8588.



NORTHERN ILLINOIS UNIVERSITY

## Office of Research Compliance, Integrity & Safety

*Division of Research & Innovation Partnerships*

### Notice of Continuing Approval

29-Jun-2017

Sherine Elswa Biological  
Sciences

RE: Review of Application for Continuing Approval

The following application for continuation of approval of the use of live vertebrate animals in research was reviewed and approved by the Institutional Animal Care and Use Committee by designated member review on 29-Jun-2017:

Title: ***Targeting the tumor microenvironment in cancer***

ORC #: **LA12-0007**

This approval will be effective for one year from **16-Jul-2017** until **15-Jul-2018**. It is your responsibility as principal investigator to implement the research/course as specified in your IACUC protocol. If there is a change in the project, a need for additional animals, or if the project will last beyond that date, you will need additional approval and should contact the Office of Research Compliance, Integrity and Safety at 753-8588.