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Enhancing The Efficacy Of Thymidylate Synthase Inhibitors By Genetic Modulation Or Pharmacological Inhibition Of Bone Marrow Derived Cells In The Tumor Stroma

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DEDICATION

I must give all the honor and glory to God, my strength and endurance comes from God's grace and mercy. I am blessed for I have reached the finish line of this journey, only to embark on another one provided that I trust in God's guidance. I will continue to look towards the heavens, searching for my purpose, so that I may perform God's work until the day I obtain God's heavenly approval.

With much admiration, I dedicate my thesis to my parents, Kirk and Vivian Tisdale for loving me unconditionally and for supporting all of my life adventures.

To my Daddy, a quiet man yet the rock of the family, who provided the foundation on which I stand.

To my Mommy, a woman of many words and actions, who always knows how to light my fire.

Thank you, for introducing me to God.

Thank you for disciplining and encouraging me.

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ABSTRACT

The impact of tumor associated stromal cells in tumor formation, progression, and response to therapy has led to a paradigm shift in cancer therapy. For many years, tumors were considered as a mass consisting only of actively proliferating cancer cells and therapies were design to target these cells. However, research in the last two decades has shown that tumors are not only comprised of a heterogeneous population of neoplastic cells, but they are also comprised of and infiltrated by a heterogeneous population of non-tumor cells that contribute to tumor progression and potentially affect the efficacy of tumor-directed chemotherapies. Tumors are now considered as complex organ consisting of both cancerous and noncancerous cells interacting with each other within the tumor microenvironment to promote malignancy.

For decades, 5- fluorouracil (5-FU), an inhibitor of the enzyme thymidylate synthase (TS), has been used in the clinical management of colorectal cancer. Although it has been beneficial to some patients, its use has been severely limited by cytotoxic side effects due to its lack of specificity, affecting both rapidly proliferating cancer and healthy normal cells, and acquired resistance by cancer cells over time. To circumvent these limitations, we propose that genetic modulation of non-tumor or stromal cells in the microenvironment might enhance drug efficacy and reduce drug-induced cytotoxicity.

Genetic modulation of stromal cells may be accomplished by utilizing ribonucleic acid interference (RNAi) technologies. With RNAi, we were able to suppress intracellular protein levels of TS prior to therapy and chemo-sensitized cells to TS inhibitors. *In vivo*, we found that the use of constitutive H1 promoter RNAi vector systems to sensitize hematopoietic stem cells in $Apc^{Min/+}$ mice, was problematic and toxic. However, using a mCMV constitutive RNAi vector which was not toxic in an *in vivo* model system, we found that we can sensitize stromal cells to the cytotoxic effects of 5-FU. To specifically direct chemo-sensitivity to 5-FU to cells in the tumor microenvironment we utilized the promoter of osteopontin, a gene that was only upregulated in hematopoietic cells in the tumor microenvironment, to drive the expression of a TS silencing shRNA. This allowed us to enhance chemo-sensitivity to 5-FU specifically within the tumor stroma.

In addition to enhancing the efficacy of TS inhibitors by modulating bone marrow derived cells, we also targeted mast cells by incorporating a pharmacological drug, Cromolyn that inhibits mast cells function, and combined this with 5-FU therapy. We had previously found that mast cells are resistant to 5-FU and are recruited into the tumor microenvironment upon systemic treatment of ApcMin/+ mice with 5-FU. We hypothesized that inhibition of mast cells in combination with 5-FU treatment might enhance anti-tumor efficacy of 5-FU or block the recurrence of tumors post-therapy. The results showed that administration of Cromolyn after systemic treatment with 5-FU decreased recurrence of tumor post therapy. These findings suggest that modulation of the tumor stromal cells by genetic modification or by pharmacological methods have

the potential to enhance the anti-tumor efficacy of TS inhibitors. It will be necessary to assess the long-term effects and the mechanistic underpinnings of these studies to realize their clinical utility.

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LIST OF ABBREVIATIONS

5-FU 5-Fluorouracil

APC ..... Adenomatous polyposis coli

BMDCs ..... Bone marrow derived cells

CAFs ..... Cancer associated fibroblasts

CH_2THF ..... 5, 10- methylenetetrahydrofolate

CHL ..... Chinese lung hamster cells

CPA3 ..... Carboxypeptidase

CRC ..... Colorectal Cancer

DAPI ..... 4', 6-diamididino-2-phenylindole

dTDP ..... 2' deoxythimdine – 5'diphosphate

dTMP ..... 2' deoxythymidine- 5' monophosphate

dTTP ..... 2' deoxythmidine- 5' triphosphate

dUMP ..... 2' deoxyuridine- 5' monophosphate

dUTP ..... Deoxyuridine triphosphate

ECM ..... Extracellular matrix

EGF ..... Epidermal growth factor

eGFP ..... Enhanced green fluorescent protein

FAP ..... Familiar adenomatous polyposis

FAT ..... Folinic acid, aminopterin, thymidine

FBS ..... Fetal Bovine Serum

FcεRI ..... FC epsilon RI

FdUMP ..... Fluorodeoxyuridine monophosphate

FGF ..... Fibroblast growth factor

HAT ..... Hypoxanthine, aminopterin, thymidine

HGF ..... Hepatocyte growth factor

HGPRT ..... Hypoxanthine-guanine phosphoribosyl transferase

HSCs ..... Hematopoietic stem cells

hTS ..... Human TS gene

i.p. ..... Intraperitoneal

IgE ..... Immunoglobulin E

IL-17 ..... Interleukin 17

IL-1β ..... Interleukin 1 beta

IL-3. ..... Interleukin 3

IL-6 ..... Interleukin 6

INF-γ ..... Interferon gamma

LPS ..... Lipopolysaccride

LV ..... Leucovorin

MDSCs ..... Myeloid derived suppressor cells

MMP9 ..... Matrix metallopeptidase-9

MSCs ..... Mesenchymal stem cells

MSCV ..... Murine stem cell virus

MusTS ..... *Mus Musculus* thymidylate synthase

OPN ..... Osteopontin

OPTecTS ..... Optimized Escherichia coli TS gene

PBS ..... Phosphate buffer solution

RISC..... RNA-induced silencing complex

RNAi..... RNA interference

RTX..... Raltitrexed

SCF.....Stem cell factor

shRNA.....Short hairpin RNA

siRNA. .....Short interference RNA

TAMs.....Tumor associated macrophages

Tc..... Cytotoxic T cells

TGF-β.....Tumor growth factor beta

TME.....Tumor microenvironment

TNF-α.....Tumor necrosis factor

TP53..... Tumor protein p53

Tregs..... T regulatory cells

TS.....Thymidylate synthase

VEGF .....Vascular endothelial growth factor

wtecTS ..... Wild type Escherichia coli TS gene

CHAPTER 1

INTRODUCTION

1.1 COLON CANCER

In 2014, the American Cancer Society estimated that approximately 136,830 individuals were diagnosed with colorectal cancer (CRC) and approximately 50,310 will die from this disease. CRC is the third most diagnosed and the second cause of cancer related deaths in the United States. CRC begins with the development of polyps in the colon or large intestines, with low incidents

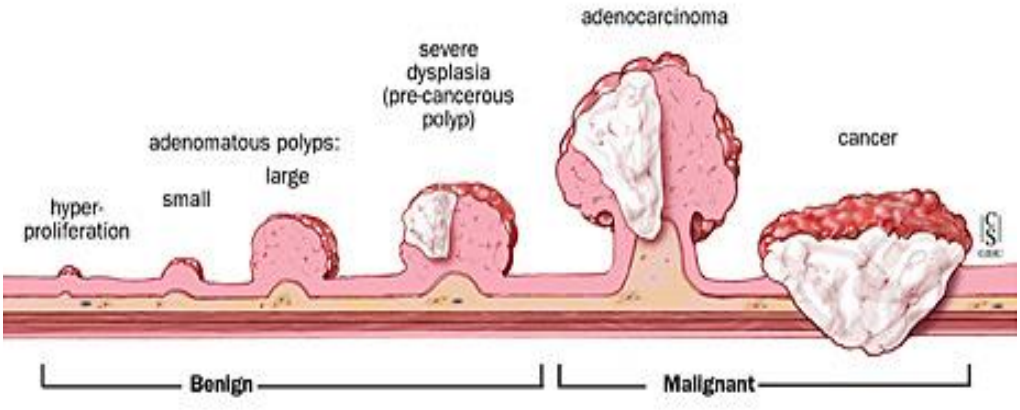


Figure 1.1: **Stages of Colorectal Cancer:** Progression of polyp development into cancer on the lining of the intestinal wall. (Johns Hopkins Cancer Center, Internet)

occurring in the small intestines. Studies have shown that the formation of polyps normally occur in a period of ten to twenty years, during which a non-cancerous

growth forms on the inner lining of the colon or the large intestine (Stryker, Wolff et al. 1987; Winawer and Zauber 2002). These non-cancerous adenomatous polyps are likely to become cancerous upon continuous growth, developing into an adenocarcinoma, a malignant tumor of glandular cells (Figure 1.1). The progression from adenomatous polyps to adenocarcinomas is the result of mutations, which occur in tumor suppressor genes such as the adenomatous polyposis coli (*APC*) and tumor protein p53 (*TP53*). Mutation of the *APC* gene occurs in the early stage of CRC development, and is responsible for familial adenomatous polyposis (FAP), an inherited form of CRC (Jasperson, Tuohy et al. 2010). The *APC* gene encodes a multifunctional protein that regulates the expression of β-catenin in the Wnt signaling pathway, cell adhesion, proliferation, and apoptotic pathways (Coppede, Lopomo et al. 2014). Typically, the *APC* protein forms a cytoplasmic complex with other proteins in the Wnt signaling pathway. This complex targets β-catenin by ubiquitination for proteasome degradation keeping its intracellular levels low. Conversely, loss of *APC* function causes accumulation of β-catenin in the cytoplasm, and its translocation into the nucleus where it assists in the activation of genes essential for cell proliferation, resulting in abnormal growth and adenomatous polyps.

Mutations in TP53 typically occur after the *APC* mutation, as the polyp continues to grow and become cancerous. TP53 is a nuclear protein that initiates cell cycle arrest to initiate repair or apoptosis in response to DNA damage (Vogelstein, Lane et al. 2000). Loss of TP53 function, which is critical for sensing and repairing damaged DNA, allows cancerous cells to escape the cell cycle

checkpoint and proceed with unregulated cell division to produce daughter cells with mutated DNA. This mutation promotes the transition from an adenomatous polyp to an adenocarcinoma.

Approximately 96% of colorectal cancers are adenocarcinomas (Stewart, Wike et al. 2006). Common symptoms include bleeding from the rectum, discomfort in the lower abdomen, constipation and diarrhea, and loss of appetite. Fortunately, early screening via a colonoscopy allows visual examination of the colon so that adenomas or adenocarcinomas can be excised from the colon and large intestine. Although the advancement of colonoscopies has increased CRC survival (Edwards, Ward et al. 2010), there is room for improvement to decrease of CRC related deaths. To date, the 5-year survival rate is approximately 89.9% for localized CRC, 70.5% when CRC spreads to regional lymph nodes, and 12.9% if CRC has metastasized to distant organs (Surveillance, Epidemiology, and the End Results (SEER) Program). The majority of CRC related deaths result from metastatic CRC cells entering into the lymph nodes and blood vessels, and spreading to distant organs such as the liver and/or lungs. Current treatments are not efficient in blocking this process. In addition, contributing to the inadequacies of our anti-cancer treatments is the reoccurrence of the cancer with more aggressive properties, or the development of resistance against chemotherapy, such as 5-Fluorouracil (5-FU) that is commonly used to treat CRC. Thus, in order to combat and eliminate this disease, it is important to develop new anti-cancer therapies or utilize current anti-cancer therapies in

combination with novel therapies to enhance their efficacy while minimizing their toxicities.

1.2 HALLMARKS OF CANCER

In order develop effective anti-cancer therapies and to resolve the problematic side effects resulting from their lack of specificity for cancer cells, it is important to understand the complexity of cancer development. Currently, eight properties define the hallmark of cancer cells. These properties provide a framework for the development of novel therapies. These hallmark properties include: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction (Hanahan and Weinberg 2011). The unregulated production of growth signals provides cancer cells the ability to acquire the hallmark of sustained proliferative signaling. This causes an imbalance in homeostasis, wherein cancer cells uncontrollably produce growth factors for themselves. However, it is not sufficient for cancer cells sustain proliferation because cellular checkpoints within cell division can inhibit the continuous growth necessary to develop a tumor mass. Thus, obtaining the ability to evade the regulation of growth suppressors, through mechanisms such as TP53 is necessary for tumor progression. Growth suppressors negatively regulate cell proliferation contingent on DNA abnormalities and the lack of normalcy due to stress caused by

nucleotide, oxygen, and energy levels within or outside the cell. Mutations in growth suppressors disrupt induced apoptosis.

Alternatively, the cancer cell's quest for survival may also increase the expression of anti-apoptotic regulators that contribute to resistance to apoptosis. Under normal conditions, the balance of pro- and anti- apoptotic proteins regulates programmed cell death. The abnormalities in cancer cells creates imbalance in favor of inhibiting the trigger of death. Resisting cell death does not guarantee cancer cells the ability for unlimited proliferation, but obtaining the hallmark of immortality does. Immortality is achieved by up regulating the expression of telomerase, an enzyme responsible for elongating telomeres, resulting in extended protection of chromosomal DNA. With each cell division, the telomere length decreases, until it reaches a point at which the cell goes into a silencing phrase or program cell death. Hence, immortality ensures the tumors capability to expand. However, as the tumor expands it must ensure the ability to sustain a healthy environment to maintain its growth. Tumor progression requires sources of energy, the import of nutrients, and the export of waste, which is accomplished by increasing energy metabolism and inducing angiogenesis. The newly discovered hallmark of reprogramming energy metabolism allows cancer cells to survive by up regulating the transport of glucose into the cell where it is broken down to generate energy and metabolites to compensate for the increase in cell growth and division. Angiogenesis is the process of developing new blood vessels from existing vessels that allow the import and export of the necessary nutrients and waste. The ability to induce angiogenesis sustains the growth of the

tumor in a localized region; however, the ability to travel to another organ requires the activation of invasion and metastatic characteristics. Normally aggressive cancers alter the expression of cell-to-cell adhesion molecules; giving cancer cells the capacity to invade nearby blood vessels in an attempt to spread to a distal organ. The accomplishment of metastasizing to a distal organ is contingent on the cancer cell's ability to detached from the primary tumor, survive in circulation, extravagate and adapt to the microenvironment of the distal organ to survive. Finally, the property of evading immune surveillance is a significant hallmark that allows cancer cells to avoid destruction by the immune system, which served to eliminate rogue cells. Delineation of these hallmark properties has provided clarity to cancer development and its capability to resist therapies. Cancer research has evolved from the perspective of exclusively targeting and understanding the development of neoplastic cells, to the forefront of studying the intricate relationship that develops between both neoplastic and non-neoplastic cells derived from bone marrow to create the tumor microenvironment (TME).

1.3 TUMOR MICROENVIROMENT

Since the recognition of the TME, a paradigm shift in cancer therapy has occurred, such that the tumor is perceived to be an organ or complex tissue (Egeblad, Nakasone et al. 2010), composed of a heterogeneous populations of both malignant and non-malignant cells and extracellular matrix, which function abnormally to promote and sustain a pro-tumorigenic environment, i.e. the

hallmarks of cancer (Coussens and Werb 2002; Chantrain, Feron et al. 2008; Whiteside 2008; Hanna, Quick et al. 2009; Peddareddigari, Wang et al. 2010; Hanahan and Coussens 2012). This heterogeneous population of non-malignant cells within a tumor includes, but is not limited to the following cells: tumor associated macrophages (TAMs); myeloid derived suppressor cells (MDSCs),

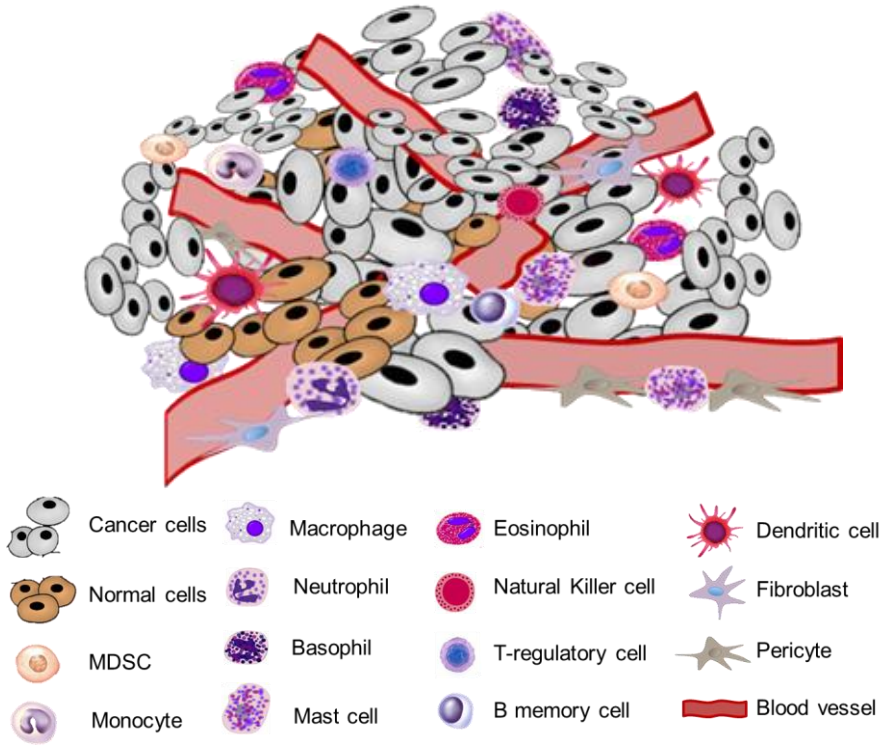


Figure 1.2: **Tumor Microenvironment**: Infiltration of bone marrow derived cells into the tumor microenvironment (TME). The tumor stroma is comprised of many immune cells that influence the tumor response to anti-cancer therapies.

mast cells, and cancer associate fibroblasts (CAFs), monocytes, endothelial cells, and neutrophils (Figure 1.2).The TME has the potential to activate TAMs into two subgroups: M_1 (anti-tumor) or M_2 (pro-tumor) macrophages (Gordon

2003; Mantovani, Sica et al. 2007). MDSCs have been shown to cooperate in the non-responsiveness of T-cells to promote tumorigenesis (Nagaraj and Gabrilovich 2008). Mast cells exhibit the ability to secrete many pro-tumorigenic growth stimulatory factors, which can trigger the angiogenic switch to turn on angiogenesis (Coussens, Raymond et al. 1999). In response to tumor growth, CAFs are secrete a host of growth factors such as the epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and hepatocyte growth factor (HGF) (Kalluri and Zeisberg 2006). These non-cancerous cells provide a pro-inflammatory environment by secreting cytokines, chemokines, and growth factors essential for the initiation and maintenance of tumor growth. In addition to the infiltration of these heterogeneous population of cells within the tumor, the composition of the extracellular matrix, for example, laminin, collagen, fibronectin, and proteoglycans can promote tumor progression (Peddareddigari, Wang et al. 2010) and provide resistance to drug therapies due to abnormal tissue organization that provides a hindrance to drug delivery (Egeblad, Nakasone et al. 2010). For example, type 1collagen plays a significant role in regulating tumor sensitivity to various anti-neoplastic therapies (Loeffler, Kruger et al. 2006).

These examples represent minuscule information on the intricate relationship between cancer and non-cancerous cells. These studies confirm that the neoplastic cells are not solely acting alone, but they collaborate and co-evolve with various immune cells to orchestrate tumor growth and development. These tumorigenic properties increase as the tumor matures causing

morphological changes to occur within the TME to become supportive. Hence advanced stages of cancer exhibit altered tumorigenic properties in comparison to the early stages of cancer. The roles of these immune cells need intense investigation, to have a better understanding of the supportive role of the TME at the different stages of tumor development. In addition, this will contribute to the knowledge of how the TME provides protection against chemotherapies, providing insight into the development of more effective ways to target the tumor.

1.4 THE ROLE OF MAST CELLS IN TUMOR BIOLOGY

As previously described, the TME consists of bone marrow derived cells that contribute to tumor formation and potentially play a vital role in resistance to current chemotherapies. In particular, there is a growing interest in the role of mast cells in cancer development, and recent studies have identified functions of these cells in tumor biology. Historically, mast cells are typically associated with immunoglobulin E (IgE)-mediated response to an allergic reaction or an asthmatic incident. The IgE mediated response causes the activation of mast cell degranulation, which results in the crosslinking of antigens and IgE molecules that are bound to the high affinity receptors, FC epsilon RI (FcεRI) located on the cell surface of mast cells (Gould, Sutton et al. 2003). This immune response provokes inflammation resulting in recruitment and activation of additional immune cells to the local site. Thus, mast cells are effectors cells that exert immune signals to regulate and recruit other immune cells. These granulocytes cells are subdivided into two subgroups; mucosal and connective mast cells. The

differences between the subgroups is dependent the content of granules, which occurs during maturation. Mast cell precursors are released from the bone marrow as immature cells and migrate into various tissues within the body to undergo maturation and differentiation. Maturation and differentiation influenced by the infiltrated tissue environment resulting in a heterogeneous population of mast cells throughout the body. Mast cell granules have three subgroups: pre-formed granules, *de novo* synthesized granules, and cytokines and chemokines. The pre-formed granules include histamine, heparin, and the proteases tryptase, chymase, and carboxypeptidase A3 (CPA3). The second group is the *de novo* synthesized lipid mediators, which includes: LTC_4 and PGD_2, and the third category consists of the various cytokines and chemokines, such as but not limited to tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and matrix metallopeptidase-9 (MMP9) (Theoharides, Kempuraj et al. 2007). Upon degranulation, the pre-formed granule mediators are immediately release within minutes; while the de novo synthesized mediators are released slowly within a period of three to twelve hours later. In addition, the mechanism for releasing the mediators can occur by either complete or selective degranulation (Dvorak and Kissell 1991; Theoharides and Kalogeromitros 2006). The role of mast cells in tumor formation and immunity is poorly understood, and accumulating evidence has shown controversial roles in tumor biology. Mast cells can either display anti-tumor or pro-tumor responses depending on the type of cancer and the stage of the tumor

development (Groot Kormelink, Abudukelimu et al. 2009; Ribatti 2013; Oldford and Marshall 2014).

Mast cells have been shown to mediate anti-tumor response to cancer in *in vivo* model systems. In *Apc*$^{Min/+}$mice, a model of colon carcinogenesis, genetic ablation of mast cells resulted in larger and increased number of intestinal tumors compared to wild type littermates, suggesting that mast cells can suppress intestinal tumor development (Sinnamon, Carter et al. 2008). In addition, several studies suggest that IgE mediated activation of mast cells within tumor results in an anti-tumor immune response (Jensen-Jarolim, Achatz et al. 2008; Teo, Utz et al. 2012; Singer and Jensen-Jarolim 2014). Mast cells can also regulate the activity of immune cells. For example, mast cells can promote recruitment of MDSCs into the tumor and activate their expression of IL-17, which in turn mobilizes and suppresses the pro-tumorigenic activity of T-regulatory cells within the tumor. Collectively, these studies suggest that mast cells induce anti-tumor responses and play a role in promoting inflammation to recruit additional leukocytes into the tumor region where they activate or regulate immune cell activity and induce cytotoxic activity.

Alternatively, mast cells can display a pro-tumorigenic response. Studies have shown that pro-inflammatory mediators from mast cells can promote tumor proliferation and provide a suitable environment to sustain tumor growth and development. Gounaris and colleagues showed that mast cells for polyp development in *Apc*$^{Min/+}$mice (Gounaris, Erdman et al. 2007). In addition poor prognosis has been correlated to high tumor infiltration of mast in many human

cancers such as melanoma, lung, gastric, colorectal, and prostate carcinomas (Groot Kormelink, Abudukelimu et al. 2009). An in vitro study utilizing the human mast cell line, LAD2 demonstrated the capacity of mast cells to regulate the invasiveness of cervical cancer cells, yet cervical cancer cells were able to stimulate the degranulation of mast cells, suggesting an intimate communication between tumor and inflammatory immune cells (Rudolph, Boza et al. 2008). Another study using a HPV16 transgenic mouse model of squamous cell epithelial carcinoma showed that chymase released from tumor infiltrating mast cells activated MMP9 resulting in extracellular remodeling (Coussens, Raymond et al. 1999). This study also demonstrated that mast cells infiltrated tumors prior to the angiogenic switch. To validate the role of mast cells in angiogenesis and tumor remodeling, another study using a Myc-induced pancreatic islet tumor model showed infiltration of mast cells within twenty-four hours of Myc activation. The recruitment of mast cells resulted in the expansion of Myc-induced tumors (Soucek, Lawlor et al. 2007). Together, these studies show that mast cells perform pro-tumorigenic roles by contributing to tumor remodeling, angiogenesis, and immune suppression.

The pro-tumorigenic roles of mast cells affect the efficacy of anti-cancer therapies by contributing to tumor progression and assisting in tumor remodeling and tumor sustainability. Currently, the efficacy of anti-cancer therapies is dependent on its direct deliverable contact with cancer cells within the tumor where its cytotoxic action can destroy or reduce tumor burden. However, tumor remodeling can inhibit delivery of anti-cancer therapies to promote relapse. It is

important to decipher the pro-tumorigenic roles of mast cells to develop strategies to enhance the efficacy of anti-cancer therapies.

1.5 THYMIDYLATE SYNTHASE AND ITS INHIBITOR 5-FU

Thymidylate synthase (TS) is an enzyme that catalyzes the reductive methylation of 2' deoxyuridine- 5' monophosphate (dUMP) to 2' deoxythymidine-5'-monophosphate (dTMP) and dihydrofolate using 5,10-methylenetetrahydro-folate (CH_2THF) as a methyl donor (Danenberg 1977; Carreras and Santi 1995). Subsequently, dTMP is sequentially phosphorylated into 2'-deoxythimdine–5'diphosphate (dTDP), and then into 2' deoxythmidine- 5' triphosphate (dTTP), an essential component for DNA replication and repair in actively proliferating cells. Importantly, this pathway provides the sole intracellular source of dTTP, thus, TS has been an important target of chemotherapeutic agents. In addition to its catalytic function, TS is an auto-regulatory protein that binds to its mRNA to repress its translation and has been shown to interact with the mRNAs of TP53 and c-myc (Chu and Allegra 1996). TS inhibition results in depletion of dTMP and accumulation of dUMP leading to increased levels of deoxyuridine triphosphate (dUTP) that is mis-incorporated into DNA, resulting in DNA fragmentation and ultimately cell apoptosis (Longley, Harkin et al. 2003; Garg, Henrich et al. 2010).

TS inhibitors are typically substrate analogs or analogs of the folate co-factor. The most widely used TS inhibitor is the 5-FU, a fluoropyrimidine that has been widely used in the clinical management of colon and head and neck cancers. 5-FU is structurally similar to uracil; however, a fluorine atom replaces a hydrogen atom at the C-5 position. After administration, 5-FU is transport into cells, where

it is converted into the powerful TS inhibitor fluorodeoxyuridine monophosphate (FdUMP). FdUMP has the same affinity to TS as dUMP: however, when bound to TS, the fluorine atom at the 5' position cannot be displaced, and it forms a stable ternary complex with TS and 5,10-CH_2THF resulting in TS inhibition. TS inhibitors based on folate analogs include tomudex (raltitrexed), liposomal GW1843U89, and capecitabine (Rose, Farrell et al. 2002).

Expression of TS after exposure to anti- therapy, such as 5-FU has shown to increase proteins levels, a consequence of inhibiting TS activity, thus allowing translation of the mRNA and production of the protein(Van der Wilt, Pinedo et al. 1992).

Although 5-FU has been used for many decades in the clinical management of cancers, its use has been limited by drug induced toxicities and acquired resistance by cancer cells. Cancer resistance to TS inhibitors can occur through a number of mechanisms. TS can be over expressed in tumors due to gene amplification or mutations that may cause resistance. TS auto-regulates its gene expression, by binding to its mRNA. However, upon treatment with TS inhibitors, bounded TS is released from its mRNA allowing its translation, which in turn increases intracellular TS protein levels (Chu and Allegra 1996). The effectiveness of 5-FU depends on the availability of 5,10-CH_2THF. The active metabolite, FdUMP forms an unstable binary complex with the enzyme, resulting in poor inhibition. However, in the presence of 5,10-CH_2THF stabilizes the ternary complex inhibiting the catalytic function of the enzyme (Aherne, Hardcastle et al. 1996). Thus, tumors expressing low levels of 5,10-CH_2THF are

resistant to 5-FU, however, addition of the drug Leucovorin (LV), increases intracellular levels of 5,10-CH_2THF resulting in enhanced sensitivity (Matherly, Czajkowski et al. 1990). Another mechanism of resistance is the salvage pathway which allows for the recovery of free bases, such as thymidine that are byproducts of degrading DNA, to be recycled for nucleotide biosynthesis through the action of the enzyme, thymidine kinase (Kinsella, Smith et al. 1997). The salvage pathway can compensate for the lack of thymidylate production resulting from TS inhibition. Acquired resistance cancan also result from mutations in TP53 wherein damaged DNA resulting from mis-incorporation of FdUTP is unrecognized allowing cells to escape apoptosis (Ahnen, Feigl et al. 1998). On the other hand, cancer cells with wild type TP53 can still be resistant to 5-FU therapy by altering the regulation of the cell cycle. In the presence of 5-FU resistant cells had increased tendency to arrest in G_1 and G_2 phases, allowing DNA repair prior to replication; unlike the parental cell line that had a tendency to arrest in the S phase (De Angelis, Svendsrud et al. 2006). In addition to acquired resistance, 5-FU therapy is limited by drug induced toxicities associated with its lack of specificity, targeting both actively dividing cancer as well as healthy normal cells. Some side effects from 5-FU include alopecia, myelosuppression, and mucositis. Considerable attempts have been made to enhance the effectiveness of 5-FU, while lowering its toxic side effects. These include the combination of 5-FU chemotherapy with targeted therapies. The impact of 5-FU on stromal cells is only beginning to be explored. Targeting both the tumor and tumor microenvironment might enhance our ability to kill colorectal cancer cells.

We hypothesize that stromal cells are also direct targets of 5-FU and that their chemo-sensitivity to 5-FU might determine tumor sensitivity to the therapy. Targeting the microenvironment is genetic modification or pharmacological inhibition of pro-tumorigenic activities of bone marrow derived cells infiltrating the tumor microenvironment, might be viable strategies to enhance anti-cancer the anticancer efficacy of 5-FU.

1.6 PARADIGM SHIFT IN ANTI-CANCER THERAPIES

Targeting both the tumor as well as the tumor stroma is a paradigm shift in our approach to enhance the efficacy of anticancer therapies while enhancing selectivity and minimizing toxicity and relapse. Clinical trials targeting the extracellular matrix (ECM), endothelial cells and pericytes, fibroblasts, and innate immune cells have shown progress in combating cancer (Joyce 2005), however, these strategies are only beginning to be developed. The recognition of tumors as a complex collection of cancer cells interacting and recruiting normal non-cancerous cells has altered our perception of cancer development, which has also alter how we must combat this disease. Traditionally, anti-cancer therapies only targeted actively proliferating cancer cell by surgical removal of the tumor mass that may be combined with radiation or chemotherapy, or a combination of both. These treatments have only been moderately effective. Since the establishment of the critical role of the tumor microenvironment in cancer initiation and progression, new anti-cancer therapies have emerged (Hanna, Quick et al. 2009) that can be harnessed to enhance the efficacy of prominent

existing chemotherapeutic drugs such as 5-FU. Furthermore, targeting stromal cells might provide an opportunity to enhance the specificity of cancer chemotherapies. Thus, combining genetic modulation or pharmacologically inhibition of bone marrow derived cells (BMDCs) within the TME with 5-FU therapy may be beneficial in reducing drug toxicity or the occurrence of relapse.

1.7 GENETIC MODULATION OF BONE MARROW DERIVED CELLS

The plasticity of BMDCs has the potential to constitute a pro-tumorigenic microenvironment for cancer cells. A review by Chantrain proposes three mechanisms by which bone marrow cells support the initiation and progression of cancer: first, through the recruitment of cancer cells to enrich the microenvironment, second, by infiltration into the tumor stoma to suppress immune response, and third, by promoting the development of the pre-metastatic niche (Chantrain, Feron et al. 2008). Numerous studies have demonstrated an array of pro-tumorigenic responses from BMDCs infiltrating the into primary tumor microenvironment permitting tumor growth (Egeblad, Ewald et al. 2008; Roorda, ter Elst et al. 2009; Fowler, Mundy et al. 2012; Kidd, Spaeth et al. 2012; Srivastava, Andersson et al. 2012; Zou, Zheng et al. 2012). Tumors have suppressed immune responses to recruit and activate MDSCs, which have been associated with malignancy and poor prognosis (Srivastava, Andersson et al. 2012). In addition, BMDCs can influence the development of the vasculature of

the tumor tissue, hampering the administration and reducing the effects of anticancer drugs (Mizukami, Sasajima et al. 2012). The ability of BMDC to circumvent immune response and reduce efficacy of anti-neoplastic drugs is the subject of ongoing investigation. It is important to understand the role of BMDCs in tumor stoma to determine if genetic modulation of these cells can enhance efficacy of antitumor drugs. Transplantation of genetically modificed BMDCs may be used to alter gene expression in stromal cells to enhance tumor response to pre-existing therapies. For example, the knockdown of the Wnt signaling inhibitor Dickkopf1(DKK1) in bone marrow derive mesenchymal stem cells (MSCs) repressed myeloma development in mice (Fowler, Mundy et al. 2012), and MSCs engineered to express lymphotoxin induced tumor regression (Zou, Zheng et al. 2012). Genetic modification of BMDCs might be an effective tool to enhance the efficacy of anti-neoplastic therapies since they can influence tumor resonse to therapy by their ability to suppress immune responses.

1.8 PHARMACOLOGICAL INHIBITION OF BMDCs

An alternative to targeting the TME is by inhibiting the pro-tumorigenic activities of BMDCs, such as mast cells, pharmacologically. Mast cells have been shown to play critical roles in tumor initiation, progression, and response to therapy. Thus, effective anti-cancer therapies may need to target mast cells in addition to cancer cells. Several studies have used the pharmacological stabilizer, sodium cromoglicate (Cromolyn), a drug known to inhibit mast cell degranulation. Initially Cromolyn was used to treat acute inflammation which occurs during an asthma attack (Altounyan 1975). However, recent studies have

shown a reduction in tumor burden mice after administration of Cromolyn (Gounaris, Erdman et al. 2007; Soucek, Lawlor et al. 2007). Based on these results, we have considered the possibility that Cromolyn might play a synergistic role in anti-cancer therapy. However, the mechanism by which Cromolyn inhibits mast cells is not fully understood. It is thought to stiffen the cellular membrane, which indirectly inhibits the influx of chloride into the cell that in turn inhibits the ability of the cell ability to trigger.

1.9 EXPERIMENTAL MODELS

A. In-vitro cell model

Mus musculus thymidylate synthase (MusTS) was stably transfected into a TS-deficient Chinese hamster lung (CHL) cell line RJK88.13 (Nussbaum, Walmsley et al. 1985). Stable transfectants were selected in media lacking thymidine in the presence of the nucleoside transport inhibitor dipyridamole. Stably transfected cells were grown in mass culture. The CHL(MusTS) cell line was used to target TS specifically to assess the sensitizing effects of diminished TS expression in response to 5-FU therapy.

B. Transgenic mouse model

The C57BL/6J-*Apc*$^{Min/+}$ mouse has been used as an *in vivo* model for the initiation and progression of intestinal adenomas. These mice are heterozygous for a mutant allele of the APC tumor suppressor gene, which genetically predisposes them to the spontaneous development of adenomas within the

intestinal tract (Moser, Pitot et al. 1990). Tumors develop in these mice at an early stage due to the loss of the remaining wild-type APC allele, which leads to insufficient expression of APC (Powell, Zilz et al. 1992; Su, Kinzler et al. 1992). The APC mutation is similar to that the germline mutation found in the human hereditary form of colorectal cancer, familial adenomatous polyposis (FAP) (Levy, Smith et al. 1994). Although FAP accounts for less than 2% of colorectal cancer incidence, mutations in the APC gene have accounted for 80% of sporadic colon tumors (Kinzler and Vogelstein 1996). Most of the adenomas that develop in these mice are located in the small intestines with a few developing in the colon, while in humans tumors develop exclusively in the colon. Nevertheless, the *Apc*$^{Min/+}$mouse model is useful for understanding the initiation of tumor development and progression, and is a useful tool for developing or examining the effects of therapeutic agents *in vivo*.

C. Syngeneic mouse model

The transplantable syngeneic CT26 tumor model in Balb/c mice is another model to examine the role of microenvironment in tumor response to 5-FU therapy. CT26 colon adenocarcinoma cells are injected into the flank of Balb/c mice where tumors are allowed to develop prior to therapy. CT26 cells were generated in Balb/c by treatment with the carcinogen N-nitroso-N-methylurethane-(NNMU) where they were isolated and to grow *in vitro* (Brattain, Strobel-Stevens et al. 1980). The tumorigenic property of this cell line was sustained when transplanted into Balb/c mice, thus providing the ability to assess tumor growth

rates. By using this model, it allows for rapid tumor growth, which can be excised to quantify BMDC infiltration and expression of mRNA levels within the tumor. Thus, this syngeneic mouse model is practical for studying the response of BMDCs after exposure to different combinations of chemotherapeutic agents.

1.10 REVIEW OF LITERATURE (GENETIC MODULATION OF BMDCs)

A. BMDCs infiltrate the intestinal tumor microenvironment in *Apc*$^{Min/+}$mice.

In previous studies, Davis et al. showed that bone marrow can be successfully transplanted and engrafted into *Apc*$^{Min/+}$ mice. Prior to transplantation, the recipient *Apc*$^{Min/+}$ mice were lethally irradiated with 950 rads to deplete existing hematopoietic system. Age matched donor marrow from C57BL/6-UB1-eGFP mice expressing the enhanced green fluorescent protein (eGFP) under the control of the human ubiquitin promoter was extracted from the femur and tibia. The bone marrow is directly transplanted into recipient mice, or grown in culture and fractionated to obtain hematopoietic stem cells (HSCs). Transplantation of whole marrow or purified HSCs was performed by tail vein injection. Full engraftment occurs within four to six weeks after transplantation. Tissue sections were collected at eight weeks post transplantation to examine the engraftment of donor eGFP bone marrow by confocal microscopy. The results showed that donor derived eGFP marrow infiltrated both normal and tumor regions of the small intestine and colon. Infiltration of the eGFP bone

marrow was greater in tumor regions (75-80%) within the small intestines and colon as compared to normal, non-tumor regions (10%) (Davis, Price et al. 2011). These results suggest that genetically modified bone marrow derived cells transplanted into *Apc*$^{Min/+}$ mice will successfully infiltrate the tumor regions of the intestines and colon.

B. BMDCs expressing E. coli TS exhibits resistance to TS inhibitors.

Previous studies have demonstrated that the an Escherichia coli TS gene whose codon usage has been optimized for expression in mammalian cells (OPTecTS) can confer high levels of resistance to TS inhibitors (Fantz, Shaw et al. 2000; Shaw, Berger et al. 2001). Shaw et al. optimized the *E. coli* TS gene by introducing mutations in the cDNA resulting in stabilization of the protein in mammalian cells. OPTecTS was cloned into a Harvey murine sarcoma-based retroviral vector and stably transfected into a TS-deficient CHL cell line (Nussbaum, Walmsley et al. 1985); wild type ecTS (wtecTS) and humanTS (hTS) were used as controls. Stably transfected cells expressing OPTecTS and the controls were treated with various concentrations of the TS inhibitor, raltitrexed (RTX) in the presence of dipyridamole to determine the concentration that inhibited the growth of fifty percent of the treated cells (IC_{50}). The results showed that cells transfected with OPTecTs were 11-fold more resistant to RTX than cells expressing hTS while cells expressing wtecTS were slightly more resistant compared to cells expressing hTS. In addition, result showed greater than 75% and greater than 95% of bone marrow cells transduced with OPTecTS were

resistant to the TS inhibitors RTX and U89 respectively, at concentrations where 100% of non-transduced cells were inhibited.

C. The effects of chemo-resistant marrow on tumor response to TS Inhibitors.

In previous studies, chemo-resistant marrow expressing the OPTecTS was transplanted into *Apc*$^{Min/+}$ mice. Retroviral vectors based on the murine stem cell virus (MSCV) promoter were constructed to express OPTecTS, while the MSCV empty vector was used as controls. Lethally irradiated *Apc*$^{Min/+}$mice were reconstituted with chemo-resistant (OPTecTS) or control BMDCs, and and then subjected to systemic treatment of a combination therapy of 5-FU and RTX or PBS as control treatment. After the treated mice were sacrificed, intestinal tissues were isolated and the tumor burden was determined. The results showed that in mice transplanted with mock transduced marrow, systemic treatment with 5-FU caused a statistically significant decrease in tumor burden by approximately 75% from 32±19 (PBS) to 8±5 (5-FU/RTX) (p=0.008). In contrast, in mice transplanted with drug resistant marrow expressing OPTecTS, tumors were not as responsive to the therapy exhibiting a statistically insignificant reduction in tumor burden from 35±22 (PBS) to 19±23 (5-FU/RTX) (p=0.183) (Pena et al, unpublished data). These results showed that genetically modified chemo-resistant bone marrow does influence tumor response to chemotherapy. These preliminary data provides the basis for our working hypothesis that (1) tumor response to chemotherapy is influenced by the sensitivity of tumor associated

BMDCs and (2) it is possible that BMDCs can be genetically modified to chemo-sensitize both BMDCs and tumors to chemotherapy.

1.11 REVIEW OF LITERATURE (INHIBITION OF BMDCs)

A. Effect of systemic 5-FU therapy on tumor burden in *Apc*$^{Min/+}$ mice

Tucker et. al. subjected *Apc*$^{Min/+}$ mice to systemic treatment consisting of three cycles of 40 mg/kg 5-FU administered intraperitoneal *(i.p.)* daily for five consecutive days (Tucker, Davis et al. 2002). Mice were either immediately sacrificed after the 5-FU regimen, or allowed to recover for 6-weeks post therapy. At the end of the treatment regimens, intestinal tissue was removed and tumor burden was assessed. The results showed that 5-FU reduced tumor numbers by approximately 50% ($p<0.001$) compared to age matched control groups treated with PBS. In addition, weekly blood samples were collected to assess the effect of 5-FU on leukocytes. The results showed a reduction in leukocyte counts after exposure to 5-FU, and a return to normal or higher levels upon cessation of 5-FU therapy. When mice were allowed to recover for six weeks post therapy, there was no significant difference in tumor numbers between mice treated with PBS or 5-FU. This suggested that 5-FU therapy was only cytostatic to the tumor cells and after removal of therapy; the tumors relapsed to the same level as that in untreated mice. This study showed that 5-FU can inhibit tumor growth, but it also showed that it can affect the leukocyte population in tumors and systemically in mice. The increase of leukocytes post

therapy, particularly in the tumor stroma may contribute to the growth of tumors post therapy. Thus, it is important to identify and further investigate the full impact of 5-FU not just on leukocytes but also on other BMDCs in the microenvironment such as mast cells, to determine their effect on tumor response to therapy and recurrence post therapy.

B. Examining the effects of 5-FU on mast cells and precursors

A previous study from our lab examines the effects of 5-FU on subsets of BMDCs, including mast cells, T regulatory (Treg) cells, and MDSCs in the tumor stroma in the *Apc*$^{Min/+}$ mouse (Acharya, G., et.al, unpublished data). Briefly, 14-15 week old mice were injected *i.p.* with a single dose of 50 mg/kg 5-FU. The mice sacrificed at 3,5,7,9 and 12 days post therapy. Tumors, spleen, and bone marrow were collected, digested to create a single cell suspension and the infiltrating BMDCs were quantified by flow cytometry. The results showed that MDSCs were selectively sensitive to 5-FU while mast cells were resistant and migrated to the tumor stroma in response to 5-FU while the Tregs were only mildly sensitive to 5-FU. Analysis of apoptotic and proliferative indices of mast cells and MDSCs showed that in mast cells, these indices were unaffected by treatment with 5-FU. At 5 days post therapy, the number of mast cells infiltrating the tumor stroma increased; this was accompanied by a corresponding decrease in mast cell population in the spleen, suggesting that mast cells might migrate from the spleen to the tumor stroma in response to 5-FU and molecular signals from the residual tumor. In contrast, MDSCs had a high proliferative index that

most likely rendered them vulnerable to 5-FU. The number of MDSCs decreases by three-fold within 5 days of 5-FU administration in tumor stroma, spleen, and bone marrow. However, they returned to pre-treatment levels within 10 days.

In another study, the effect of 5-FU on mast cell precursors was determined in 8-week old C57BL/6 mice (Ophir, Berenshtein et al. 1993). Mice were injected intravenously once with 0–150 mg/kg 5-FU. BMDCs were collected at 2 and 4 days after injection and cultured in enriched media for 14 days to stimulate the proliferation of mast cell precursors. Toluidine and alcian blue staining identified mast cells. In response to 50 mg/kg the sub lethal dose of 150 mg/kg 5-FU, BMDCs retrieved on day 2 after 5-FU injection yielded 12.2±0.9% and 2.6±0.3% mast cell precursors, while day 4 yielded greater than 80% and 60% mast cell precursors, respectively. Normal levels of mast cells were observed by day 8 for the sub lethal dose. Data from the sub lethal dose indicated that mast precursors were continuously activated or cycling after 5-FU injection. Thus, there was gradual replacement, instead of recovery of mast cells in the bone marrow compartment. These preliminary data collectively provides evidence which support our working hypothesis that (1) tumor response to chemotherapy may be influenced by the sensitivity of tumor associated mast cells, and may interfere with tumor development, (2) effect of 5-FU on mast cells may play a synergistic role to enhance its anti-tumor efficacy, or (3) inhibition of mast cells may prolong tumor recurrence.

1.12 RESEARCH GOAL

The preliminary data showed that tumor response to TS inhibitors was influenced by the sensitivity of tumor associated BMDCs to the therapy. Genetic modification of BMDCs to make them resistant to TS inhibitors also made tumors resistant to the therapy. The goal of this project is to develop a therapeutic strategy to target both cancer cells and non-cancer tumor stromal cells to enhance the efficacy of TS inhibitors. I will examine the ability of reducing intracellular TS levels by utilizing small RNA interference technologies against TS to enhance sensitivity to TS inhibitors. Genetically modified BMDCs that are sensitized to TS inhibitors are predicted to enhance the cytotoxic effects of 5-FU within the tumor upon infiltrating the TME while protecting normal non-cancerous cells from drug-induced toxicity.

In addition, I will test the hypothesis that pharmacological inhibition of mast cell activity will also inhibit their pro-tumorigenic role within the TME will act synergistically with 5-FU as well as prolong tumor relapse post therapy. My overall goal is to target the TME to enhance the cytotoxicity of 5-FU to cancer cells and to reduce tumor recurrence post therapy.

1.13 RESEARCH INNOVATION

The realization that tumor stromal cells have profound influence on tumor behavior and tumor response to anti-neoplastic therapies is leading to a paradigm shift in cancer therapy. These findings suggest that effective therapies should also target these cells in addition to tumor cells. I will explore the

possibility that altering the sensitivity of the cells in the microenvironment will also alter tumor response to therapy. I will use the Osteopontin (OPN) promoter that is primarily upregulated and highly activated within tumor stromal cells to drive the expression of a TS shRNA sequence to sensitize the infiltrating BMDCs and the tumor prior to chemotherapy. The use of a promoter that is only activated in the tumor stroma, will not only enhance tumor sensitivity to the therapy but will also specifically direct tumor sensitivity to tumor cells while reducing toxicity to normal cells. This strategy to enhance the efficacy and the specificity of anticancer therapies can be applied not just too intestinal tumors but to other classes of therapeutic agents in different types of tumors.

In addition, to modifying BMDCs I will use a drug known to specifically inhibit mast cell degranulation during and post post 5-FU therapy to determine if mast cells inhibition can synergize with 5-FU anticancer efficacy or prolong tumor recurrence of tumor after chemotherapy. The novelty of inhibiting mast cells is the utilization of the drug Cromolyn that has been used in treating asthma and is readily available on the market. Even though this drug has displayed success in reduction of various tumors, the combination of Cromolyn and 5-FU needs further investigation.

CHAPTER 2

DEVELOPING STRATEGIES TO INCREASE CHEMO-SENSITIVITY OF CELLS TO THYMIDYLATE SYNTHASE INHIBITORS [1]

[1] Developing Strategies to Increase Chemo-sensitivity of Cells to Thymidylate Synthase Inhibitors, Nikeya L. Tisdale, Karen W. Barbour, Maria Marjorette O. Peña. To be submitted.

2.1 ABSTRACT:

Thymidylate synthase (TS) is an essential enzyme necessary for DNA replication and repair; it is responsible for the synthesis of thymidine. Typically, cancer cells exponentially proliferate at higher rates, resulting in elevated TS activity. Thus, using TS inhibitors has been promising in treating cancer. Although, like any drug TS inhibitors do exhibit problematic pitfalls that include harming normal non-cancerous cells, the occurrence of cancer resistance and relapse to therapy. Attempting to circumvent these limitations, we used RNAi vectors to suppress cellular levels of TS. Reduction of TS expression did enhance sensitive to therapy, however the toxicity of the RNAi vector made it impossible to examine the effects *in vivo*.

2.2 INTRODUCTION:

Thymidylate synthase (TS) is an enzyme that catalyzes the reductive methylation of dUMP to produce dTMP and dihydrofolate (Danenberg 1977; Carreras and Santi 1995). Subsequent, phosphorylation converts dTMP to dTTP, an essential precursor for DNA synthesis. This catalytic activity provides the sole intracellular *de novo* source of dTMP, thus TS is essential for actively dividing cells. Due to this, TS is an optimal target for anti-cancer therapies(Wilson, Danenberg et al. 2014).Examples of TS inhibitors are 5-fluorodeoxyuridnine (FdUMP), Raltitrexed, and 5-Fluoruouracil (5-FU).

The most commonly TS inhibit is 5-FU which has been used in the clinical management of many cancers. Inhibition occurs after 5-FU is transported into cells and converted into several metabolites. One metabolite is FdUMP, a powerful TS inhibitor. It disrupts DNA synthesis by forming a covalent ternary complex with TS and the substrate 5,10-methylenetetrahydrofolate. Inhibition results in the accumulation of dUMP, which is subsequently phosphorylated into dUTP. The incorporation of dUTP into DNA, triggers DNA fragmentation and ultimately cell death. Although, 5-FU is clinically used to treat cancer there are some drawbacks to this therapy, such as acquired resistance and the lack of specificity. Acquired resistance requires increase doses of 5-FU needed to kill cancer cells; however, the dose administered is limited by its lack of specificity resulting in toxicity to normal cells.

In addition, to its catalytic function, TS negatively auto-regulates its intracellular protein level, by binding to its mRNA to block translation. To mitigate the effects of 5-FU, TS protein bound to its mRNA are release to permit translation leading to induction of TS protein, exhaustion of 5-FU, lowering cell response and development of resistance to 5-FU. (Chu and Allegra 1996). Another, disadvantage of 5-FU therapy is its lack of specificity, targeting both actively dividing cancer and healthy non-cancerous cells. This leads to side effects such as alopecia, myelosuppression, and mucositis, due to sensitivity of non-cancerous cells in the hair follicles, the bone marrow compartment, and the intestinal lining which are always cycling and are vulnerable to 5-FU. Thus, there have been many attempts to enhance its efficacy while lowering toxic side by

developing new therapies or technologies to be used in conjunction with 5-FU (Natoli, Lupertz et al. 2013).

RNA interference (RNAi) is a novel technology that specifically lowers cellular expression of a target gene of interest. It involves using short interfering double stranded RNA (siRNA) or vectors driving the expression of short hairpin sequences (shRNA) that are complementary to a target mRNA sequence. Dependig on whether the siRNA is a perfect or imperfect match, binding to the mRNA can cause its degradation or inhibit its translation, both resulting in lowering the expression of the encoded protein (Hammond, Caudy et al. 2001; Ku and McManus 2008). This technology has been beneficial in regulating gene expression in cancers. A study targeting cyclin D1 with a silencing sequence resulted in sensitivity to 5-FU (Seo, Jeong et al. 2013). More specifically a number of studies have utilized RNAi against human thymidylate synthase in cells to show that depletion of TS mRNA and protein levels can circumvent the mechanism of resistance that occurs after exposure to TS inhibitors (Schmitz, Chen et al. 2004; Yang, Cloud et al. 2006).

Although the use of RNAi against TS results to sensitize cells to TS inhibitors is a promising strategy, these studies have only been performed in cancer cell lines; the efficacy of this strategy and tumor response to this therapy needs to be examined in mouse models in vivo. Targeting the TS siRNAs to cells in the tumor strom to sensitize tumors to the therapy has not been explored and provides a unique strategy to enhance antitumor efficacy.

The goals in this chapter are 1) to identify siRNA sequences that can down regulated mouse TS expression in CHL (musTS) cell lines, 2) to determine if the siRNAs can sensitize cells to TS inhibitors. We sought to chemo-sensitized BMDCs to TS inhibitors by also using RNAi technologies against *Mus musculus* thymidylate synthase (Mus TS), to establish a chemo-sensitive TME within the $Apc^{Min/+}$mouse model. Our results confirm that constitutive reduction of TS protein levels enhances 5-FU efficacy. Yet, our attempts to observed chemo-sensitive BMDCs response to therapy in $Apc^{Min/+}$mice produced unfavorable results, demonstrates some negatives of using RNAi for therapeutic applications.

2.3 MATERIAL AND METHODS:

Mus musculus TS expressing vector

A 1 kilo-base cDNA corresponding to LUC3-7 *Mus musculus* TS mRNA was cloned into pBR322, a generous gift from Dr. Lee Johnson at Ohio State University (Perryman, Rossana et al. 1986). This plasmid was used as a template to PCR amplify mouse TS. The forward primer 5' AAA AAA AGC TTT TTG TCG CTG ACT ACA CTG 3' inserts a HindIII site at position -37 and the reverse primer 5' TTT GTT AAC TTT TTT TTT TTA AAC AGC CAT TTC CAT 3' inserts a HpaI site directly after a nine nucleotide poly A tail. The PCR amplified fragment was cloned into pSV2CAT with the HindIII/ HapI sites, removing CAT and replacing it with the 961 base pair amplified MusTS fragment. The resulting plasmid, PSV2CAT-MusTS was stably transfected into the TS deficient CHL cell line to generate the CHL (MusTS) cell lines expressing mouse TS.

Generating CHL (Mus TS) cell line

Cell line RJK88.13, a TS deficient derivative of V79 Chinese hamster lung (CHL) cells, was a generous gift from Dr. Robert Nussbaum from the University of Pennsylvania(Nussbaum, Walmsley et al. 1985). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning Cellgro, Manassas, VA) containing 4 g/L glucose, L-glutamine, sodium pyruvate, penicillin (100 U/mL), streptomycin (100 mg/ml), 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals Inc., Flowery Branch, GA) and supplemented with 10 uM thymidine at 37°C with 5% CO2 in a humified incubator.

To generate CHL (MusTS) cell line the vector pSV2 (No CAT) expressing mouse TS, PSV2CAT-MusTS was stably transfected into CHL cell line with Liptofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Stable transfectants were selected in media lacking thymidine in the presence of 5 mM dipyridamole, a nucleoside transport inhibitor. Selected colonies were grown in mass culture.

Characterization of siRNAs against mouse TS in cells

siRNAs against mouse TS were designed using software by Dharmacon RNA Technologies (Layette, CO) . The siRNAs were reconstituted in siRNA buffer (Dharmacon RNA Technologies) (100µM stock solutions). The sequences of two siRNAs against the *Mus musculus* TS mRNA sequence are: TS001 (GGA GCA GAG TAC AAA GAT A) and TS003 (GGG TAT AAT CCA CAT CCA A).

Twenty-four hours prior to siRNA transfection 1 x 10^6 cells were plated in duplicate in 2 ml of DMEM with 10% FBS in a 6 well plate. Cells were transfected with either of the siRNA sequences using Dharmafect 1 transfection reagent following manufacturer's instructions (Dharmacon RNA Technologies). Cells were collected post-siRNA exposure for immunoblot analysis.

Sensitivity to siRNA transfected cells to 5-FU

5 -Fluorouracil was purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were made by dissolving in phosphate buffered saline (PBS) and filter sterilized using a 0.22 uM syringe filter. Two treatments with siRNA were necessary to obtain sufficient down regulation of mTS during 5-FU treatment. The first exposure induced the reduction of mTS protein level, while the second exposure maintained the reduced protein levels for the duration of the treatment. To evaluate the cytotoxic effects of 5-FU in combination with siRNA treatment, 0.5 x 10^5 CHL(MusTS) cells were plated in 1 mL DMEM with 10% FBS in triplicate in 6 wells pates. Twenty-four hours later cells were transfected with 2 nM TS001 or scramble siRNA as control and allowed to incubate for forty-eight hours. After down regulation of TS, cells were treated with increasing concentration of 5-FU (0-1000 nM) and incubated for five days. Twenty-four hours after the addition of 5-FU, cells were subject to 4 nM or 10 nM siRNA or scramble siRNA. The second exposure ensured that TS was downregulated during the remainder of 5-FU treatment. An additional control were cells treated with 5-FU with no siRNA added.

After 5 days, the cells were washed with Hanks buffered salt solution (HBSS), trypsinized, and counted in the presence of trypan blue to distinguish dead cells, using a hemocytometer.. The number of cells surviving at the end of the treatment were expressed as a percentage of the number of cells surviving in the absence of 5-FU.

Construction of plasmid for constitutive down regulation of MusTS

A double strand MusTS silencing oligo sequence was inserted into linearized pSuper RNAi vector to express short interfering RNA against MusTS (Oligo Engine, Seattle, WA). The silencing oligos contained the TS001 siRNA sequence (target sense sequence), but with the addition of a hairpin loop sequence TTC AAG AGA, the anti-sense sequence TAT CTT TGT ACT CTG CTC C, and the restriction sites Bglll or HindIII on the forward or reverse oligo respectively, resulting in two 60-nucleotide oligos (Integrated DNA Technologies, Coralville, IA). The oligo strands were annealed together by mixing equal molar concentrations, heating at 90ºC for 4 minutes, followed by annealing at 70ºC for 10 minutes, then gradually cooling to room temperature within 30-45 minutes. The annealed oligos were then cloned into the BglII and HindIII restriction sites in pSuper RNAi vector where their expression was under the control of the constitutive RNA Polymerase III H1 promoter.

The resulting vector plasmid, pSuper-MusTS001 (8-10 ug DNA) was transfected into CHL (MusTS) cells using Liptofectamine 2000 reagent. After 24-

48 hours, cells were selected in 2 ug/mL puromycin (Invitrogen, Carlsbad, CA) to generate a stable cell line.

Immunoblotting Assay and Quantification

Total protein extracts were obtained using M-PER protein lysis buffer (Thermo Fisher, Waltham, MA) following manufacturer's instructions. Gel electrophoresis and membrane transfer were performed following standard procedures. Membranes were incubated in rabbit polyclonal anti-mouse TS antibody (sc134130, Santa Cruz Biotechnology; 1:2000 dilutions in PBS-T/ 1% milk) overnight at 4ºC, washed with PBS-T, and incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (BioRad; 1:1000 in PBS-T) for 1 hour at 20°C. The same membrane was with mouse monoclonal anti-mouse actin antibody (β-actin-HRP ab49900, Abcam,) (1:1000 in TBS-T/1% skim milk, 1 h, 20°C). Bands were visualized using enhanced chemiluminescence plus (ECL Plus; GE Healthcare) and a Konica SRX-101A film processor. Band intensity was quantified using ImageJ software.

Mice

Animal experiments were conducted in accordance with the guidelines and approval of the USC Institutional Animal Care and Use Committee. Mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred and maintained at the Mouse Experimentation Core Facility of the Center for Colon Cancer Research at the University of South Carolina (USC), Columbia, SC.

C57BL/6-UB1-eGFP mice transgenically express the enhanced green fluorescent protein (eGFP), under the control of the human ubiquitin promoter. These mice were used as donors for bone marrow transplantation (BMT), to reconstitute lethally irradiated aged matched C57BL/6J-ApcMin/J mice (*Apc*$^{Min/+}$).

Bone Marrow Isolation

C57BL/6-UB1-eGFP donor mice were anesthetized by inhalation of 2% isoflurane in oxygen prior to sacrifice by cervical dislocation. Bone marrow (BM) cells were flushed from the femur and tibia using a 21-gauge needle into PBS containing 2% FBS. To quantify, red blood cells were lysed in ammonium chloride solution and mononucleated cells were counted using a hemocytometer.

Transduction of CHL (Mus TS) cell line and Bone Marrow Derived Cells

CHL(Mus TS) cells were incubated in viral supernatant from GP+E86 producer cells stably transfected with pSuper retroviral vectors expressing MusTS siRNA and scrambles siRNA containing 8ug/ml polybrene for 48 hours. Stable transductants were selected in 2ug/ml puromycin.

For bone marrow transduction, donor mice were injected i.p,with 150 mg/kg 5-FU 48 hours prior to bone marrow isolation. Harvested marrow were cultured in DMEM 20% FBS, recombinant murine interleukin 3 (IL-3, 20 ng/ml), recombinant murine interleukin 6 (IL-6, 20 ng/ml), and recombinant murine stem cell factor (SCF, 50 ng/ml) in suspension culture dishes (Corning Incorporation, Corning, NY) for 2 days. After 2 days, bone marrow cells were harvested and co-

cultured with GP+E86 producer cells in the presence of 8ug/ml polybrene for an additional 2 days.

Methylcellulose Assay

To evaluate chemo-sensitivity of transduced mononuclear cells. Approximately 15000 cells plated in 1ml of methylcellulose in the present of RTX (0-25nM) and 5μM dipyridamole. The number of progenitors was determined by counting colonies after 7 to 14 days.

2.4 RESULTS:

2.4.1 Identifying an effective siRNA target sequences against MusTS

CHL(MusTS) were with two siRNAs TS001 or TS003, which were complementary to nucleotides 487-505 or 958-976, respectively. Cells with the siRNAs for 24-120 hours, their abilities to suppress TS expression were assessed. Western blot analysis showed a decrease in protein levels over time using both siRNAs (Figure 2.1a). Quantification of protein levels conveyed that TS001 and TS003 suppressed TS expression by 63% and 82%, respectively, within 24 hours, 14%, and 31%, respectively, by 48 hours (Figure 2.1b). Thus, TS001 was more effective at inhibiting protein expression and was used in further studies.

2.4.2 Enhancing sensitivity to 5-FU by using TS001 silencing sequence

To determine if downregulating TS expression with TS001 siRNA will sensitize cells to 5-FU, we treated cells with a second dose of TS001 siRNA

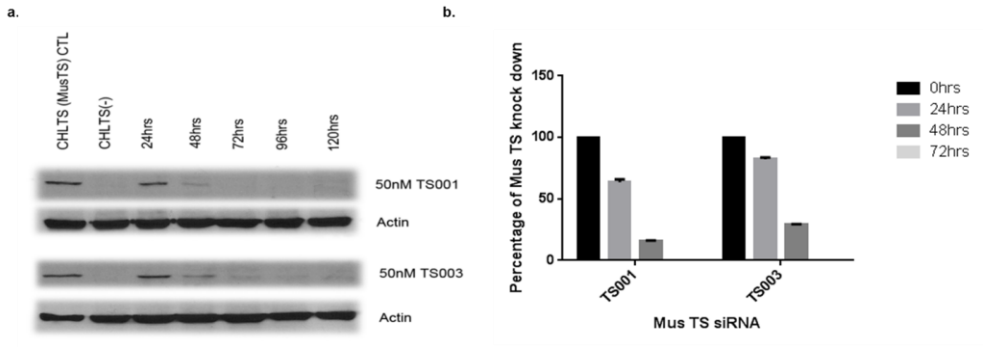


Figure 2.1: **Down Regulation of MusTS with siRNA**: a. Western blot analysis showing suppression of Mus TS protein in CHL (Mus TS) cells during a time course of 24-120 hours when exposed to 50nM of either TS001 or TS003 silencing sequences. Actin was used as the loading control. b. Quantification of Mus TS protein represented in percentages after exposure to Mus TS silencing sequences at various times.

ensure that TS expression was repressed for the duration of the experiment. Failure to maintain a sufficient level of siRNA made cells more resistant to 5-FU (data not shown). Cells were initially treated with 2 nM of the TS001 prior to 5-FU treatment. Twenty-four hours post 5-FU exposure, additional TS001 siRNA added to a final concentration of 4 or 10 nM were sufficient to maintain suppression of MusTS protein levels over the 5-day treatment (Figure 2.2a). The results from the growth inhibition curves also showed that cells treated with TS001 siRNA had lower IC_{50}values. Cells exposed to either 4 nM or 10 nM final concentration of TS001 in combination with 5-FU had IC_{50} values of 1.00 nM or 0.12 nM that were approximately 2.5- and 20- fold more sensitive, respectively,

as compared to the control cells with an IC_{50} of 2.37 nM. Western blot analysis showed down regulation of MusTS protein levels only in cells treated with TS001 siRNA (Figure 2.2b). Quantitation of the bands indicate that MusTS levels were

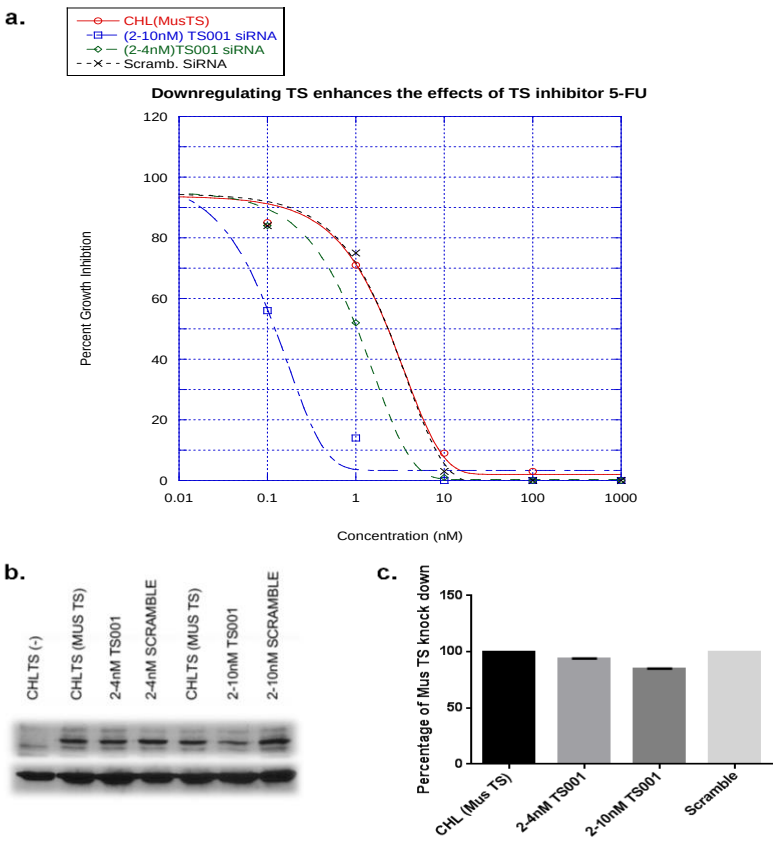


Figure 2.2: **Growth Inhibition Assay with siRNA**: a. Growth inhibition curve displaying enhancement of sensitivity towards 5-FU in CHL (MUS TS) cells treated with various concentration of siRNA Mus TS sequence. IC_{50} values were 0.12nM, 1.00nM, and 2.37nM for cells treated with 10nM, 4nM, 0nM of TS001 siRNA respectively. b. Western blot analysis of Mus TS protein of cells in the absent of 5-FU and either treated with or without Mus TS silencing target sequence (TS001). Cells treated with a final concentration of 10nM TS001 siRNA displayed the greatest reduction of Mus TS protein. Actin was used as the loading control. c. Quantification Mus TS protein after 5 days of transiently transfected with TS001 siRNA and exposure to 5-FU treatment.

reduced to approximately 93% and 85% in cells treated with 4nM or 10nM TS001, respectively, as compared to cells with no siRNA or those treated with scrambled siRNA (Figure 2.2c). These results showed that downregulation of intracellular TS levels can sensitize cells to 5-FU. To translate these results in vivo, it is important to develop expression vectors that will maintain reduced MusTS levels in targeted cells constitutively as a mechanism to sensitize cells to TS inhibitors.

2.4.3 Effects of constitutive expression of TS silencing sequence TS001

To investigate the effects of continuous expression of TS001 we constructed pSuper retroviral vectors, which contains the RNA polymerase III promoter, H1 to drive shRNA expression. The use of a retroviral vector, will allow expression of silencing MusTS shRNA constitutively to maintain sensitivity to TS inhibitos *in vitro* and *in vivo* without having to add additional siRNA. Transduction of CHL(MusTS) cells line or bone marrow derived cells with the retroviral vectors showed that constitutive expressesion of TS001 shRNA rendered cells more sensitive to 5-FU as compared to the cells transduced with control empty vector or scrambles shRNA (Figure 2.3a). We observed IC_{50} values of 33.07 nM, 2.69 nM, and 44.89 nM for cells expressing pSuper vector control, TS001 shRNA, or the scrambled shRNA, respectively. Thus, cells expressing TS001 shRNA were approximately 13-16-fold more sensitive to 5-FU as compared to cells expressing scrambled shRNA or empty vector. Western blot analysis showed that MusTS levels were downregulated only in cells expressing

the TS001 shRNA retroviral vector (Figure 2.3a). MusTS levels in these cells were 40% less as compared to the control cells (Figure 2.3b). This reduction of

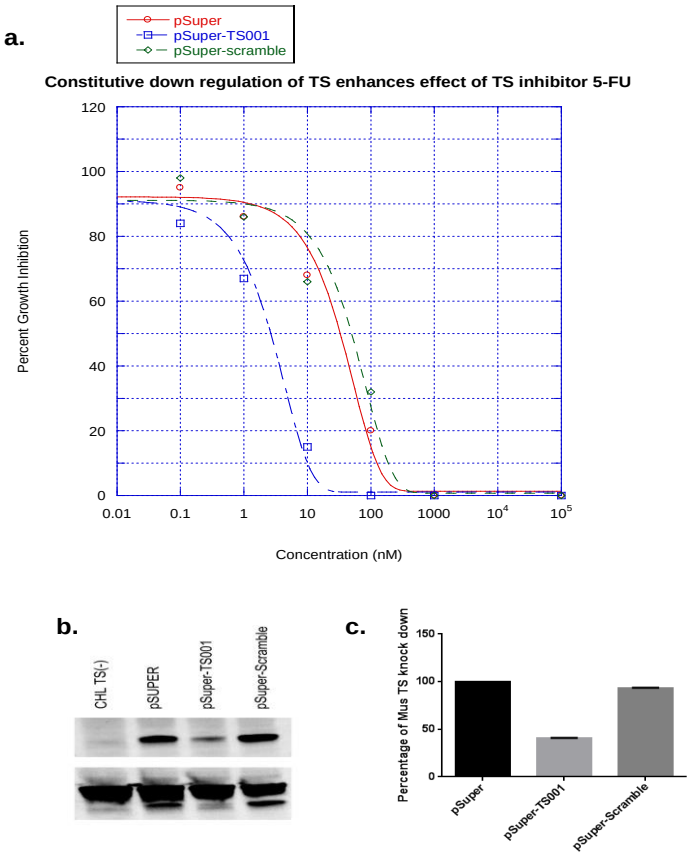


Figure 2.3: **Growth Inhibition Assay with pSuper Constitutive Vector**: a. Growth inhibition curves of CHL (Mus TS) cells that were transduced with retroviruses to express the pSuper vectors or a silencing or a scramble sequence against Mus TS. All cells were exposed to increasing concentration of 5-FU to determine IC_{50} values. CHL (Mus TS) cells expressing the silencing sequence display more sensitivity to 5-FU. IC_{50} values are 33.07nM, 2.69nM, 49.48nM for pSuper vector control, pSuper-TS001, and pSuper-scramble respectively. b. Western blot analysis of CHL (Mus TS) transduced with pSuper retroviral vector expressing either TS001 shRNA or the scramble sequences. Reduction of Mus TS protein is only observed in cells transduced with the silencing sequence. c. Quantification of the Mus TS protein shows the shRNA sequence reduces the expression of Mus TS protein by approximately 40%.

MusTS protein confirmed that the shRNA retroviral vectors were functioning appropriately and that we have constructed an efficient shRNA vector against MusTS that is able to sensitized cells to 5-FU by suppressing protein levels. Next we transduced bone marrow cells to determine if these vectors can sensitize bone marrow cells to TS inhibitors. Transduced bone marrow progenitor cells where plated in methylcellulose in the presence of increasing concentrations (0 nM-25 nM) of the TS inhibitor, raltitrexed. Approximately 50% growth reduction

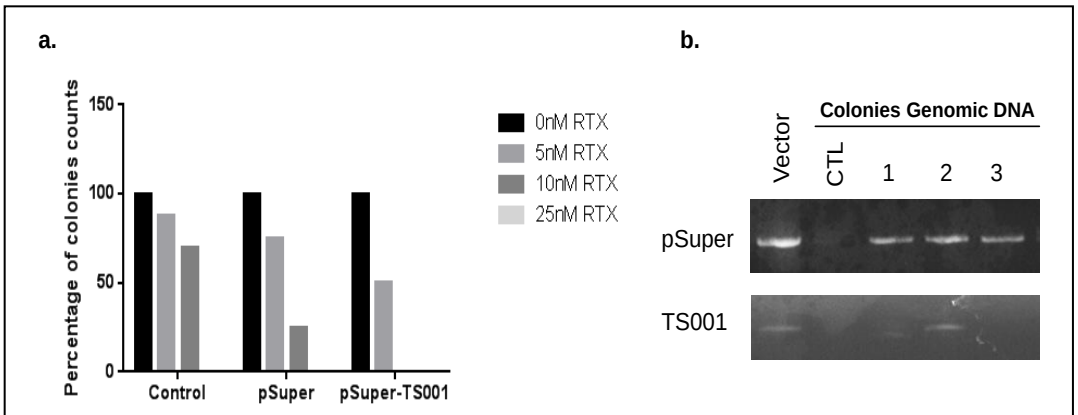


Figure 2.4**: Examining Sensitivity of Transduce BMDCs expressing pSuper vectors:** a. Colonies counts of bone marrow derived cells transduced to express the retroviral vector pSuper or pSuper-TS001 treated with increasing concentration of raltitrexed. Bone marrow expressing the Mus TS shRNA shows increase sensitivity to raltitrexed for exposure to 10nM concentration no colonies formed, however colonies developed in both the control groups. b. PCR amplification of colonies that were in the absent of raltitrexed to confirm expression of retroviral vector pSuper of pSuper-TS001.

was observed in BMDCs that were constitutively expressing the MusTS shRNA in the presence of 5 nM RTX when only 88% or 75% growth reduction was observed in non-transduced or vector controls, respectively. In addition, colony growth at 10 nM RTX was observed in only the control groups but not in BM cells transduced with TS001 shRNA (Figure 2.4a), supporting the idea that the

addition of an shRNA against MusTS is effective in sensitizing BMDCs to TS inhibitors. These results confirmed our ability to enhance the efficacy of TS inhibitors with the utilization of RNA interference technology. The question then is whether we can apply these strategies to sensitize the tumor microenvironment *in vivo*.

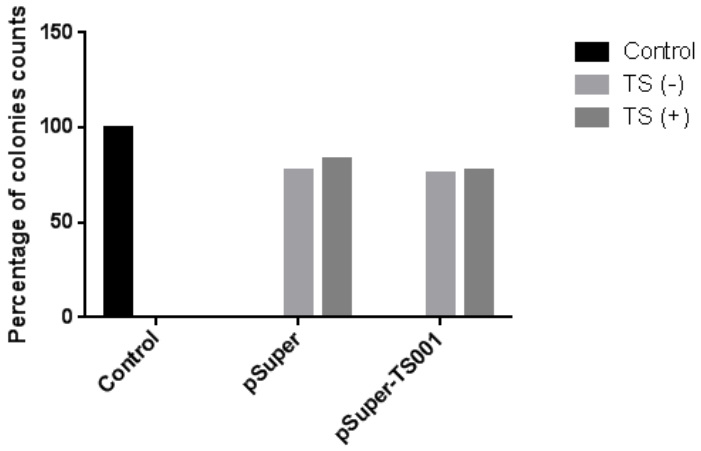


Figure 2.5: **Non-recovery of MusTS expression in BMDCs expressing pSuper vectors:** Colonies counts of bone marrow derived cells transduced to express the retroviral vector pSuper or pSuper-TS001 in the present or absent of thymidine. The addition of thymidine does not recovery the colonies counts for bone marrow that expresses either pSuper or pSuper-TS001 vectors.

To determine if the sensitization of bone marrow cells by TS001 shRNA in vitro can be recapitulated in mouse models in vivo, BMDCs transduced with pSUPER-TS001 shRNA were transplanted into lethally irradiated *Apc*$^{Min/+}$ mice. However, reconstituting mice with BMDCs expressing either pSuper vector control or pSuper shRNA resulted in immediate death of the recipient mice. Since mice subjected to transduced bone marrow did not survive to engraftment, we

were unable to examine the effects of chemosensitive BMDCs *in vivo*. To determine if the constitutive down regulation of TS was the cause of the unsuccessful attempts to reconstitute irradiated mice, BMDCs transduced with the retroviral vectors were plated onto methycellulose in the presence or absence of thymidine and growth of progenitor cells were assessed and compared to non-transduced cells. The results showed that the number of colonies did not differ significantly with the addition or lack of thymidine (Figure 2.5). Thus, the failure of thymidine to rescue transduced BMDCs from toxicity suggested that the retroviral vector pSuper was most likely problematic and toxic. To resolve this issue we used a different RNAi vector expressing system.

2.5 DISCUSSION:

In this study, we investigated effect of diminished MusTS expression on the sensitivity of CHL (MusTS) or transduced bone marrow cells to TS inhibitors using siRNA technology. We identified an siRNA sequence that reduced translation of MusTS mRNA by approximately 37%. Transfection of the siRNA or transduction of its corresponding shRNA in both CHL(MusTS) or bone marrow cells effectively sensitized cells to the TS inhibitor 5-FU.

Initially, to observed the response to therapy transiently transfecting cells with the2nM TS001 siRNA, displayed a resistance phenotype when expose to 5-FU (data not shown). Plausibly reasoning for resistance is due to the delayed decrease in protein levels, due to using low concentration of the siRNA. However, resolving this problem by exposing the cells to higher concentration of

the siRNA could drastically affect proliferation and hamper cells response to therapy. Therefore, we decided to expose the cells to the low concentration of siRNA before therapy for the delay decrease in TS expression, providing sensitivity, and then gradually increase the concentration of the siRNA for the duration of the therapy to inhibit in additionally translation of the mRNA to reduce TS expression. Thus, to evaluate siRNA technology capability of sensitizing cells to TS inhibitors, it was necessary to establish slight reduction of protein level before therapy followed by a prolonged disruption in the ability to translate the mRNA, for the duration of the therapy. Indeed, we found that transfecting cells with a low concentration of the siRNA prior to 5-FU exposure induce sensitivity. However, to accomplish long-term reduction of protein expression, the addition of more siRNA was needed twenty-four hours after 5-FU treatment, resulting in a final concentration of either 4nM or 10nM of the siRNA, which ensuring prolong down regulation of the protein, resulting in enhanced sensitivity towards 5-FU. Our results show an equivalence of either a 2 or20- fold increase in chemo-sensitivity, of cells transiently transfected with either 4nM or 10nM TS001 siRNA respectively in the present of 5-FU.From these results, we established that siRNA technology is able to sensitized cells to chemotherapy. Nevertheless, is problematic and is not conductive for *in vivo* studies. The problem of providing efficient and permanent reduction of Mus TS gene expression was resolved only by the addition of more siRNA, yet this is a solution for in vitro studies is not conceivable in the attempt to sensitizing the TME in the $Apc^{Min/+}$mouse model.

Therefore, it is necessary to use an RNA interference system that is vector base, and which we utilized the H1 promoter in the pSuper retroviral vector to drive the expression of the Mus TS shRNA to provide a permanent reduction in the Mus TS gene expression. The utilization of this retroviral vector allowed us the ability transduced CHL (Mus TS) and mouse bone marrow derived cells to express the shRNA against TS, such to develop and enhance sensitivity to TS inhibitors. Promising result from CHL (Mus TS) cells expressing Mus TSshRNAdisplayed13-fold increase in chemo-sensitivity towards 5-FU. We observed approximately a 40% reduction of protein expression, which is strikingly greater than the protein reduction observed from siRNA technology. Although, promising this 40% reduction in protein expression that occurred in CHL (Mus TS) cells, may translate into proliferation problems when expressed by BMDCs. For the delivery of Mus TS shRNA sequence within the TME of $Apc^{Min/+}$ mice is contingent on the ability of bone marrow derived cells to proliferate, imperative for infiltration into the TME.

To assess this issue, retroviral particles were used to transduce bone marrow derived cells to examine the effects of stably incorporating the pSuper vector into the genome of these. The genetic modulate bone marrow derived cells expressing the Mus TS shRNA was used for both concurrent *ex vivo* and *in vivo* chemo-sensitive studies, for both studies required time for either colony formation or reconstitution engraftment of $Apc^{Min/+}$mice that were subjected to irradiation to completely deplete existent bone marrow. Our *ex vivo* results of transducing mouse bone marrow derive cells to express the MusTS shRNA

sequence showed promising effects, of chemo-sensitivity toward raltitirexed. However, the occurrence of fatalities in *Apc*$^{Min/+}$mice reconstituted with bone marrow expressing Mus TS shRNA seems to hinder cells ability to proliferate, due to cytotoxic effects. The data in which we attempt to recover cellular thymidine level with the addition of exogenous thymidine in BMDCs implicates that the pSuper vector is indeed toxic. The inability to recover colony formation in either vector control or shRNA expressing BMDCs proves that the pSuper vector system is problematic.

Additionally, this *in vivo* result suggests that down regulation of Mus TS gene expression needs to regulate. TS is an essential enzyme for cell proliferation and to continuously suppress it's expression is sure to have devastating outcomes, thus, in the future we intend to use an inducible promoter, one that activates only in the tumor microenvironment. In hopes to alleviate, any problem caused by the constitutive down regulation of Mus TS.

Overall our data suggest that the down regulation of MusTS expression prior exposure to chemotherapy is beneficial in reducing the concentration of 5-FU required to inhibit cellular proliferation. These results are the effect of the mechanistic degradation or inhibition of translating of Mus TS mRNA that is target by RNA interference technology. The process of subjecting cells first to target Mus TS RNA interference disrupts cellular translation of the TS protein. The reduction in cellular protein levels is advantageous for requirements of inhibiting the catalytic activity of the protein have lessened. In addition the additive effect of utilizing RNA interference in combination with TS inhibitors

eliminates the induce activation of protein production, for MusTS mRNA is targeted for degradation prohibiting translation.

Clearly, many problems can occur with utilizing RNA interference technologies. However, used appropriately it may be beneficial in a variety of clinical disease processes.

CHAPTER 3

CHARACTERIZATION OF THE ABILITY OF THE MURINE OSTEOPONTIN PROMOTER TO DRIVE THE GENE EXPRESSION SPECIFICALLY IN BONE MARROW DERIVED CELLS IN THE TUMOR MICROENVIRONMENT[2]

[2]Characterization of the Ability of the Murine Osteopontin promoter to Drive Gene Expression Specifically in Bone Marrow Derived Cells in the Tumor Microenvironment, Nikeya L. Tisdale, Sapana N. Shah, Celestia Davis, Maria Marjorette O. Peña. To be submitted.

3.1 ABSTRACT:

Osteopontin (OPN) has been implicated as a cytokine that plays an important role in cancer progression. It is secreted by a number of cells and jas been shoen to activate the function of many immune cells. Studies investigating the activity of the osteopontin promoter showed that it contains many enhancer sites, which are influenced by inflammatory stimuli. Here, we show that the regions encompassing (-777/-740 to +79) of the OPN promoter is responsive to inflammatory stimuli and can specifically be activated only in the tumor microenvironment of $APC^{Min/+}$ mice. In addition, we have validated that the OPN promoter has the capacity to drive the expression of an RNAi sequence. The results of these studies indicate that the OPN promoter may be useful in directing the expression of chemo-sensitizing genes specifically in stromal cells in the tumor microenvironment. These will be useful in testing our central hypothesis that by sensitizing tumor stromal cells, it is possible to sensitize cancer cells to the therapy.

3.2 INTRODUCTION:

Endogenous gene silencing is mediate by microRNA (miRNA). The development and maturation of miRNA begins in the nucleus, where miRNAs are transcribe as part of long primary transcripts (pri-miRNAs), which contain multiple embedded hairpins. Drosha/Pasha, RNASE III enzymes cleaves these

pri-miRNAs into individual stem loops within the nucleus. These individual stem loops are precursor miRNAs (pre-miRNAs), which are approximately 70nt length double strand sequences (Yeom, Lee et al. 2006). Pre-miRNAs are exported out of the nucleus by the protein Exportin 5 into the cytoplasm, to further, undergo processing by RNASE III enzymes, Dicer and RNA-induced silencing complex RISC to obtain gene silencing (Ku and McManus 2008). Dicer removes the stem loop structure to produce a ~21-23nt long double stranded sequence, which is then subsequently incorporated into the RNA-induced silencing complex (RISC)(Hammond, Caudy et al. 2001). The RISC uses one strand of the double strand RNA as a guide to target the mRNA of interest for degradation or inhibition of translation, thus resulting in lowering expression of the encoded protein (Ku and McManus 2008). This mechanism of gene silence provides the template for exogenous mediated gene silencing accomplished by small interfering (siRNAs) or short hairpin RNAs (shRNAs).

The widely utilized commercial synthetic siRNAs are ideal to examine the functional roles of genes in upstream and downstream processes during disease development. Upon entry into the cytoplasm of cells, the cytoplasmic enzymes of miRNA machinery process siRNAs to achieve inactivation of genes. Even though it has been advantageous, the utilization of siRNAs has limitations that include the inability to enter slowly dividing cells and the inability to sustain continuous suppression of gene expression. To circumvent these issues the development of shRNA viral expression vectors are modeled after pre-miRNAs. Typically, to provide constitutive repression of a gene, shRNA viral vectors

employed RNA polymerase (pol) lll promoters, H1 or U6 to control the expression of the shRNA sequence(Paddison, Caudy et al. 2002; Rossi 2008) . However, these vectors can be problematic, causing RNAi toxicity and they lack the capacity to be tissue specific (Boudreau, Martins et al. 2009) as previously discussed in Chapter2.

Thus, modifications to shRNA expression vectors were developed to be more similar to pri-miRNA, by embedding the shRNA sequence into an endogenous miRNA backbone (shRNA-miRs), such as miR30 (Matveeva, Nazipova et al. 2012; Fellmann, Hoffmann et al. 2013). The advantage of this feature is that it enables the expression of the shRNA to be controlled by a RNA Polymerase II promoter. Additionally, this model system exploits the entire miRNA processing machinery resembling the natural maturation of gene silencing within a cell. This particular system eliminates the need to use a pol III promoter to drive the expression of a silencing sequence. Thus, we decided to use this model system to circumvent the problems that occurred when utilizing the constitutive retroviral vector pSuper, which uses the H1 promoter to drive the expression of the silencing sequence. We show that using the murine cytomegalovirus promoter (mCMV) a polymerase II promoter driving the expression of TS silencing sequence is an ideal expression system that will allow us to examine the systemic effects of reducing TS expression in mice.

The processes of gene expression is regulated by several influential factors such as frequency of transcription of a gene, post-transcription modification of messenger RNA, translation, and maturation or degradation of

protein. These regulatory processes enforce the balance of gene expression, which controls cellular function or development. Regulation of gene expression can be tissue specific, which is typical during developmental progression and the processes of wound healing or cancer. Reproducing and using the regulatory features of tissue specific gene expression can be beneficial for targeting therapies, hence alluding to the capacity to target only the tumor and its microenvironment.

In devising the approach for specific targeting, it was essential to identify a gene that is only activate and expressed in the TME; and then use this gene's promoter to drive the expression of an RNAi sequence against thymidylate synthase. We identified Osteopontin (OPN) as a potential candidate, since studies have shown that it plays a vital role in the progression of cancer, and is highly expressed in several tumors.

The emerging evidence pertaining to OPN involvements in cancer development has led to its consideration as a biomarker (Cao, Li et al. 2012). OPN is a secretory extra cellular matrix (ECM) protein that is secrete by a variety of cells such as endothelial, epithelial, and activated immune cells. Results have implicated the involvement of OPN in the interactions that occur between cancer cells and its tumor stroma (Gotoh, Sakamoto et al. 2002). OPN is highly expressed in a variety of human cancers. In particular, in human gastric cancer OPN has been shown to be highly expressed in gastric cancer lesions, mildly expressed in the tumor stroma, and is negatively expressed in the normal regions of tissue sections(Ue, Yokozaki et al. 1998; Junnila, Kokkola et al. 2010).

In addition, a study comparing the alteration of gene expression in normal versus tumor intestinal tissues revealed elevated levels of OPN in the polyps of *APC*$^{Min/+}$ mice (Chen, Hao et al. 2004).

Moreover, several studies have indicated that pro-inflammatory stimuli induce the transcription of OPN gene (Guo, Cai et al. 2001; Denhardt, Mistretta et al. 2003; Zhu, Denhardt et al. 2005). Guoet.al. demonstrated that the OPN promoter activity increased in the presence of lipopolysaccharide (LPS) and nitric oxide (NO). Furthermore, examination of OPN promoter identified several regions as pro-inflammatory response elements such as ras- activated enhancers that promoted the transcription of the gene in metastatic transformed cells or in cells exposed to hypoxic conditions(Guo, Zhang et al. 1995; Zhu, Denhardt et al. 2005). In addition, identification of positive and negative regulatory cis- acting elements in various regions of the OPN promoter influences tissue specific expression (Higashibata, Sakuma et al. 2004). The premises of these studies indicate that the OPN promoter is an ideal candidate to use to target specifically the TME.

Once, again all roads leads back to the TME stressing the importance of infiltration of BMDCs and their function in tumor progression and resistance to various chemotherapies. Important players in tumor progression that also, exhibit immune suppressive activities include mast cells; myeloid-derived suppressor cells (MDSCs) and regulator T cell (Tregs). Mast cells are effectors cells that release an array of pro-inflammatory mediators, which have shown to influence the migration and function of many hematopoietic derived cells in cancer

progression (Ribatti 2015). Crosstalk between mast cells and MDSCs was observed in a CT26 cancer model and was shown to enhance the suppressive activities of MDSCs (Danelli, Frossi et al. 2015). MDSCs are a heterogeneous population of immature myeloid cells that are activated by the pro-inflammatory stimuli produced within the tumor or by mast cells. Activation of these cells results in suppression of T cell activation, which is necessary for antitumor immunity, permitting the immune system to be acceptance to tumor progression (Marvel and Gabrilovich 2015; Parker, Beury et al. 2015). MDSCs production of interleukin 17 (IL-17) have been shown to reinforce it suppressor functions by indirectly attracting regulatory T cells into the tumor (Yang, Zhang et al. 2010).In the presence of 5-FU, depletion of MDSCs have shown to result in two outcomes. First, the beneficial outcome results in the enhancement of T cell dependent antitumor activity (Vincent, Mignot et al. 2010). The other outcome supports tumor growth due to the secretion IL-1B by MDSCs, which recruits Tregs to produce IL-17 to assist with angiogenesis (Bruchard, Mignot et al. 2013). The effect of 5-FU on tumor infiltrate mast cells response has not been elucidate, however reduction in mast cell progenitors followed by a rapid recovery was observed in the bone marrow and spleen of mice (Hokari, Tsuboi et al. 2011). These studies highlight the importance for why chemotherapy sensitivity needs to be established in these particular BMDCs populations. Hence, reprogramming of these cells to be genetically more sensitive to therapy may be beneficial to how these BMDCs respond to therapy.

Thus, we sought to determine if utilizing the inducible characteristic of the OPN promoter could drive the expression of an RNAi sequence against Mus TS specifically within the tumor microenvironment to establish chemo –sensitivity of these cells to TS inhibitor. To accomplish this we demonstrated the OPN promoter (-740/ -777 to +79) regions are responsive to pro-inflammatory stimuli and are capable of driving the expression of mCherry a fluorescent protein only within the tumor microenvironment of *APC*$^{Min/+}$mice. In addition, we validate that the -740 to +79 OPN promoter region is capable of driving the expressing of RNAi sequence against MusTS to enhance cellular sensitivity to 5-FU. The premises of our studies show that the OPN promoter can indeed provide specific chemo-sensitivity in the TME.

3.3 MATERIAL AND METHODS:

Lentiviral vector

We obtain the pZip lentiviral vector from Transomic Technologies. Dr. Greg Hannon and colleagues from Cold spring Harbor Laboratory developed this system, which is comprised, of an ultramiR scaffold shRNA, fluorescent marker Zsgreen, and the selectable marker puromycin constitutively driven by the mCMV promoter to establish efficient repression of a target gene. The MusTS shRNA sequence expressed by this vector is the following: TGCTGTTGACAGTGAGCGACAAGACCTTTCCCAAAGCTCATAGTGAAGCCA CAGATGTATGAGCTTTGGGAAAGGTCTTGGTGCCTACTGCCTCGGA. The lentiviral vectors were stably transfected into CHL (MusTS) cells following

manufacturer's instructions. Selection of stable transfectants was done by incubating transfected cells with puromycin antibiotics and visual verification of Zsgreen expression in cells fluorescence microscopy and flow cytometry.

Vector Construction

Vector pXP1-OPN (-777 to +79) was a generous gift from Dr. David T. Denhardt from Rutgers University. Regions -777 to +79 and -749 t0 +79 were PCR amplified using the forward primers: GGT ACC ATG CAT CTG CTC CAA CAG AGC AAC AAG GTT C or GGT ACC ATG CAT TCT CTA AAG GTC AGT GGA GGC AGG, respectively, and the reverse primer: AAG CTT GAT ATC ACC GGT CTT GGC TGG TTT CCT CCG AGA ATG. Amplified regions were cloned into the retroviral vector pMSCV that contained the mCherry fluorescent protein. These retroviral vectors were transfected into viral producer cell line GP+E86.

The pZip lentiviral vectors were used to construct lentiviral vectors containing the OPN promoter. The MSCV promoter was replace with the OPN promoter by recombination cloning following manufacturer's protocol for (Clonetech Infusion Cloning kit). Manufactures protocol for transfection was use to establish stable expression of the vectors in CHL (MusTS) cell line (Transomic Technologies). Confirmation of stable expression was acquired by exposing transfected cells to puromycin antibiotics and visual verification of Zsgreen expression in cells.

Transducing bone marrow derived cells

Dr. Boris Kantor of the Vector Core at the University of South Carolina produced the viral particles for the lentiviral vectors in the pZip backbone. . These viral particles were used to transduce bone marrow for reconstitution of lethally irradiated mice.

Lethal irradiation and reconstitution of *Apc*$^{Min/+}$or Balb/c mice

Four-week-old recipient *Apc*$^{Min/+}$ mice were lethally irradiated utilizing the JL Shepard Cesium-137 irradiator by administering 190 rads/min for a total of 950rads. 2–3 × 10^6 genetically modified mono-nucleated cells were transplanted into the lethally irradiated mice by tail vein injection within eight-hours of irradiation. Transplanted mice were administered sterile water containing 0.004% Baytril antibiotic (Bayer Animal Health Division, Shawnee, KS) for two weeks post-transplantation to prevent infection. These mice were used to determine if the OPN promoter is only activated within the intestinal tumor regions of a mouse model. Confirmation of successfully engraftment of genetically modified BMDCs was obtained 6 to 8 weeks post transplantation by bleeding mice and isolating genomic DNA from leukocytes. Genomic DNA was PCR amplified with primers to confirm incorporations of the mCherry gene.

Reconstituted 15-week old *Apc*$^{Min/+}$ mice were humanely sacrificed by cervical dislocation. Their intestines were isolated, flushed with 1xPBS and cut open longitudinally. The tissues were fixed in 4% paraformaldehyde overnight. The fixed intestines were then swiss-rolled and embedded in paraffin cassettes

and left to dry overnight. 3-5 micron sections were mounted on standard microscopy slides and nuclei were stained with Dapi.

Four-week-old recipient Balb/c mice were lethally irradiated using the JL Shepard Cesium-137 irradiator by administering 950 rad at 190 rads/min. Transplantation and verification of engraftment of genetically modified marrow was performed as described above.

Flow Cytometry

BMDCs were isolated from the femur, tibia, and blood from recipient mice. The single suspensions were incubated in ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM Na_2CO_3, 0.1 mM EDTA, pH 7.4) for 5 minutes on ice, in the dark to lyse the red blood cells, followed by centrifugation at 2000 rpm for 5 minutes at room temperature and the supernatant discarded. Excess lysis buffer was removed by washing the pellet twice with cold PBS containing 2% FBS and then centrifuged at 2000 rpm for 5 minutes. The cells were counted using a hemocytometer, and approximately 2 million cells were stained with antibodies against the cell surface markers CD45 for leukocytes, CD8a and CD8a for T cytotoxic cells, CD34 and CD117 for mast cells, CD4 and Foxp3 for T regulatory cells, and GR1 and CD11b for MDSCs for 1 hour at 4°C. To determine apoptosis index antibodies against Annexin V and Viability Dye eFluro 780 were used to stained particular cell populations. Antibodies against Ki67 determined the proliferation index for the different cell population. All antibodies for flow analysis obtained from Ebioscience. Excess antibodies were removed by washing cells

once with cold PBS containing 2% FBS and then centrifuged at 2000 rpm for 5 minutes. The cell pellet was then resuspended in 500µL of PBS containing 2% FBS for flow cytometry analysis using a Beckman Coulter FC-500 flow cytometer.

Immunofluorescence

The sections were deparaffinized, rehydrated, and then incubated in a microwave oven at 95ºC with 0.01 M citrate buffer, pH 6.0 (Diagnostic Biosystems, Pleasanton, CA) for 10 minutes for antigen retrieval. Sections were blocked with 5% BSA for one hour. To identify apoptotic cells, the samples were incubated for 2 hours at 37ºC. with a mixture of primary antibodies against cleaved PARP-1 (Rabbit polyclonal, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA USA) and a cell-type specific cell surface marker such as CD11b for monocytes, Ly-6 for granulocytes, or c-kit for mast cells (all the antibodies are rat monoclonal used at 1:200 dilution, all from Abcam, Cambridge, MA) in 1% BSA in PBS. Samples were wash twice 1XPBS and stained with respective secondary antibodies conjugated to Cy-3 (surface markers) or Cy-5 (Parp-1) for1 hour at 37ºC. The sections were again washed with 1XPBS and then stained with DAPI. Stained sections were treated with ProLong Gold anti-fade reagent (Invitrogen, Frederick, MD) prior to applying the cover slips.

Confocal Microscopy

The slides containing sections stained with the various combinations of primary antibodies were imaged using Zeiss LSM Meta 510 confocal microscope. For each antibody combination, approximately 100 field view images were captured at 40 X magnification. For each field view image, cells that stained positive for the specific cell surface markers were counted and scored as apoptotic if they also stained positive for cleaved PARP-1. Apoptotic indices were calculated as the percentage of cells that stained for both the cell surface marker and cleaved PARP against the total number of cells that stained positively for the specific cell surface marker.

3.4 RESULTS:

3.4.1 Examining the capacity of mCMV promoter to drive the expression of an RNAi sequence against TS *in vitro* and *in vivo*

To determine if the murine polymerase II promoter is able to drive the expression of an RNAi sequence against MusTS, we stably transfected the pZiplenti-viral vectors into the CHL(Mus TS) cell line to observe suppression of TS protein levels. Cells that were stably transfected with the pZip-mCMV-TS shRNA vector displayed a 67% expression of the protein and a 0.43 fold reduction in TS mRNA levels in these cells (Figure 3.1) when compared to the non-transfected cells. In addition, we observed a slight reduction of protein and

mRNA expression in the vector control cells, which was of concern due to the possibility that the vector might also prove to be toxic to transduced cells in an in vivo model. Thus, we determined the effect of expressing the lentiviral vectors on the growth rate of transduced cells as compared to control cells and non-transduced cells. Twenty thousand cells were plated and the growth rate was monitored over 6 days. The results showed that there were no differences in the growth rates between these cells (Figure 3.1) providing assurance that the lentil-viral vector system did not impair cellular proliferation and, thus may not be detrimental in an *in vivo* model system. To determine of the 33% reduction in MusTS level was sufficient to sensitize cells to 5-FU, we performed a growth inhibition assay. The results showed that there was no increase in the sensitivity to 5-FU of cells that expressed the RNAi sequence against MusTS as compared to control cells expressing the empty vector (Figure 3.1). Despite its inability to enhance sensitivity, most importantly, it was necessary for us to determine if this vector system can be used to modulate BMDCs for successfully engraftment into tumor-bearing mice.

To examine the effects of transplanting BMDCs transduced with the pZiplenti-viral vectors *in vivo*, BMDCs were isolated from donor Balb/c mice and transduced with viral particles from either the pZip-mCMV control vector or pZip-mCMV-TS shRNA vector using a multiplicity of infection of five. This resulted in approximately 20% of the BMDCs population expressing either vector as confirmed by fluorescence activated cell sorting (FACS) analysis to detect Zsgreen protein expression. This fluorescent marker in the pZip vector backbone

was used to confirm incorporation of vector into the cell's genomic DNA and its expression within transduced cells. We used the bone marrow that was 20% Zsgreen (ie, transduced) to transplant and engraft into lethally irradiated Balb/c mice. The controls for this study were Balb/c mice reconstituted with BMDCs that expressed the vector control that contain the Zsgreen gene under the control of the mCMV promoter or GFP expressing transgenic BMDCs.

Four weeks post transplantation, genomic DNA from mice reconstituted with genetically modified bone marrow was extracted from the blood to confirm incorporation of the Zsgreen gene and the Ultramir 3' and 5' regions with the RNAi control or RNAi Mus TS sequence in the genome (Figure 3.2). This result is significant for since have identified an RNAi system that is safe to use in mice. Thus, by using the pZip-mCMV vector we confirmed that an polymerase ii promoter can drive the expression of an RNAi sequence against MusTS, and this RNAi vector system can be used to modulate BMDCs to successfully engraft in mice without exhibiting cytotoxic effects. These results suggest that we potentially have capability to direct tumor stromal specific sensitivity to chemotherapy drugs in an *in vivo* model. The potential to establish specific sensitivity is dependent on the ability of the promoter to drive the expression of the RNAi sequence. We identified the OPN promoter as a possible candidate. However, further examination of the promoter is necessary before the implementation of this RNAi vector system into *in vivo* mouse model of cancer.

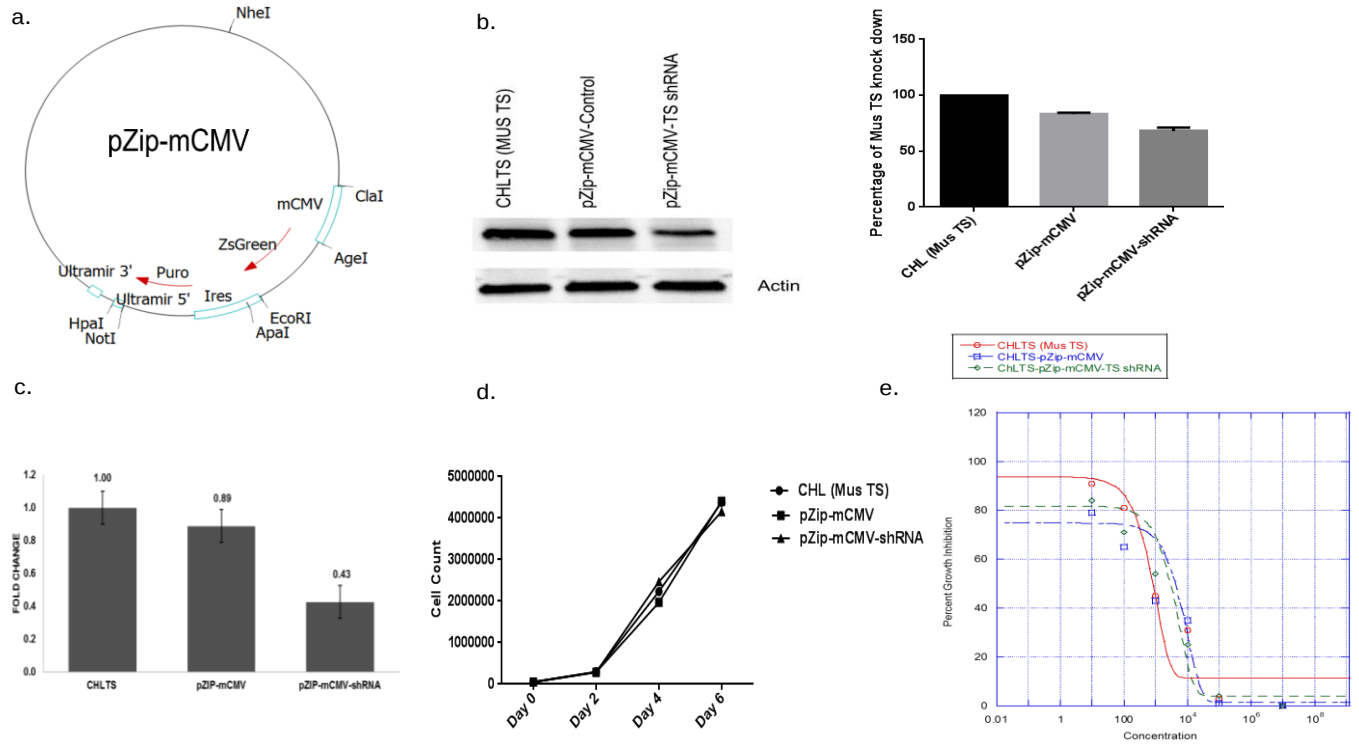


Figure 3.1: **Examining the ability of mCMV Promoter to control an RNAi sequence.** a. Depiction of the lenti-viral pZip-mCMV vector map. b. Western blot analysis of CHL (Mus TS) stably transfected with pZip lenti- viral vector containing the mCMV promoter to drive the expressing both shRNA sequence and Zsgreen or the vector control which only drive the expression of the Zsgreen protein. Reduction of Mus TS protein is only observed in cells transduced with the silencing sequence. c. Quantification of the Mus TS mRNA shows the shRNA sequence reduces the expression of Mus TS protein by approximately 33%. d. Growth curve graph indicating that the growth rate of cells expressing the shRNA sequence is comparable to control cells or the cells expressing the vector control. e. Growth inhibition assay show that pZip vectors exhibit sensitivity to 5-FU therapy.

3.4.2 Characterizing the specificity and induciblity of the OPN promoter regions (-777/-740 to +79)

To determine responsiveness to inflammatory stimuli of the OPN promoter (-777/-740 to + 79) driving the expression of the gene encoding the mCherry fluorescent gene, cells transfecyed with these constructs were treated with different inflammatory stimuli. We exmined the expression of the mCherry reprted gene by microscopy and by immunoblot analysis of mCherry protein levels to

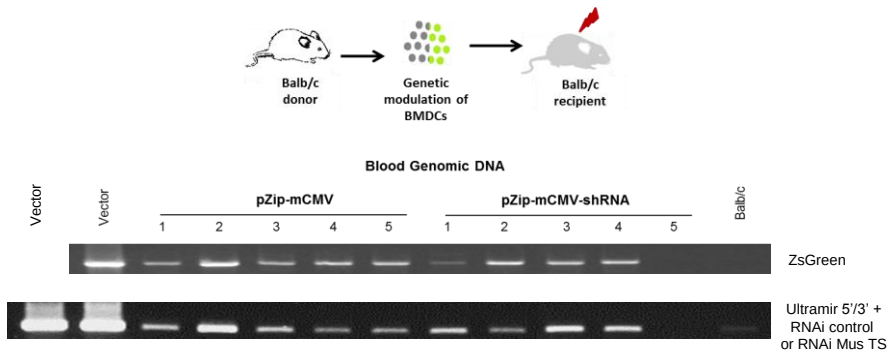


Figure 3.2: **Confirming incorporation of pZip-mCMV lenti-viral vectors *in vivo*.** a. Depiction of extracting BMDCs from donor Balb/c mice for genetic modulation of cells with lentil-viral particles to express either mCMV-Zsgreen or mCMV-ZSgreen-TS shRNA sequence to transplant into lethally irradiate Balb/c mice recipients. b. Confirmation of successful reconstitution of transduce BMDCs into recipient mice by PCR amplifying genomic DNA to detect the Zsgreen gene and either the RNAi control or RNAi Mus TS sequence located between the ultramir 3' and 5' regions.

assess the activation of the OPN promoter. Activation of the OPN promoter was accomplished by exposing cells to the systemic inflammatory stimuli LPS, the inflammatory cytokines interferon gamma (INF-γ) and interleukin 1-β (IL-1β), or condition media from cancer cell line CT26 that are thought to secrete molecules

that are typically found in the tumor microenvironment. Microscopy and western blot analysis shown in Figure 3.3 confirm an increase in visual and protein levels of mCherry only in cells exposed to inflammatory stimuli INF-γ (b), LPS (c), IL1-β (d), or conditioned media from CT26 cells (e). In addition, western blot analysis quantifying mCherry protein levels confirm that protein levels of mCherry increased after cells were exposed to the inflammatory stimuli. The results indicated that regions -777 or -740 to +79 of the OPN promoter contain sites that are responsive to inflammatory stimuli, thus permitting it to induce the expression of the mCherry transcript as compared to the untreated cells (a). Western blot analysis showed basal levels of mCherry expression in untreated cells, but at low levels that will still allow the use of these promoter regions to drive the expression an RNAi sequence against MusTS.

To further analyze these regions of the OPN promoter, we transduced green fluorescent protein (GFP) expressing BMDCs with viral particles packaged with these retroviral vectors. During transduction BMDCs were exposed to stem cell factor (SCF), interleukin 3 (IL-3), and interleukin-6 (IL-6) cytokines and then plated in methylcellulose to examine colony formation and the expression of mCherry. Colony formation is conveyed in percentage wherein 100% of the colonies formed in the control and the transduced BMDCs expressed GFP, however transduced BMDCs expressing OPN (-777/-740 to +79) fused to GFP showed that approximately 50% of the GFP colonies also expressed mCherry. Figure 3.4 depicts the percentage of colony formation as well as the visual confirmation of co-localization of GFP and mCherry expression in BMDCs. From

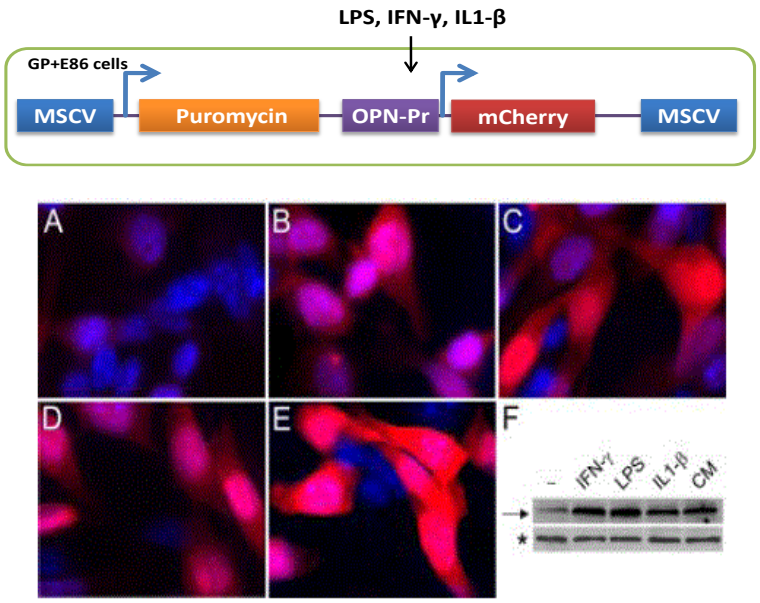


Figure 3.3: **Inducible characteristic of the OPN promoter**. Retroviral vector containing OPN promoter region -740 to +79 driving the expression of a mCherry reporter gene. A-E: GP+E86 cells stably transfected with the retroviral vector in top panel were (A) untreated or treated with (B) IFN-γ, (C) LPS, (D) IL1-β, (E) or conditioned media. (F): Western blot showing induction of mCherry in response to the extracellular factors in A-E. Actin (*) was used as an internal control for equal protein loading.

this *ex vivo* study, we are able to validate our ability to modulate the expression of BMDCs, and show that the regions -777/-740 to +79 of the OPN promoter is responsive to inflammatory stimuli. These results also implied that genetically modified BMDCs expressing OPN (-777 or -740 to +79) driving the expression of mCherry can be used to reconstitute irradiated mice since the growth of progenitor colonies showed that transduction did not affect the ability of the BMDCs to proliferate and differentiate. In addition, these results suggest that reconstitution with the modulated BMDCs will allow us to visually examine the activation of the OPN promoter by locating the expression of mCherry. Since, we have only observed activation of the OPN promoter in the presence of

inflammatory stimuli; these results indicate that expression of the mCherry protein should only occur in an inflammatory environment such as tumor regions.

3.4.3 Validating reconstitution of irradiated *Apc$^{Min/+}$* mice with modulate BMDCs

To observe BMDCs expressing OPN (-777/-740) promoters controlling the expressing of mCherry in an *in vivo* model, we lethally irradiated existing BMDCs from *Apc$^{Min/+}$* mice. Six week post transplantation genomic DNA was extracted from the blood of mice and PCR amplified to confirm reconstitution with modulate BMDCs. Amplification of genomic DNA for mCherry expression concluded that we succeed in integrating the vector into the genome of mice (Figure 3.5); however the expression of mCherry needed validation. Thus, we sacrificed reconstituted mice to observe expression of mCherry within circulating BMDCs from the blood and in the bone marrow compartment by flow cytometry analysis. Even though, transduced BMDCs express GFP, we use antibodies against CD45 for a leukocyte cell surface- marker. Flow analysis of the circulating BMDCs in the blood show that approximately 25% or 4% of the modulate BMDCs express mCherry driven by OPN promoter -740 or -777 to +79 respectively (Figure 3.6a). An increase expressing of mCherry was observe in the bone marrow compartments, showing approximately 40% and 10% for OPN promoter -740 or -777 to +79 respectively (Figure 3.6b). The expression of mCherry in the mice show that the OPN promoter is functional, and since less than 50% of the transduced BMDCs are expressing mCherry in the blood and in the bone marrow

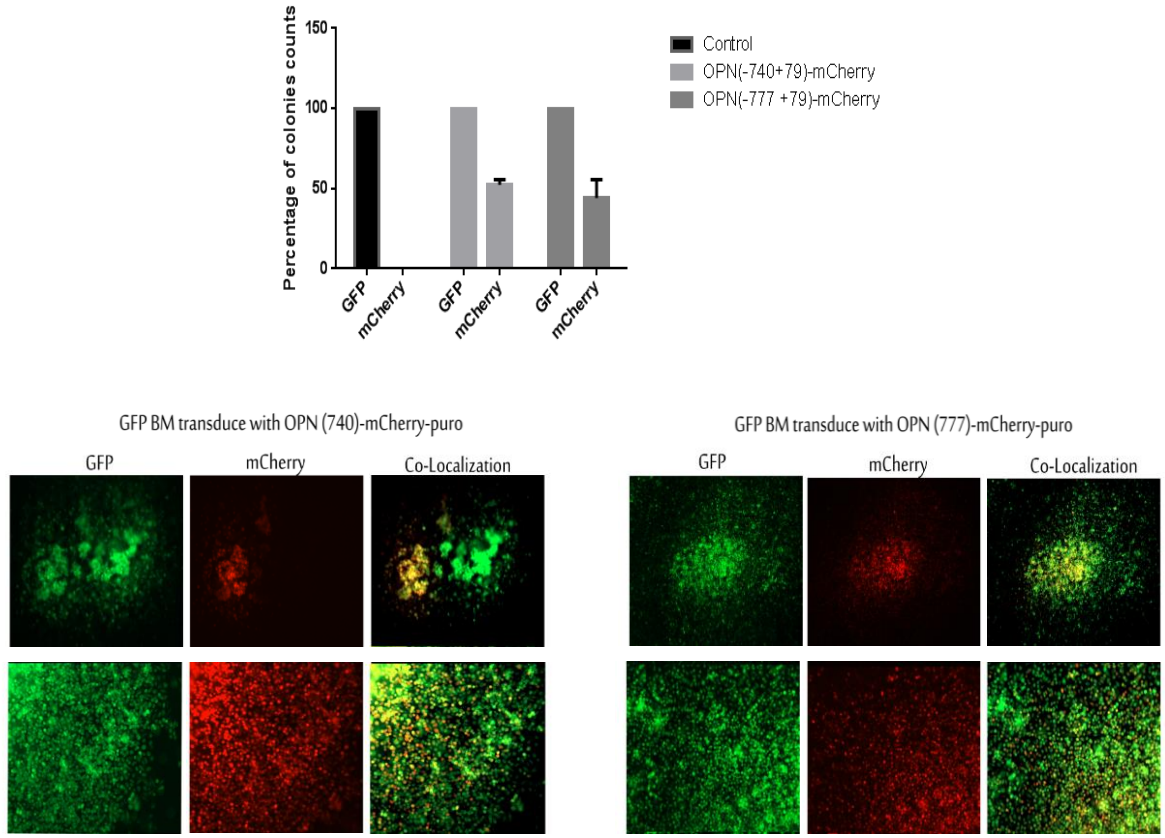


Figure 3.4: **Colony formation of modulate BMDCs expressing MSCV-OPN-mCherry vector.** a. Percentage of GFP colonies that were transduced with either OPN (740/777) - mCherry retroviral particles. 50% of the GFP colonies expressed mCherry that were transduced with OPN (740)-mCherry viral particles, and approximately less than 50% GFP colonies expressed mCherry that were transduced with OPN (777)-mCherry viral particles. b. Confocal microscopy images of methylcellulose colonies displaying separately the expression of GFP or mCherry, and the merged co-localization expression of GFP and mCherry (yellow).

compartment these results could be basal levels of OPN promoter activation, or the promoter response to systemic inflammation. This basal level of OPN promoter activation and mCherry expression may be problematic, for basal activities may cause a systemic effect when driving the expression of an RNAi sequence. It is essential that we determine which organs are infiltrated with modulated BMDCs. This will allow us to assess the basal activity of OPN promoter and determine the possibility of systemic disadvantageous for future *in vivo* assays with the addition of an RNAi sequence. In addition, we expected to

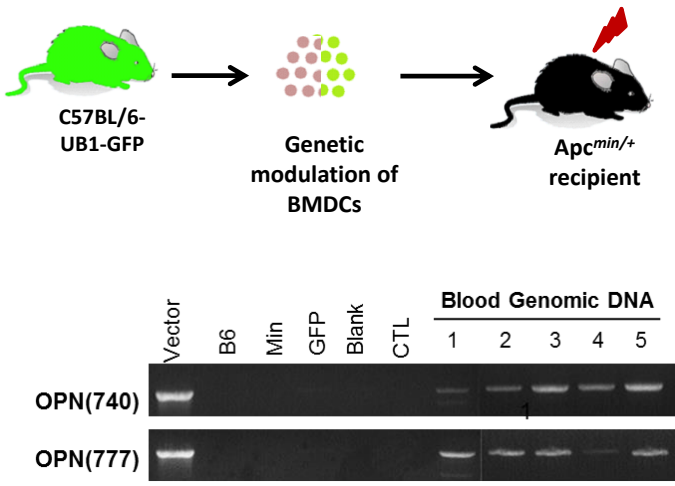


Figure 3.5: **Confirming incorporation of MSCV-OPN-mCherry vectors *in vivo*.** a. Depiction of extracting BMDCs from donor C57BL/6-GFP expression mouse to genetically modulate the cells with retroviral particles to express mCherry to transplant into lethally irradiate *APC*$^{Min/+}$ mice recipients. b. Confirmation of successful reconstitution of transduced BMDCs into recipient mice by PCR amplifying genomic DNA to detect mCherry gene.

observe basal activity of OPN promoter activation in tumor burden mice, for these mice are prone to more inflammation.

However if the expression of mCherry is not localized only in the tumor of *Apc*$^{Min/+}$ *mice*, the goal of tissue specific chemo- sensitivity will be impossible to

accomplish by using the OPN promoter. Since, we observed a greater increase of mCherry expression driven by OPN promoter (-740 to +79) region we only evaluated this region of promoter in tissue sections. Rationing that this region was to drive the expression of an RNAi sequence will cause the most harm systemically, since until now both promoter regions behave similar.

3.4.4 Examining activation of the OPN promoter in tissues of *Apc$^{Min/+}$* mice

To determine if activation of the OPN promoter and expression of mCherry in the *Apc$^{Min/+}$*mice various tissue samples, such as the spleen, liver, lung, normal and tumor intestinal regions were collected to be analyzed by confocal microscope. Confocal microscope allows us to examine the expression of GFP and mCherry. Visual confirmation of GFP confirmed the infiltration of BMDCs within different tissue, and the visualization of mCherry expression in tissues provided confirmation of the OPN promoter activity. Co-localization of GFP and mCherry expression view by yellow shows that infiltrated transduced BMDCs in specific tissue is responsive to inflammatory stimuli. Our, previous data imply that OPN is only induced by inflammatory stimuli, thus mCherry expression should only be observed in the tumor regions the intestines of *Apc$^{Min/+}$* mice. Figure 3.5 examining the various tissue section from control and experimental mice by confocal depicts images of the normal intestine regions, liver, and lung showing negative expression of mCherry in these tissues. Spleen tissue images show fluorescent background, yet we did not observe co-localization of mCherry and GFP expressions. On the other hand confocal images of the tumor regions in mice reconstituted with modified bone marrow show positive expression of

mCherry when compared with the control. We observe co-localization of GFP and mCherry expression only in the tumor regions of experimental group and not in control group tumor (Figure 3.6). This compelling data from confocal microscopy confirms that the OPN promoter is only induce in inflammatory

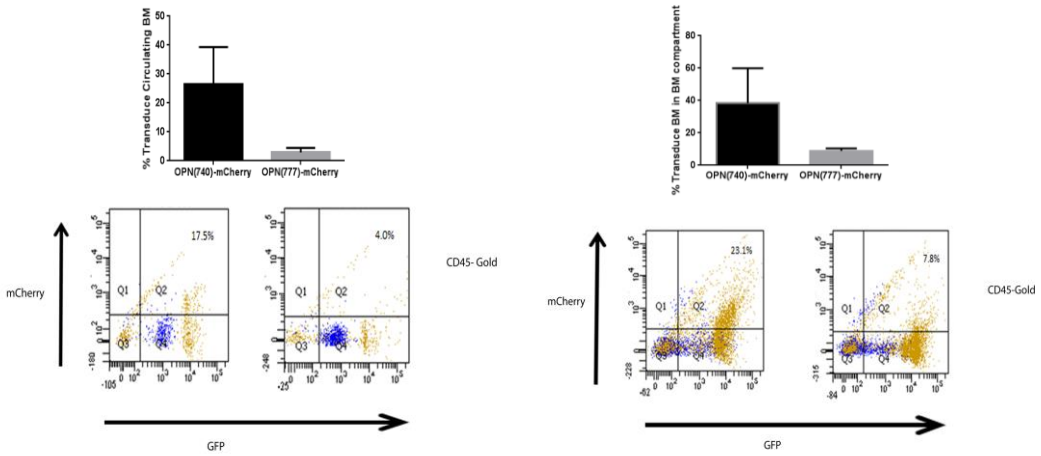


Figure 3.6: **Flow analysis of transduced BMDCs expressing MSCV-OPN-mCherry vector.** a. Approximation of the percentage of transduced BMDCs that expressed either OPN (740/777) - mCherry in the circulation of *APC*$^{Min/+}$ mice recipient from flow cytometry analysis. b. Approximation of the percentage of transduced BMDCs that expressed either OPN (740/777) - mCherry in the bone marrow compartment of *APC*$^{Min/+}$ mice recipient from flow cytometry analysis.

environment, for the expression of the mCherry was only observed in the tumor regions and nowhere else in the animal. This data validates that OPN promoter regions (-777/-740 to +79) will permit the ability to only chemo-sensitized the tumor microenvironment, but only if these regions of promoter are able to drive the expression of an RNAi sequence. .

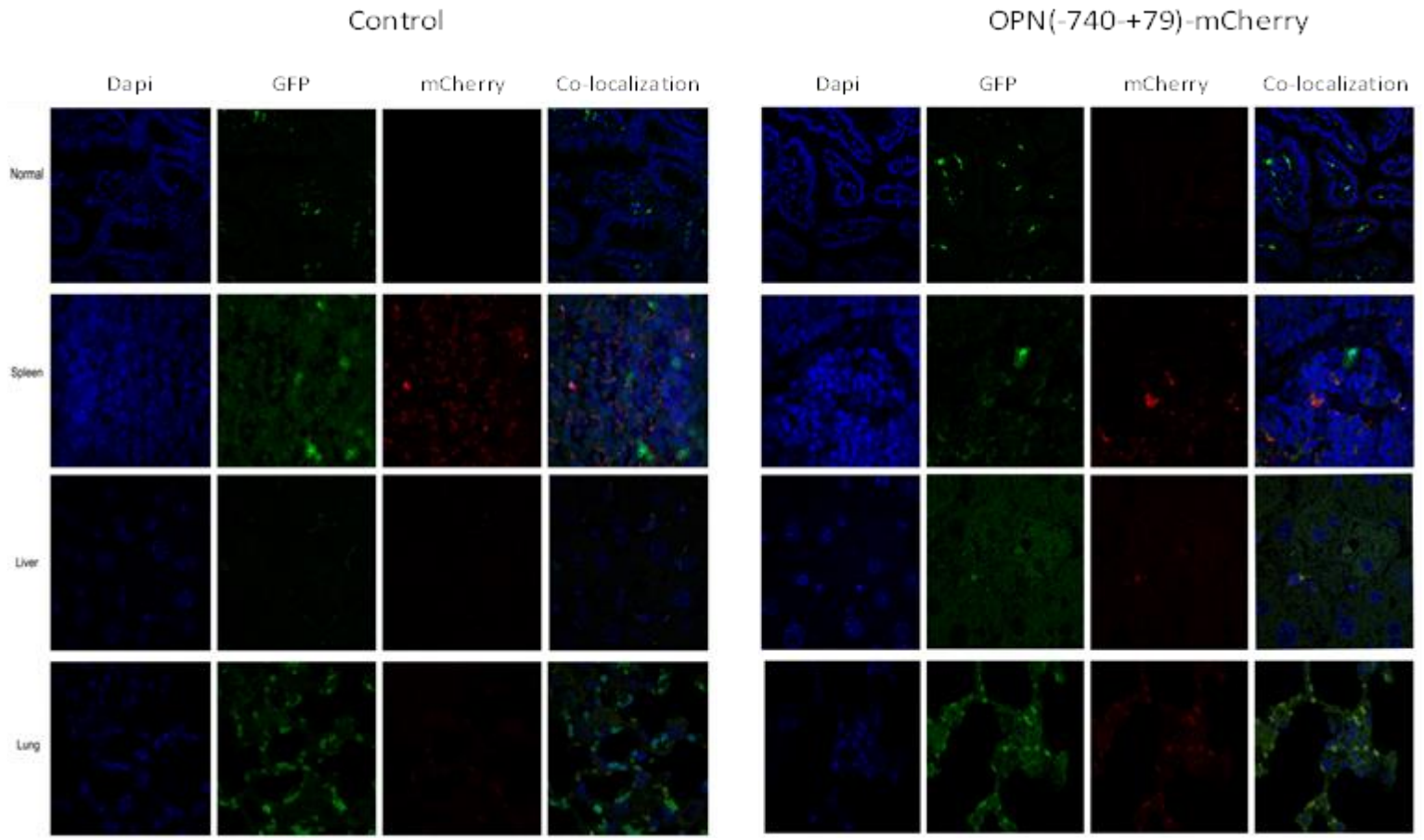


Figure 3.7: **Confocal microscopy analysis of the OPN promoter activity in various tissues.** Confocal images of tissues sections extracted from normal regions of the intestine, spleen, liver, and lungs of *APC*$^{Min/+}$ mice recipient. The nucleus stained by Dapi (blue), infiltration of BMDCs(green), expression of mCherry from BMDCs transduced with OPN(740)-mCherry (red), and co-localization (yellow). Infiltration of BMDCs occurred in both the normal intestinal and spleen tissues. Co-localization expression of GFP and mCherry was not observed in any tissue sections.

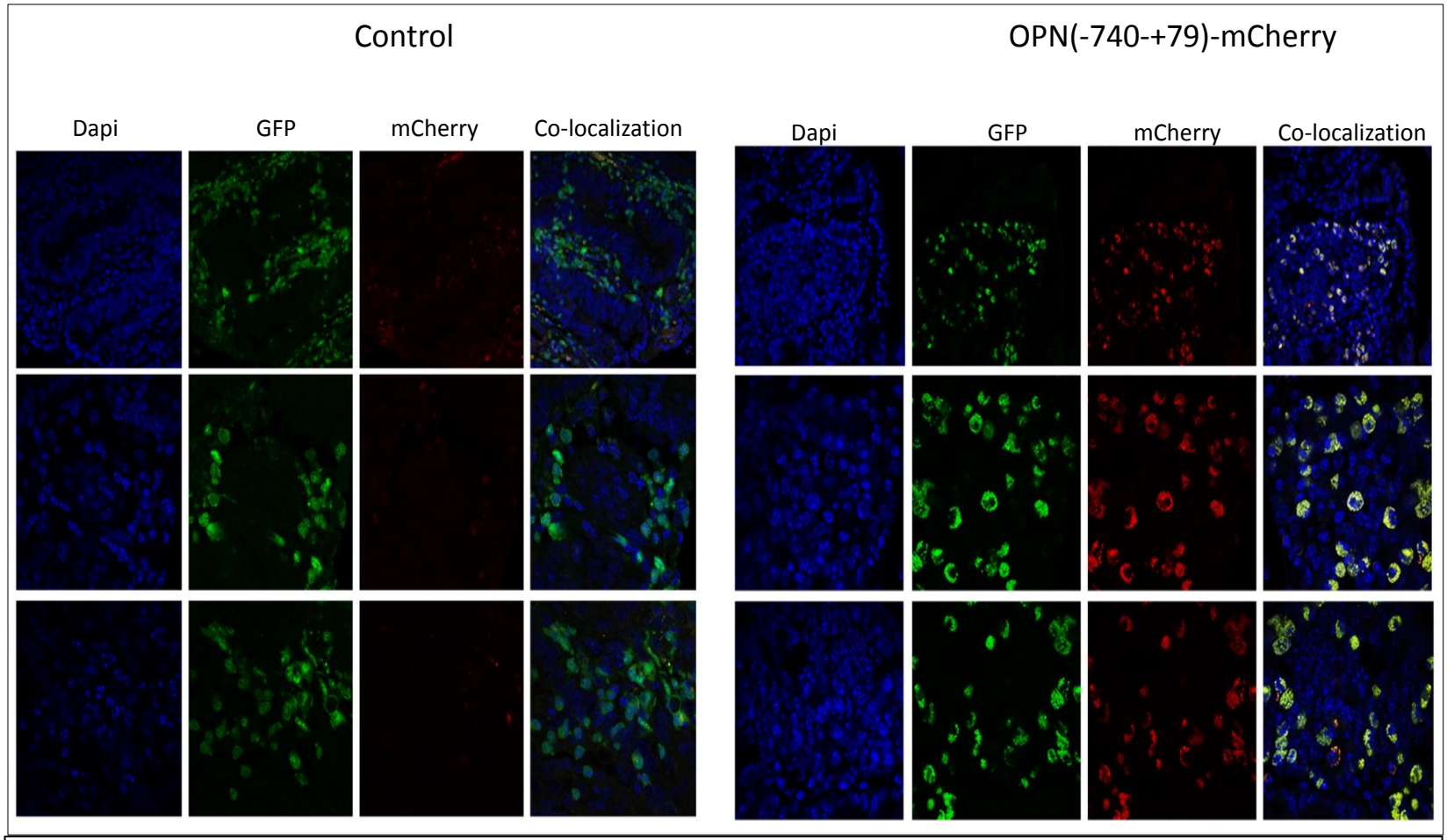


Figure 3.8: **Confocal microscopy analysis of the OPN promoter activity in the tumor region.** Confocal images of tissues sections extracted from the tumor regions of the intestine of *APC*$^{Min/+}$ mice recipient. The nucleus stained by Dapi (blue), infiltration of BMDCs(green), expression of mCherry from BMDCs transduced with OPN(740)-mCherry (red), and co-localization (yellow). Infiltration of BMDCs occurred in the tumor intestinal tissues. Co-localization expression of GFP and mCherry was only observed in the tumor section of recipients that were reconstituted with GFP BMDCs that were transduced to express OPN (740) – mCherry.

3.4.5 Ensuring OPN promoter aptitude to drive the expression of RNAi sequence against MusTS

We have shown that the OPN promoter (-777/-740 to +79) can drive the expression of the mCherry gene, but it is important to show that it has the capacity to drive the expression of an RNAi sequence. Initially, we used the pMSCV retroviral vector in which we inserted an RNAi sequence against MusTS between the OPN promoter region and the mCherry transcript. An internal ribosome entry site placed between the RNAi sequence and the mCherry gene to ensure transcription of both. We established stable expression in cell lines GP+E86 and CHL (MUS TS) in which cells treated in the presence or absence of inflammatory stimuli to observe reduction of Mus TS protein levels. Unfortunately, we were unable to observe a reduction in Mus TS protein levels (data not shown). To determine if these results were correct or if there is a problem with the construction of the vector design, we decided to evaluate the OPN promoter in the pZip lenti-viral vector. To accomplish this we removed the existing the mCMV promoter and replace it with the OPN (-740 to +79) promoter to drive, such that OPN promoter would drive the expression of Zsgreen and the RNAi sequence against MusTS. When stably expressed in CHL (Mus TS) cells, we observed an appropriate phenotype. Proteins levels of Mus TS were reduce by approximately 50% when compared to control and the vector control cells (Figure 3.9a/b). Demonstrating that the OPN promoter does has the potential to drive the expression of an RNAi sequence. Since the OPN promoter reduces protein levels, better than the mCMV promoter we need to analyze if this percentage of

reduction effected cell proliferation. Performing a growth assay, we show that cells expressing the RNAi sequence against MusTS, growth rate is not retarded

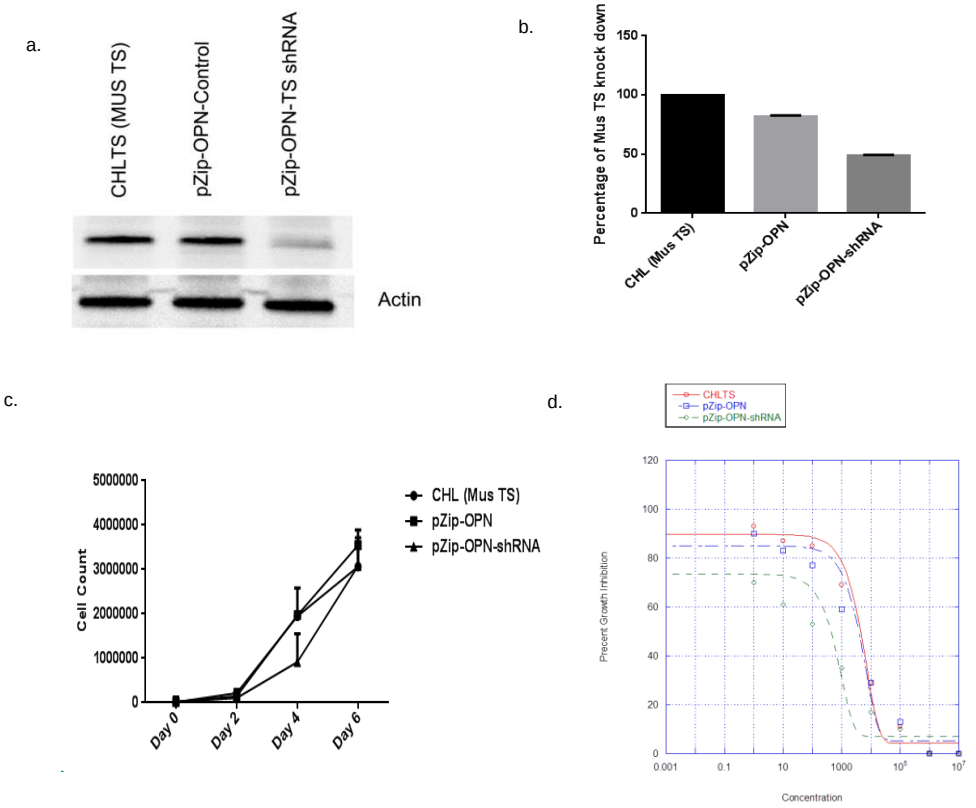


Figure 3.9**: Examining the ability of the OPN promoter to control an RNAi sequence.** a. Western blot analysis of CHL (Mus TS) stably transfected with pZip lenti-viral vector expressing either shRNA sequence or the vector control. Reduction of Mus TS protein is only observed in cells transduced with the silencing sequence. b. Quantification of the Mus TS protein shows the shRNA sequence reduces the expression of Mus TS protein by approximately 50%. c. Growth curve graph indicating that the growth rate of cells expressing the shRNA sequence is comparable to control cells or the cells expressing the vector control. d. Growth inhibition assay show that pZip vectors exhibit sensitivity to 5-FU therapy.

when compared to control cells or cells expressing the vector control (Figure 3.9c). Next we examine the ability of the OPN promoter to enhance sensitivity to

5-FU by treating cells that expressed either the pZip-OPN-shRNA or pZip-OPN control vector with increasing concentration of 5-FU to observe growth inhibition. Cells that were not transfected with either of the pZip vectors was used as an addition control to confirm that the vector control does not affect cellular response to 5-FU. Results indicate that cells that are expressing the RNAi

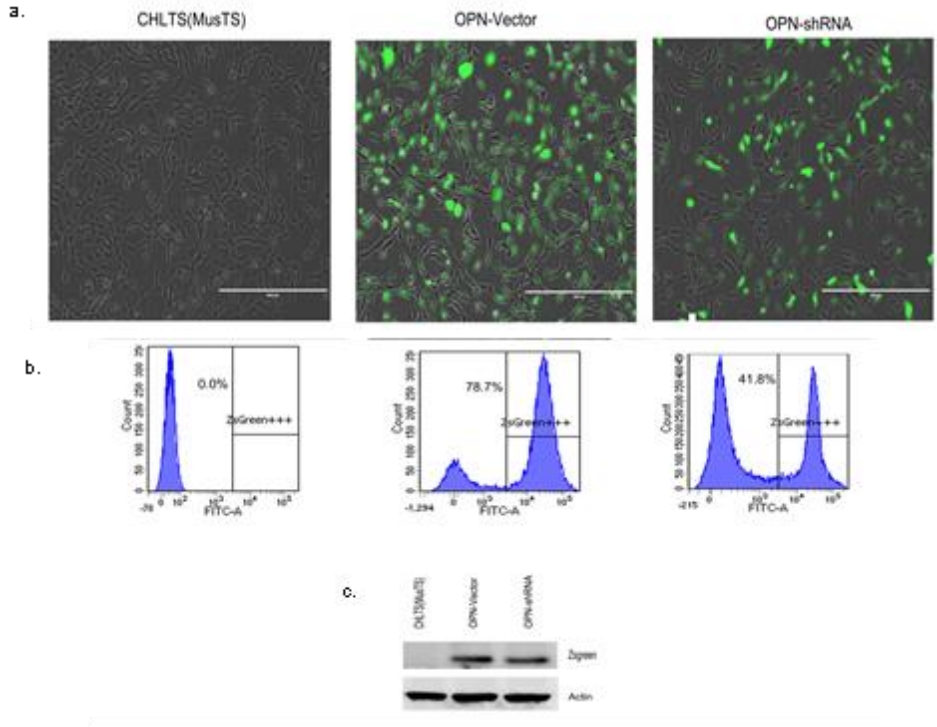


Figure 3.10: **Examining Zsgreen expression** a. Microscopy images of cells transduced to express pZip-OPN-vector and pZip-OPN-shRNA vector to observe Zsgreen expression in cells. b. Flow cytometry analysis of cells transduced to express pZip-OPN-vector and pZip-OPN-shRNA vector to quantify Zsgreen expression in cells. c. Western blot analysis to quantify protein expression of Zsgreen in transduced cells.

sequence against MusTS under the control of the OPN promoter are more sensitive to 5-FU therapy in comparison to the vector control and control cells

(Figure 3.9d). These premises further assure the advantage in using the OPN promoter to establish tissue specific chemo-sensitivity and drive the expression of RNAi technology.

Additional, we determine that the expression of the RNAi sequence does not corresponds to the expression of Zsgreen fluorescent marker under the control of the OPN promoter. With microscopy and flow cytometry analysis, we show that cells transduce to expression pZip-OPN vector control has a higher percentage of cells expression Zsgreen compared to cell transduce to express the pZip-OPN-shRNA vector (Figure 3.10a and b). Indicating that some cells in the pZip-OPN-shRNA group expresses the shRNA sequence, however the Zsgreen marker is silent. Results show that approximately 78% of the cells transduced to express pZip-OPN vector expressing the Zsgreen fluorescent protein, however only approximately 48% of the cells transduced to express the pZip-OPN-shRNA vector expresses the Zsgreen fluorescent protein. The expressing of the Zgreen protein in almost half expressed in the cell that were are expressing the RNAi sequence against MusTS, however most importantly these cells have been shown to be highly sensitivity to 5-FU treatment. This is significant result because this demonstrates that cells do not have to express Zsgreen to be more sensitive to 5-FU therapy.

3.4.6 Examining the apoptotic indices of modulate CHLT (MusTS) cells

To determine if we were able to enhance apoptosis in cells expressing the pZip-OPN-shRNA vector in comparison to the controls we treated the cells with

500nM or 10000nM of 5-FU for 5 days. These two concentration were used because 500nM of 5-FU is the concentration to 5-FU needed to inhibit 50% cell growth in cell expressing the pZip-OPN-shRNA vector previously shown in Figure 3.9c; and 10000nM of 5-FU was used because this concentration would ensure that we would observe apoptosis in cells. We decided to use flow cytometry analysis staining cells with Annexin V and viability dye antibodies. Using both of these antibodies together, we were able to obtain late apoptotic indices (Annexin V and viability dye positive cells) and early apoptotic indices (Annexin V only positive cells). In addition, we were able to determine the apoptotic indices of Zsgreen cells by identifying cells that express Zsgreen and Annexin V antibodies. The result from this study are displayed in fold changes comparing the no treated cells with the cells treated with either 500nm or 10000nM of 5-FU. For the late apoptotic indices we observe a 0.5, 0.2, and 0.7 fold change increase in cells treated with 500nM 5-FU for CHLT (Mus TS) control cells, cells transduced with pZIP-OPN vector control, and cells transduces to express pZip-OPN-shRNA vector respectively. The major fold change increase for late apoptotic indices occurred when cells were exposed to 10000nM 5-FU. These fold change increase are 5.0, 2.2, and 4.7 for CHLT (Mus TS) control cells, cells transduced with pZIP-OPN vector control, and cells transduced to express pZip-OPN-shRNA vector respectively (Figure 3.11a). Even though, we did not observe a significance in the apoptotic indices for late apoptosis we do indeed observe a significant difference in the early apoptotic indices in cells treated with the highest concentration of 5-FU. Results show increase fold changes of 26.1, 20.7, and

59.7 for CHLT (Mus TS) control cells, cells transduced with pZIP-OPN vector control, and cells transduced to express pZip-OPN-shRNA vector respectively (Figure 3.11b). These results show that cells transduced to express the pZip-OPN-shRNA vectors are highly apoptotic compared to the control, Thus confirming the RNAi sequence against MusTS is enhancing cellular sensitivity to

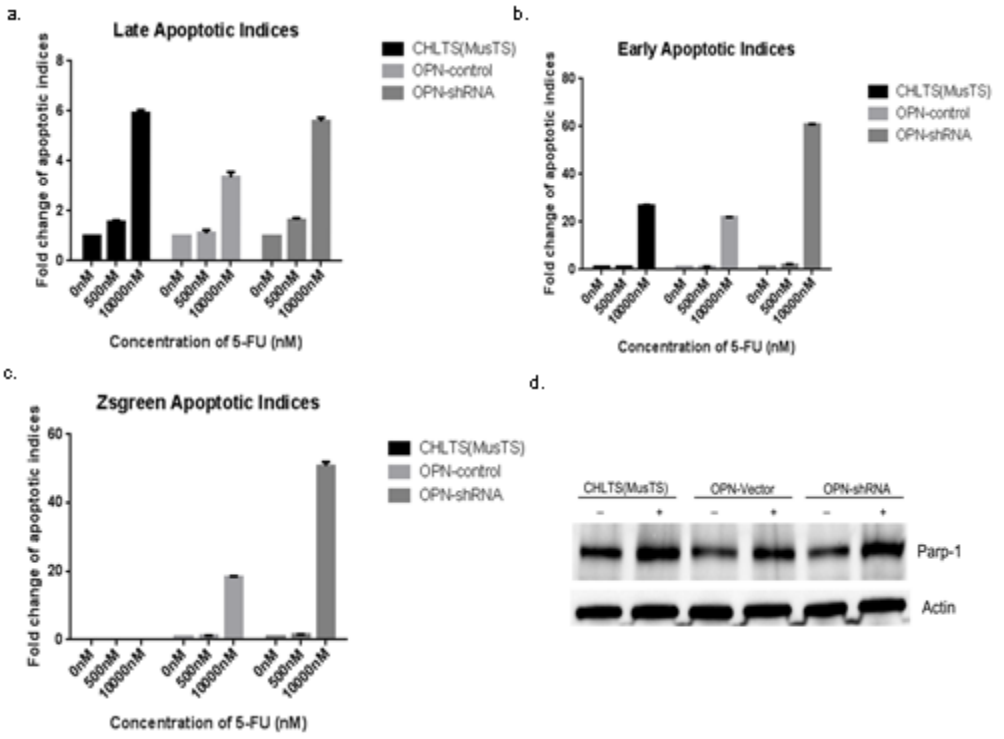


Figure 3.11: **Apoptotic indices of transduce CHLT (MusTS) cells.** a. The late apoptotic indices of cells treat with 5-FU. b. The early apoptotic indices of cells treated with 5-FU. c. The apoptotic indices of cells expressing Zsgreen. d. Western blot analysis of Parp-1 protein expressing from cells treated in the absence or present of 500nM of 5-FU.

5-FU. Upon, investigating the apoptotic indices in Zsgreen expressing cells results also show that cells expressing the RNAi sequences are highly more

apoptotic than cells that are expressing on the vector control. The fold change increase are 17.2 and 50.6 for pZip-OPN- vector control and pZip-OPN-shRNA vector respectively (Figure 3.11c). In addition western blot analysis of Parp-1 protein expression show and increase in cells treated with 500nM 5-FU when compared to cells that are not expose to the drug (Figure 3.11d). The premise of these finding confirms the ability to establish chemo-sensitivity within the TME.

3.4.7 Examining the apoptotic and proliferation indices of modulate BMDCs in Balb/c CT26 subcutaneous tumor model

To evaluate if the tissue specific chemo-sensitivity can be achieved by using the OPN promoter we performed an acute study which involve lethally irradiated Balb/c mice and reconstituted with BMDCs that expressed the pZip vector that contain Zsgreen and TS RNAi sequence driven by the OPN (-740 to +79) promoter. Controls for this study were Balb/c mice reconstituted with BMDCs that expressed the vector control that contain the Zsgreen gene under the control of the OPN promoter or GFP BMDCs. Four weeks post reconstitution mice with modulated BMDCs; genomic DNA was extracted from the blood to confirm incorporation of the Zsgreen gene in the genome (Figure 3.12). Six weeks post reconstitution CT26 colon cancer cells was injected in the flank of these mice. Tumors were allowed to grow to approximate 10-12mm diameter before mice were subjected to a single *i.p.* injection of either PBS or 50mg/kg 5-FU. Tumors average growth rates shown in Figure 3.12. We did not observe significant reduction in the growth of tumors in the experimental groups in comparison with the controls, which is expected since mice were only exposed to

a single injection of 50mg/ kg of 5-FU. Five days post therapy mice were sacrificed to collect the tumor for flow analysis to determine apoptotic and proliferation indexes in CD45+ immune cells, T cytotoxic cells (CD8a/CD8b), mast cells (CD34/CD117), MDSCs (Gr1/CD11b), and T regulatory cells

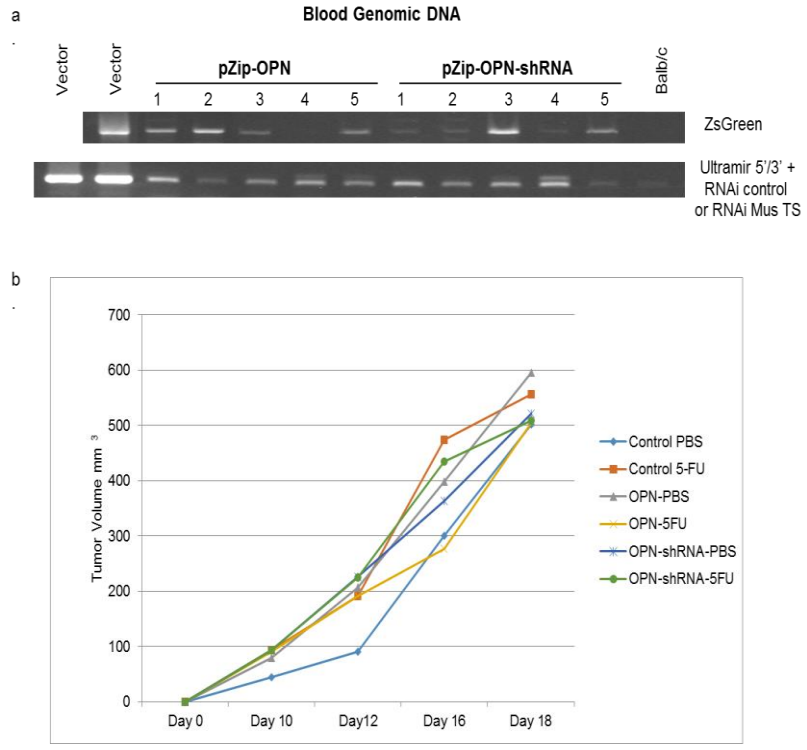


Figure 3.12: **Confirming incorporation of pZip-OPN lenti-viral vectors *in vivo***. a. Confirmation of successful reconstitution of transduce BMDCs into recipient mice by PCR amplifying genomic DNA for the incorporation of the Zsgreen and either the RNAi control or RNAi Mus TS sequence located between the ultramir 3' and 5' regions from the pZip-OPN control vector or of pZip-OPN-MusTS shRNA vector. b. CT26 cells tumor growth rate in mice that were reconstituted with modulate BMDCs or control BMDCs.

(CD4/Foxp3). We decided to obtain data five days post therapy, for previous result from our lab observed reduction in MDSCs, moderate effect in Tregs cells,

and resistance in mast cells in the tumor bed of Balb/c mice in a time course treatment study for ten days to observe BMDCs response to 5-FU in this advance colorectal model. The quantity of MDSCs and Tregs within the CT26 tumor in Balb/c mice displayed a 0.5 fold change five day after a single dose of 5-FU. Recovery for both these cells type were back to normalcy by day 7. Conversely, mast cells displayed a two folds increase within the tumors five days after 5-FU exposure and returned to basal level at day 7 (unpublished data Acharya, Grishma). Using this prior data as the initial point of interest, we expect to observe an enhancement in the reduction of MDSCs and Treg cells in response to 5-FU, but most importantly we expect to observe sensitivity of tumor mast cells to 5-FU in mice that are reconstituted with the genetically modulated BMDCs. In addition to these three cell types we decide to observe the effects of 5-FU on $CD45^{+}$ immune cells and cytotoxic T cells (Tc cells). Observing the response of the broad spectrum of immune cells, in particular CD45+ cells may provide a better depiction of the effect of 5-FU on BMDCs. Additionally, observing the effects of 5-FU on Tc cells will provide a depiction of how 5-FU disrupts antitumor immunity activities, which are typically performed by these cells.

Figure 3.13 represents the flow analysis, which provides the percentages of cells that are positive for the surface markers to identify the different cell types. In similarity with our previous acute time course study, we observed approximately 10% reduction in the MDSCs cell population five day post 5-FU subjection in the control and vector control groups when compared to the cohorts

subjected to PBS. However, the percentage of tumor MDSCs increased approximately 7% in mice transduced with BMDCs expressing the shRNA vector and exposed to 5-FU compared to the mice transduced with BMDCs expressing the shRNA vector and subjected to PBS (Figure 3.13a). Examination of the tumor Tregs show an approximately 3%, 1.5%, and 2% increase in animals treated with 5-FU for control, OPN vector control, and OPN-shRNA vector groups correspondingly compared to respective PBS animals (Figure 3.13b). Figure 3.13 c demonstrates an increase in tumor mast cells for the control and OPN-shRNA vector groups, approximately 2% and 10% respectively. However, for the OPN vector control and 5-FU treated mice, tumor mast cells were reduced approximately 1.5%. Behaving similarly to Tregs we observed an increase of tumor Tc cells in all groups, approximately 3%, 0.5%, and 5% for the control, OPN vector control, and OPN-shRNA vector mice expose to therapy correspondingly (Figure 3.13 d). Lastly, resembling the trend of tumor MDSCs we observed reduction of tumor CD45+ immune cells in both the control and OPN vector control 5-FU treated mice, approximately 16% and 5% respectively. However, an approximately 20% increase occurred in mice transduced with BMDCs expressing OPN-shRNA vector and treated with 5-FU compared to PBS cohorts (Figure 3.13 e).

Advance, flow analysis permitted examination of the total apoptotic and proliferation indices of each of these cell types. Additionally, the total apoptotic and proliferation populations further evaluation is accomplish by dividing the population into two subgroups: Zsgreen positive or Zsgreen negative apoptotic

and proliferative populations, which are depicted by the percentages bar graphs in Figures 3.14-3.18. The Zsgreen positive population identifies modulated BMDCs expressing either the OPN vector control or the OPN-shRNA vector. The double stained surface markers plus Zsgreen positive or Zsgreen negative Detection of AnnexinV antibody determined the apoptotic index, and detection of intercellular levels of ki67 determined the proliferation index for each target cell types.

Firstly, examining the of total apoptotic index of tumor MDSCs show that the percentage of apoptotic MDSCs decrease in the tumor of control mice treated with 5-FU in comparison to the PBS cohorts, however the apoptotic percentages of tumor MDSCs in mice reconstituted with transduced BMDCs expressing either lenti-viral vectors display an increase of apoptotic tumor MDSCs. There is a 3% and 16.8% increase of apoptotic tumor MDSCs in mice subjected to 5-FU in OPN vector or OPN-shRNA vector groups respectively, when compared to PBS cohorts (Figure 3.14 a). Notably the proliferation of tumor MDSCs does not decrease for any of the groups after 5-days post 5-FU treatment. The percentage of proliferative MDSCs for the PBS and 5-FU cohorts in the control group are similar; and a proliferative increase of a 4.4% and 17.5% occurred in OPN vector control and OPN-shRNA vector - 5-FU treated animals respectively, paralleled to PBS cohorts (Figure 3.14 b). Only for the 5-FU control group was there a 4.6% increase in the percentage of tumor Tregs, and no change in the percentage of proliferative tumor Tregs for this group when equated to control PBS mice. Yet there was a slight decrease of 1.5% or no variation in the percentage of apoptotic

Tregs in OPN vector control or OPN-shRNA vector reconstituted animals treated with 5-FU, however the proliferative percentage of Tregs does increase by 9% or 3.8% for each respective groups compared to PBS mice (Figure 3.15). Highlighting that the percentage of apoptotic tumor mast cells only displayed a percentage increased in mice reconstituted with BMDCs expressing the OPN-shRNA vector and treated with 5-FU by 2.6% elevation compared to the PBS cohorts. The other 5-FU treated mice for control and the OPN-vector control showed a decreased in the percentage of apoptotic tumor mast cells when compared to PBS associates. However, an increase in the total percentage of proliferative tumor mast cells occurred in OPN vector control and OPN-shRNA vector groups that were exposed to 5-FU, whereas a reduction was seen in the control 5-FU group (Figure 3. 16). Observing the percentage of tumor apoptotic Tc cells result show a decrease for both control and OPN vector control, 3.6% and 7.4% reduction respectively, however, result show an increase of 12.5% for the OPN-shRNA vector reconstituted animals when equated to the corresponding PBS treated mice. All groups displayed a reduction in the percentage of tumor proliferative Tc cells (Figure 3.17). Lastly, examining the percentage of apoptotic CD45+ immune cells in the tumor after 5-FU treatment show an increase for all groups compared to the PBS treat animals. The increase of apoptotic CD45+ immune cells is 0.5%, 1.2%, and 9.7% for control, OPN vector control, and OPN-shRNA vector 5-FU treated mice respectively. Only the proliferative percentage for CD45+ immune cells increased by 15.2% for mice reconstituted with BMDCs expressing the OPN-shRNA vector and subjected to

5-FU, whereas the decrease in the percentage of proliferative CD45+ immune cells was observed in the control and OPN-vector control 5-FU subject mice compared to PBS counterparts (Figure 3.18).

Advance analysis of the apoptotic and proliferative indices of each cell types by dividing the total percentage of indices into subgroups dependent on present or absent of Zsgreen expressing will indicate if modulate BMDCs expressing the shRNA sequence is more sensitive to 5-FU therapy. It is important to note that expression of Zsgreen does not determine correlate with expression of the shRNA sequence against TS. For we did not determine the difference of expression of TS protein levels in Zsgreen or non-Zsgreen cell types. Thus, briefly examining the of apoptotic indices of MDSCs, mast cells, Tregs cells, and Tc cells that are Zsgreen positive result show no increase the percentage of apoptotic cells after 5-FU treatment compared to PBS. However, examining the non-Zsgreen cells of each of these cell types that are transduced to express the shRNA show an increase in the apoptotic indices compared to the PBS group, thus suggesting the shRNA may be expressed in some of the non-Zsgreen cells and thus enhance cellular sensitivity to 5-FU treatment. Also, to confirm this implication analyzing the result for the CD45+ immune cells modulated to express OPN-shRNA vector, result shows that in both subgroups Zsgreen positive and negative cells after 5-FU therapy increases the percentage of apoptotic CD45+ immune cells equated to the PBS treated cohorts.

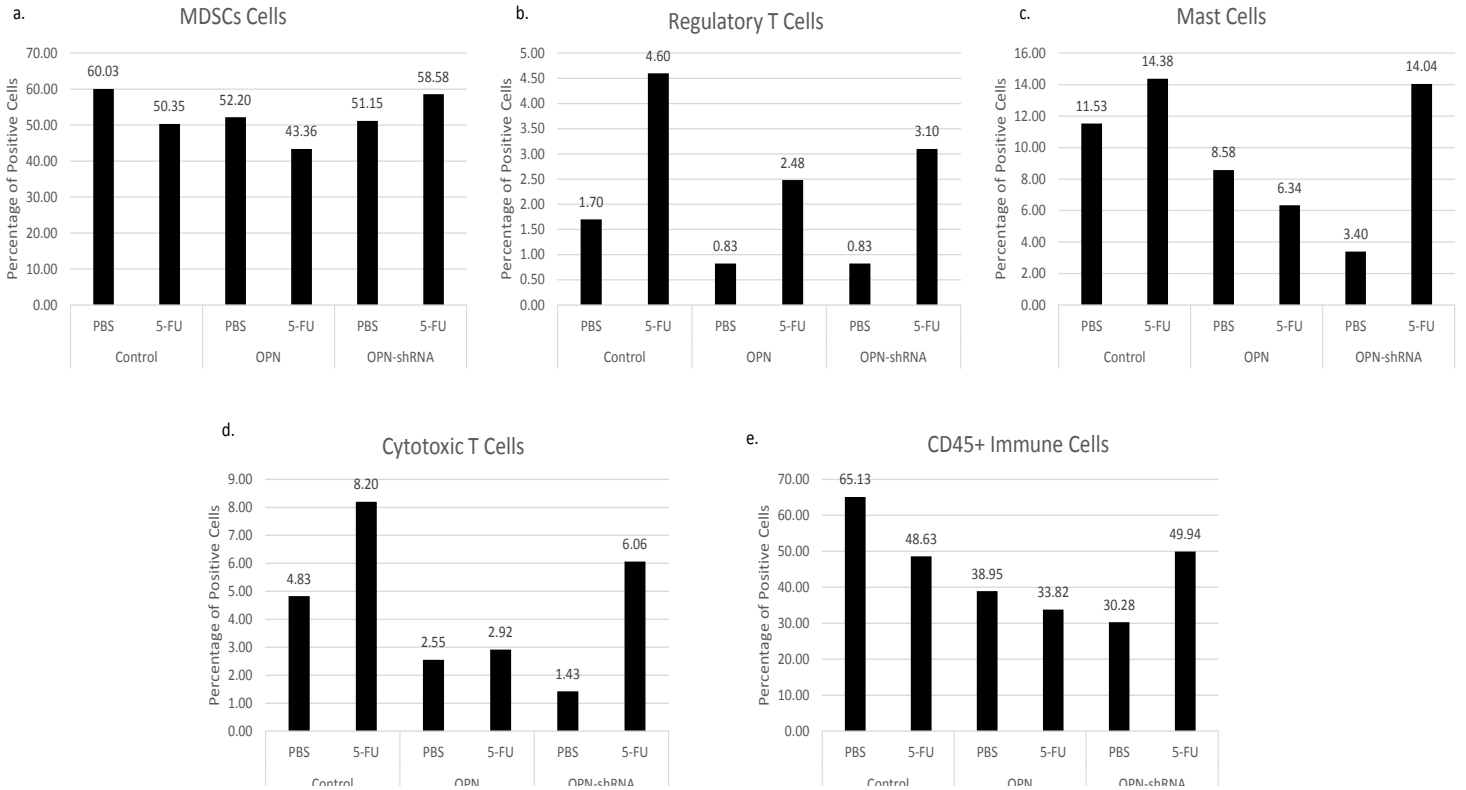


Figure 3.13: **Flow analysis of tumor BMDCs populations.** a. The total percentage of tumor MDSCs double stained with CD11b and Gr1 antibodies. b. The total percentage of tumor regulatory T cells stained with CD4 and Foxp3 antibodies. c. The total percentage of tumor mast cells stained with CD34 and CD117 antibodies. d. The total percentage of tumor cytotoxic T cells stained with CD8a and CD8b antibodies. e. The total percentage of CD45+ immune cells stained with CD45 antibody. Each graph include mice reconstituted with non- transduce BMDCs (Control), or mice reconstituted with BMDCs expressing either the OPN- vector control (OPN), or OPN-shRNA vector (OPN-shRNA). Each of these engraftment groups are subdivided into two groups of animals either exposed to PBS for the control or to 50mg/kg of 5-FU for the treatment and experimental group.

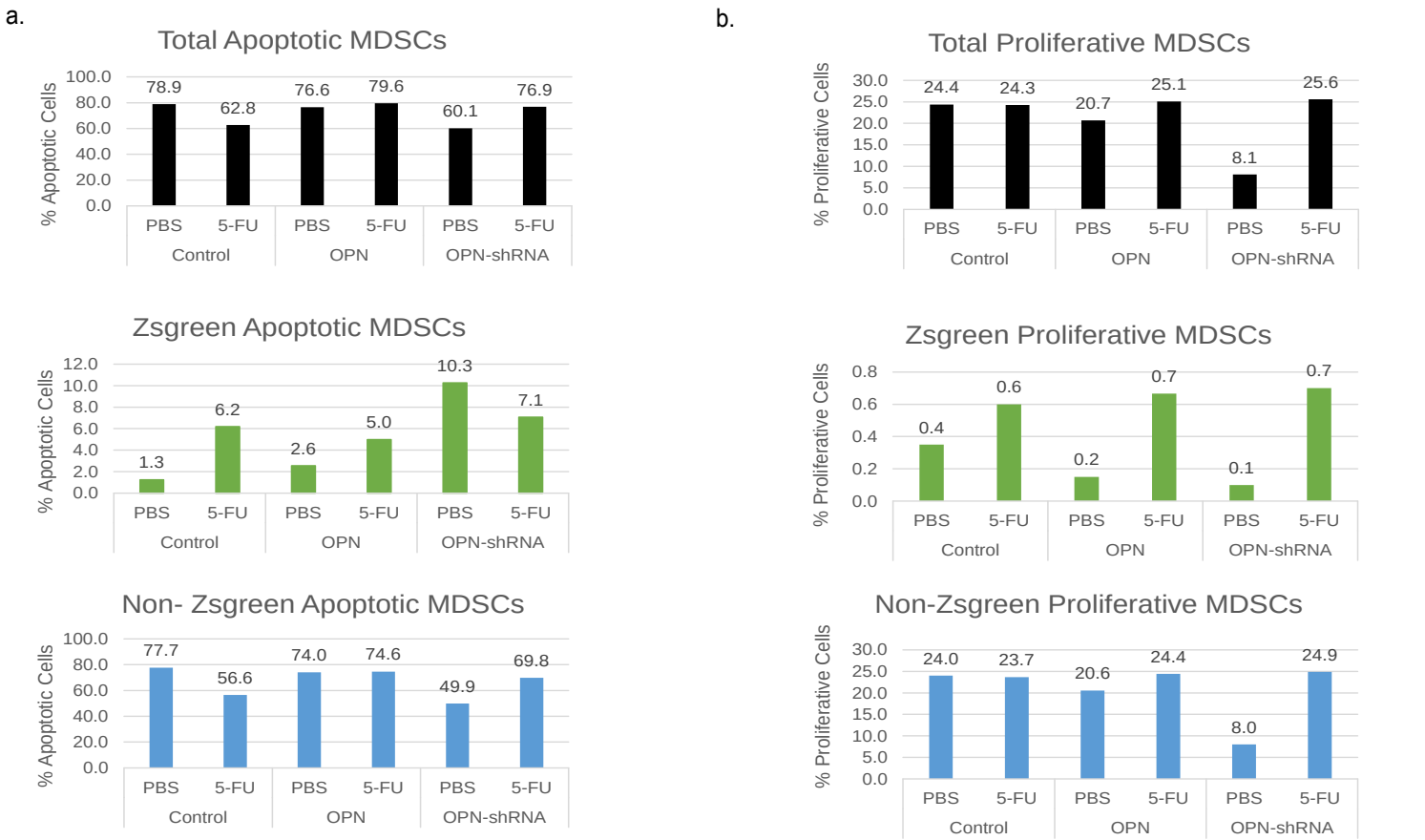
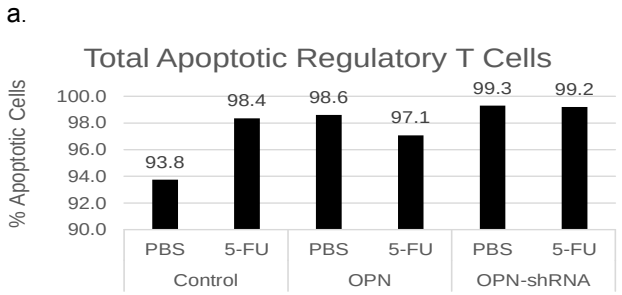
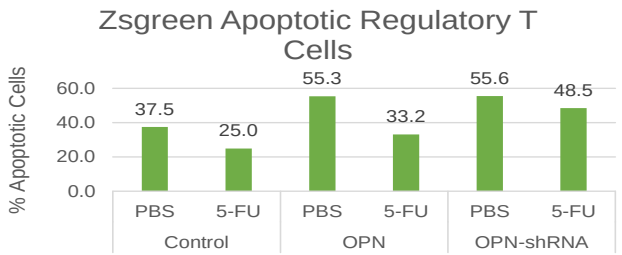
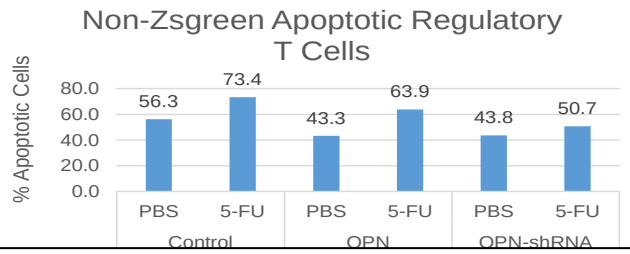
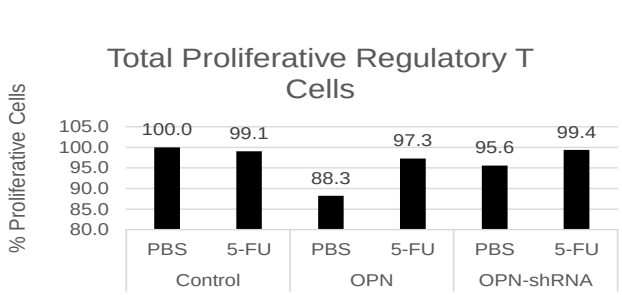


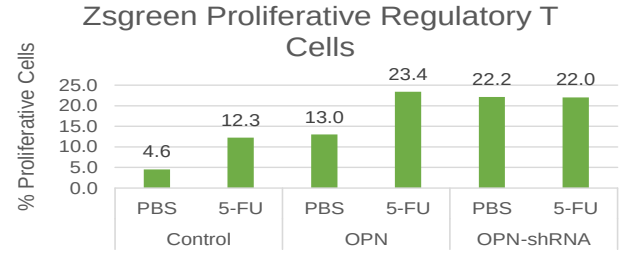
Figure 3.14: **Apoptotic and proliferation indices for tumor MDSCs.** a. The apoptotic indices of tumor MDSCs for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). b. The proliferative indices of tumor MDSCs for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). Mice reconstituted with the various BMDCs were either subjected to PBS or 50mg/kg of 5-FU.











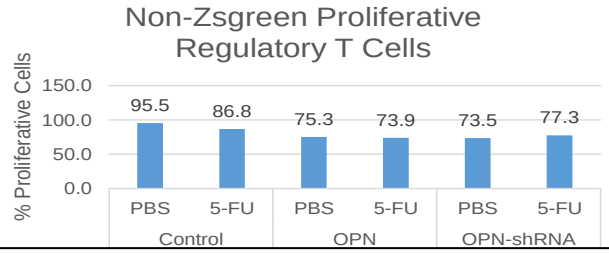


Figure 3.15: **Apoptotic and proliferation indices for tumor Tregs cells.** a. The apoptotic indices of tumor Tregs cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). b. The proliferative indices of tumor Tregs cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). Mice reconstituted with the various BMDCs were either subjected to PBS or 50mg/kg of 5-FU.

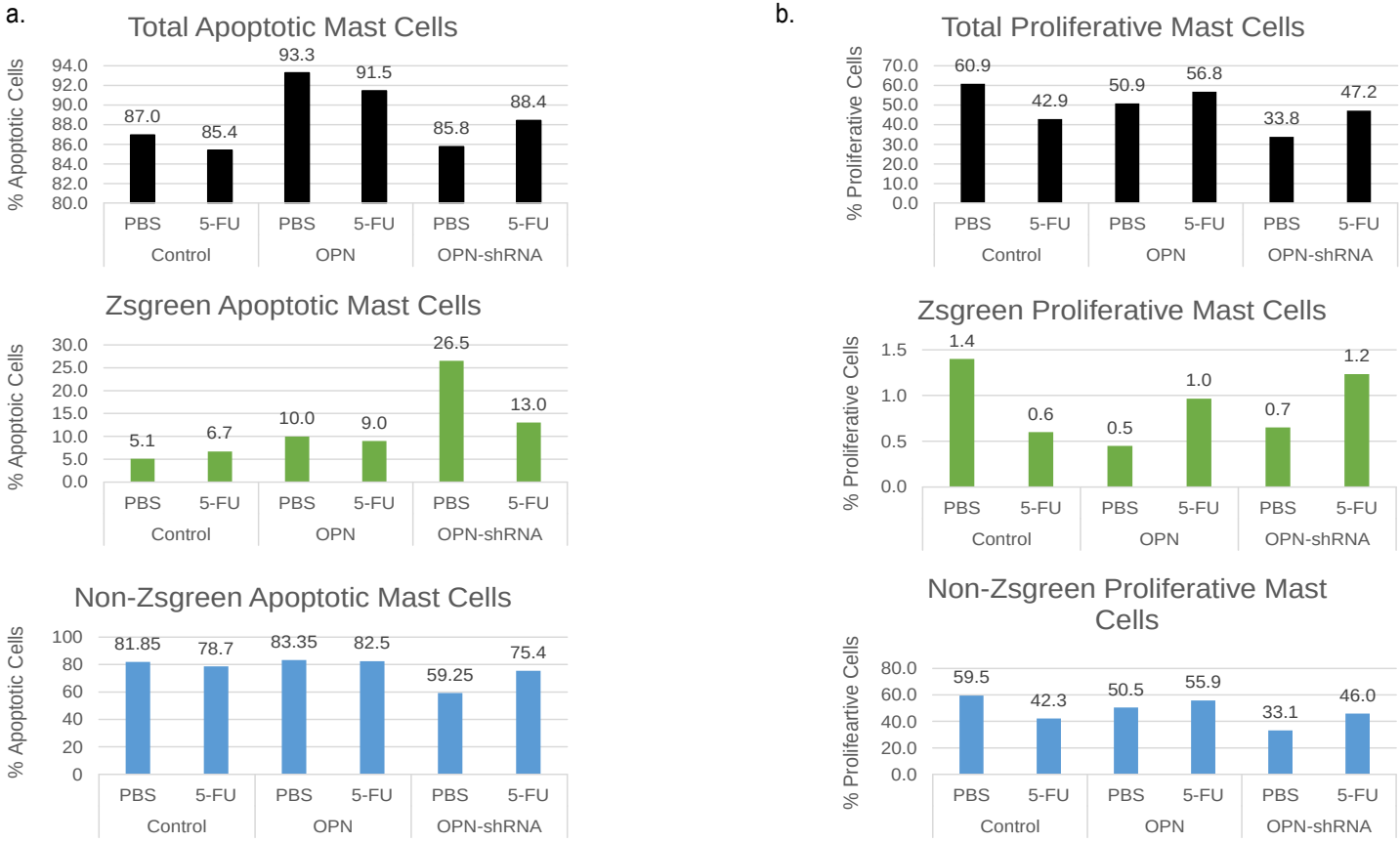
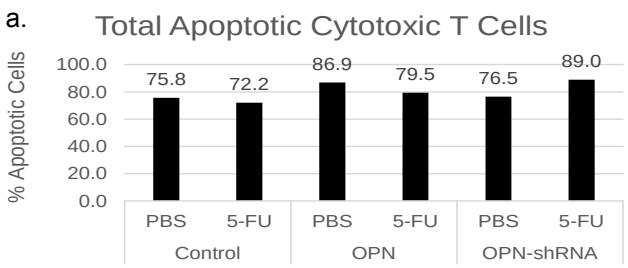
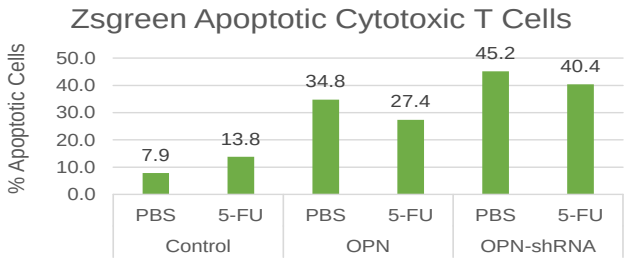
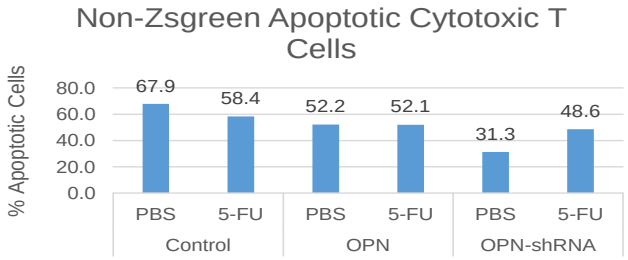
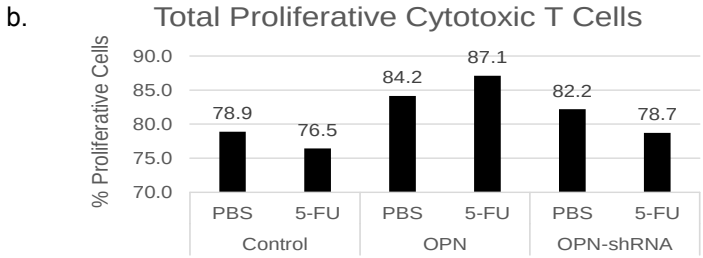


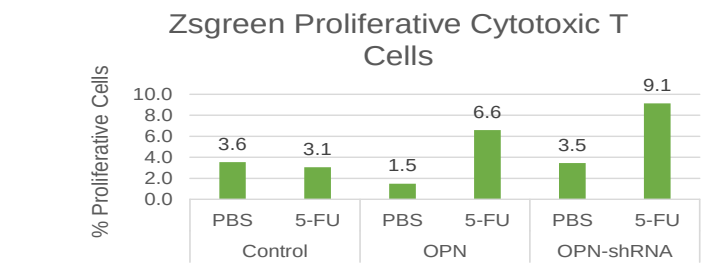
Figure 3.16: **Apoptotic and proliferation indices for tumor mast cells** a. The apoptotic indices of tumor mast cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). b. The proliferative indices of tumor mast cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). Mice reconstituted with the various BMDCs were either subjected to PBS or 50mg/kg of 5-FU.











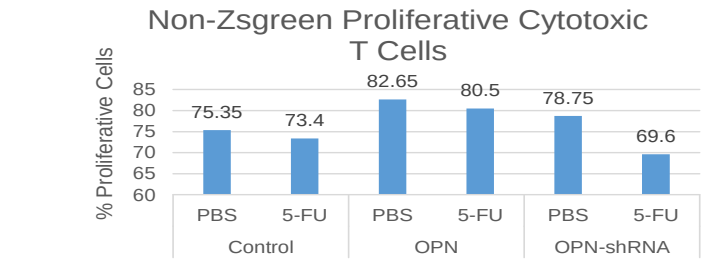
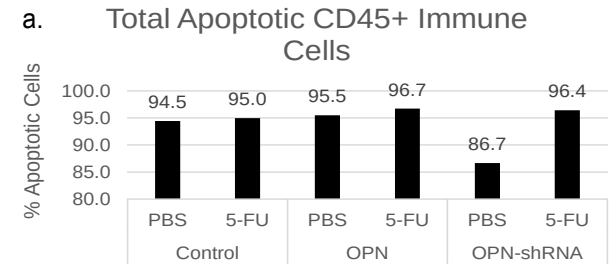
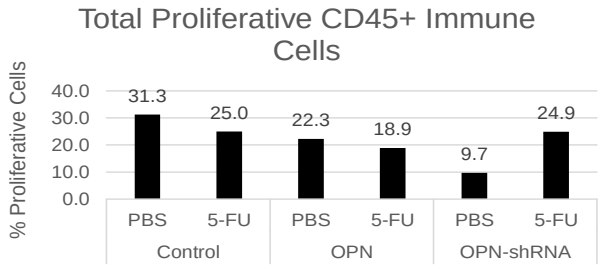
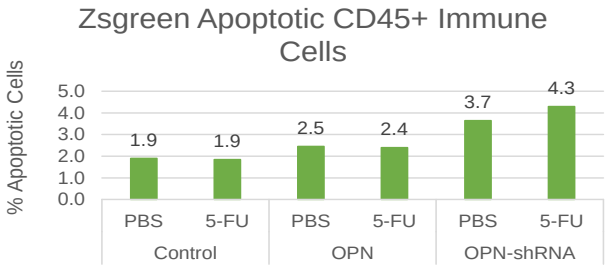
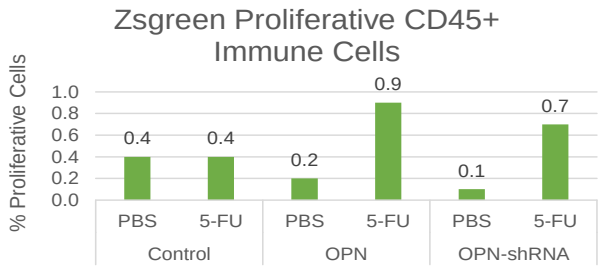


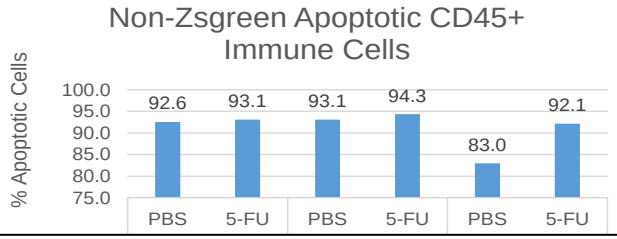
Figure 3.17: **Apoptotic and proliferation indices for tumor Tc cells.** a. The apoptotic indices of tumor Tc cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). b. The proliferative indices of tumor Tc cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). Mice reconstituted with the various BMDCs were either subjected to PBS or 50mg/kg of 5-FU.











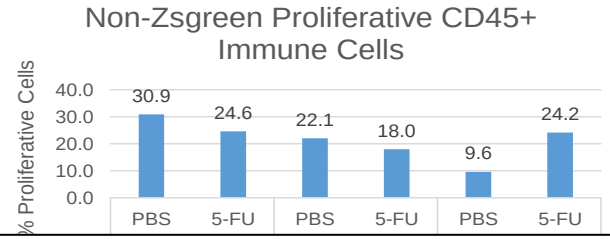
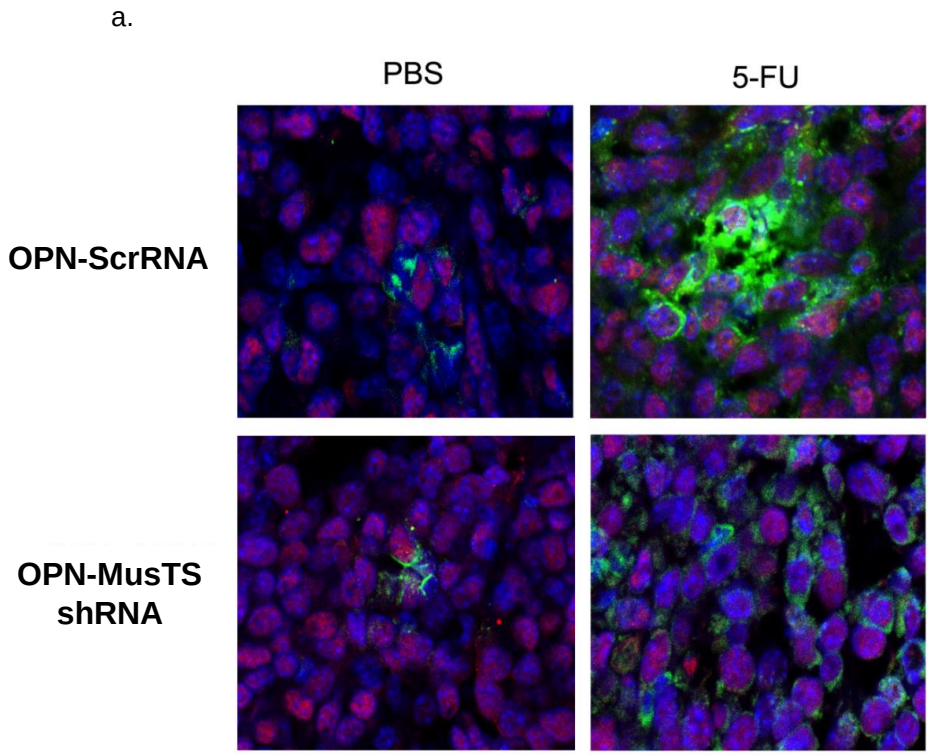


Figure 3.18: **Apoptotic and proliferation indices for CD45+ immune cells.** a. The apoptotic indices of tumor CD45+ immune cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). b. The proliferative indices of tumor CD45+ immune cells for mice either reconstituted with non-transduced BMDCs (Control) or modulated BMDCs expressing either OPN-vector control (OPN) or OPN-shRNA vector (OPN-shRNA). Mice reconstituted with the various BMDCs were either subjected to PBS or 50mg/kg of 5-FU.

Since, the percentages of apoptotic cells were higher than expected from the flow analysis. We further analyze the apoptotic indices of some cells by confocal imagery. Unlike flow analysis, we were unable to stain with two surface markers to identify the different cell types. Thus, for MDSCs we stained for both granulocytes and monocytes by using antibodies against surface markers Ly-6 and Cd11b respectively, antibodies against surface marker c-kit was used to identify mast cells, and to determine the apoptotic cells we stained with cleaved PARP-1. Thus, the combination of one surface marker and cPARP-1 antibodies were used to determine the apoptotic indices of Ly-6 positive granulocyte, CD11b positive monocytes, and c-kit positive mast cells. The apoptotic indices for each of these cells are express by the percentage of the cells stained positive for both the surface marker and a cPARP-1 antibody, which was accomplished by counting 100 field views for each cell type, and PBS treated mice, were used to determine apoptotic baseline of each cell type. Figure 3.19 shows the apoptotic indices for Ly-6 cells, and results show that 5-FU treated mice apoptotic indices increases approximately 6% and 14% for mice reconstituted with BMDCs transduce to express pZip-OPN vector control or pZip-OPN-shRNA vector respectively. This same trend is observed in CD11b positive cells showing approximately an 8% and 17% increase (Figure 3.20), and for c-kit positive cells showing approximately, a 3% and 7% increase for mice treated with 5-FU and engrafted with modulate BMDCs (Figure 3.21). In comparing 5-FU, treated animals we show that mice engrafted with BMDCs expressing the shRNA vector had more apoptotic monocytes, granulocytes, and mast cells. Even though,

these are promising results, we were unable to determine which of the Ly6, CD11b, and c-Kit positive cells were transduced to express either the pZip-OPN vector control or the pZip-OPN-shRNA vector. Due to the inability to distinguish the real signal of Zsgreen in the Fittc channel from the confocal microscopy analysis, we are unable to quantify the number of cells that are transduced. Therefore, are unable to determine if the transduced cells expressing the RNAi sequence are highly apoptotic cells when compared to the controls. However, we are able to infer this data by combining results from our flow analysis with our results from our confocal microscopy analysis. From our flow analysis, we are able to determine the percentage of cells that are positive for both Zsgreen and MDSC or mast cell markers, and by using this percentage has a reference to infer the percentage of Ly6, CD11b, or c-Kits positive stained cells from confocal microscopy analysis which maybe Zsgreen. Theoretically, this is logically since these are the same tumors samples for both flow and confocal microscopy analysis. These inference results in Figure 3.22 show the difference between cells transduced to express the pZip-OPN vector control and the pZip-OPN-shRNA vector. Results show that Zsgreen Ly6 positive cells treated with 5-FU has either a 5% or 11% increase in mice engrafted with BMDCs expressing pZip-OPN vector control or pZip-OPN-shRNA vector respectively (Figure 3.22a). Figure 3.22 b and c show the same trend observed in Zsgreen CD11b demonstrated a 7% or 13% increase, and for Zsgreen c-Kit positive cells a 0% or 5% increase for samples that expressed the pZip-oPN vector control or the pZip-OPN-shRNA vector respectively.



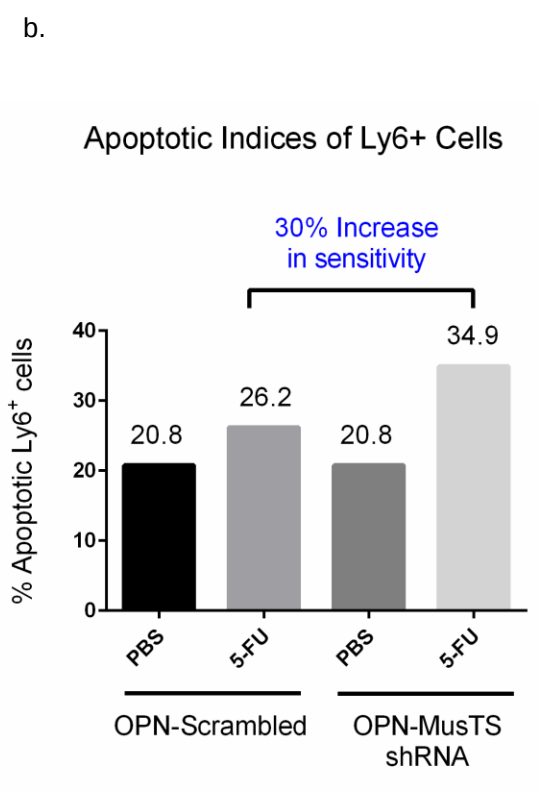


Figure 3.19: **Apoptosis induced in granulocytes at 5 days after 5-FU administration.** Tumor tissues from reconstituted Balb/c mice were stained with rat monoclonal primary antibody againstLy-6 and rabbit polyclonal primary antibody against c-PARP-1. The samples were then incubated with Cy-3-conjugated donkey anti-rat secondary antibody and Cy5-conjugated donkey anti-rabbit secondary antibody, respectively. 100 total images were taken for both PBS and 5-FU samples... a. Representative field views of confocal images of tumor sections from PBS and 5-FU treated mice are shown. Green = Gr-1 positive cells (granulocytes), Red = cleaved PARP-1 (apoptotic cells). Double positive cells indicate apoptotic granulocytes. b. Percentage of apoptotic Ly-6 cells in the tumor tissues of mice reconstituted with modulated BMDCs.

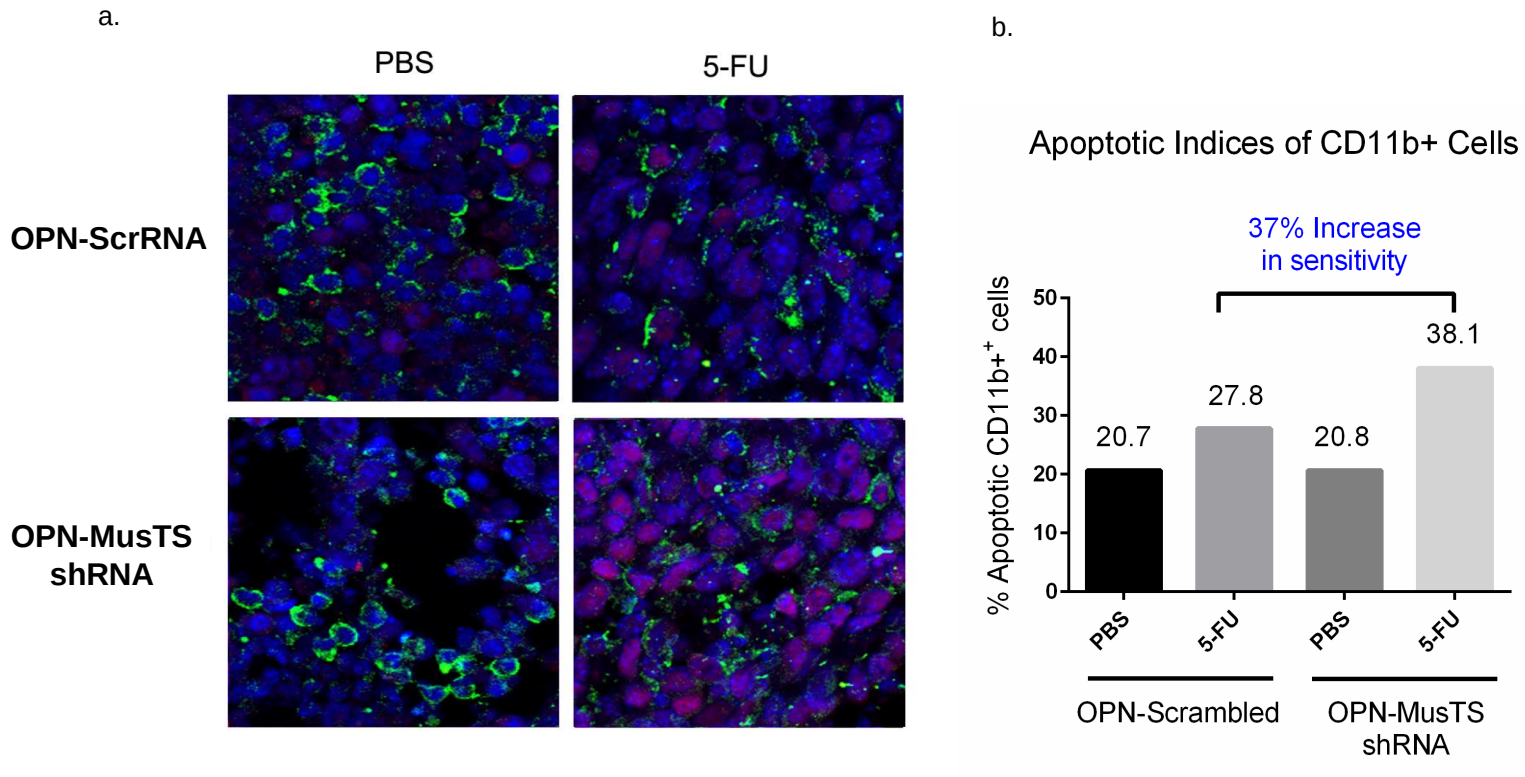
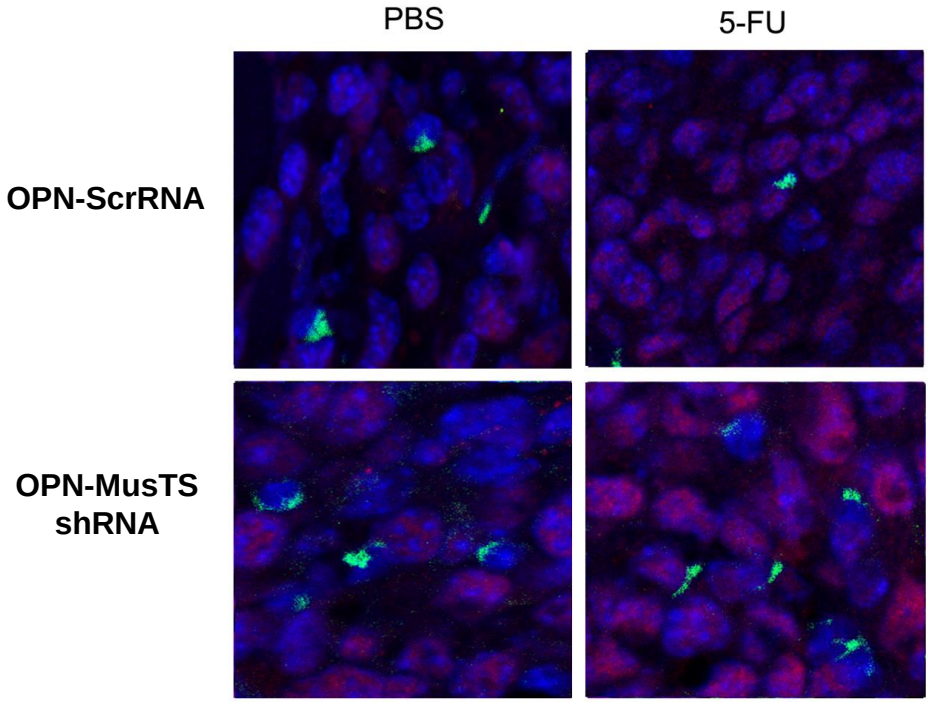


Figure 3.20: **Apoptosis induced in monocytes at 5 days after 5-FU administration.** Tumor tissues from reconstituted Balb/c mice were stained with rat monoclonal primary antibody againstCD11b and rabbit polyclonal primary antibody against c-PARP-1. The samples were then incubated with Cy-3-conjugated donkey anti-rat secondary antibody and Cy5-conjugated donkey anti-rabbit secondary antibody, respectively. 100 total images were taken for bothPBS and 5-FU samples. .a. Representative field views of confocal images of tumor sections from PBS or 5-FU treated mice are shown. Green = CD11b positive cells (granulocytes), Red = cleaved PARP-1 (apoptotic cells). Double positive cells indicate apoptotic monocytes. b. Percentage of apoptotic CD11b cells in the tumor tissues of mice reconstituted with modulated BMDCs



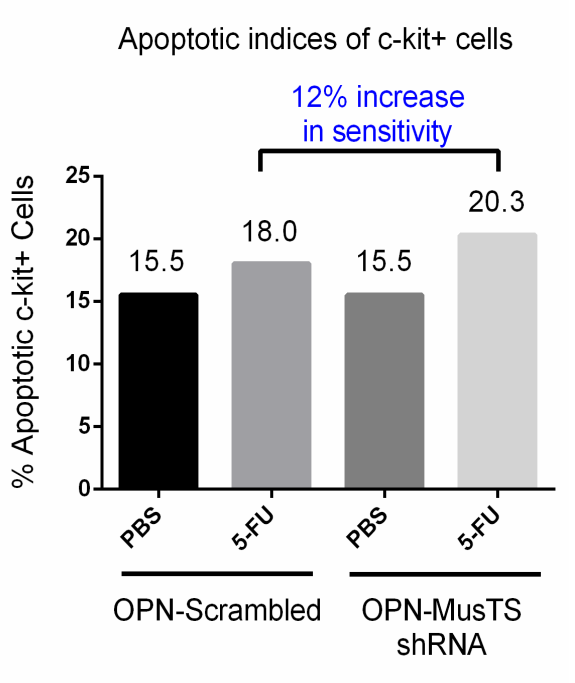


Figure 3.21: **Apoptosis induced in mast cells at 5 days after 5-FU administration.** Tumor tissues from reconstituted Balb/c mice were stained with rat monoclonal primary antibody against c-Kit and rabbit polyclonal primary antibody against c-PARP-1. The samples were then incubated with Cy-3-conjugated donkey anti-rat secondary antibody and Cy5-conjugated donkey anti-rabbit secondary antibody, respectively. 100 total images were taken for both PBS and 5-FU samples. a. Representative field views of confocal images of tumor sections from PBS or 5-FU treated mice are shown. Green = c-kit positive cells (mast cells), Red = cleaved PARP-1 (apoptotic cells). Double positive cells indicate apoptotic monocytes. b. Percentage of apoptotic c-kit cells in the tumor tissues of mice reconstituted with modulated BMDCs

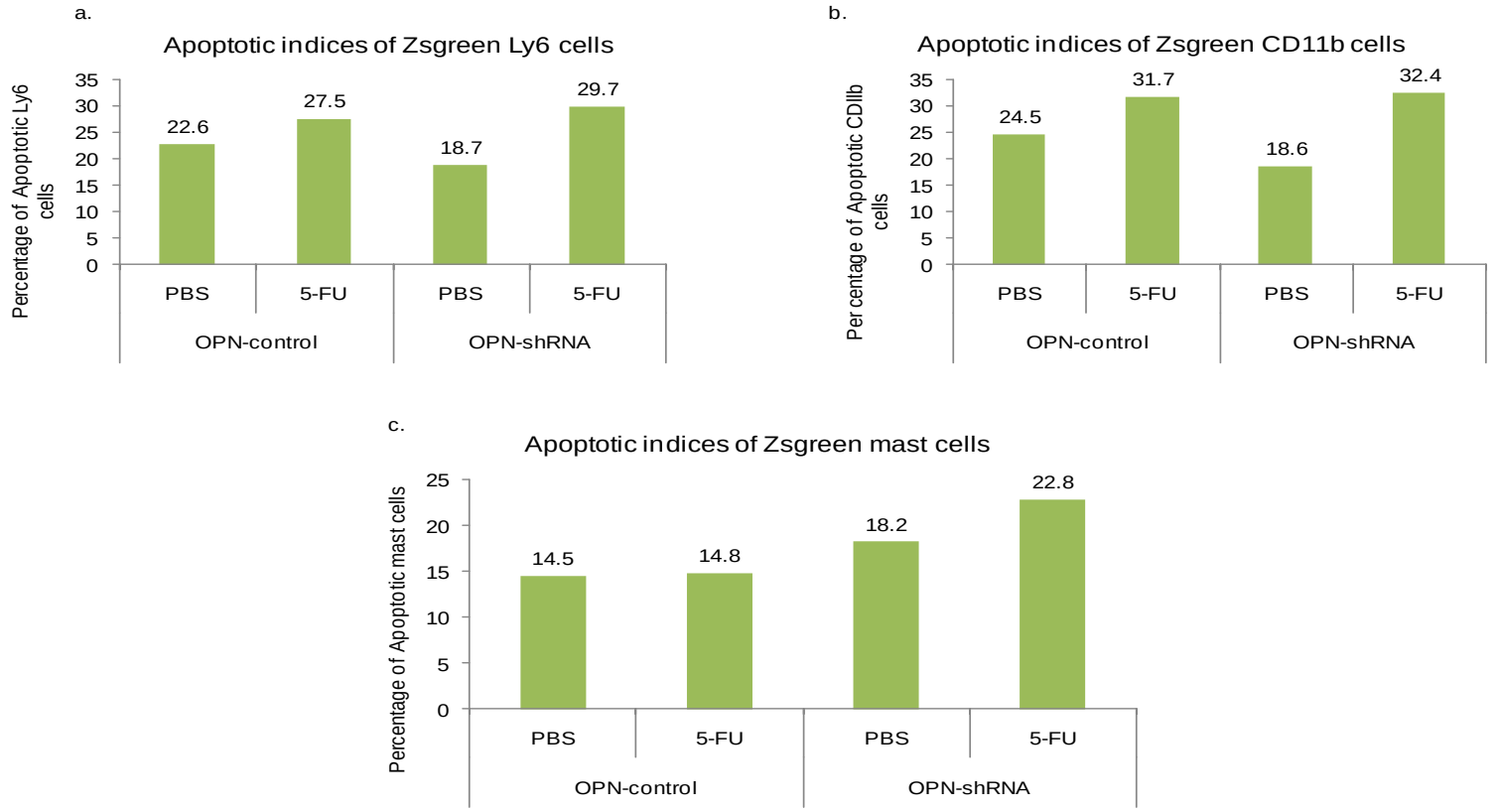


Figure 3.22: **Theoretical apoptotic indices of modulate BMDCs expressing Zsgreen**. This data is theoretical data from flow cytometry analysis taking the percentage of positive Zsgreen cells to determine the number of cell of positively stained cell by confocal analysis is Zsgreen positive. a. Theoretical percentages of apoptotic indices of Zsgreen and Ly6 positive cells from 100 field views. b. Theoretical percentages of apoptotic indices of Zsgreen and CD11b positive cells from 100 field views. c. Theoretical percentages of apoptotic indices of Zsgreen and c-Kit positive cells from 100 field views.

3.5 DISCUSSION:

To be able to establish tumor specific chemo-sensitivity to TS inhibitors it was necessary to identify an RNAi vector system that does not exhibit cytotoxic effect *in vivo*. For our previous studies, which used the retroviral pSuper vector to establish constitutive expressing of a silencing sequence suggest we had the ability to examine systemic effects reducing TS protein levels *in vivo*. However, introduction of transduce BMDCs expressing this RNAi system in $Apc^{Min/+}$ mice demonstrated detrimental results, in that none of the mice survived engraftment. The effects from our prior result points out the importance of identifying an RNAi vector system that has the capacity to be used for *in vivo* studies. Therefore, we decided to employ the pZip-mCMV lenti-viral RNAi vector system, for this system contains resemblance characteristics of endogenous pri-miRNA. Mechanistically allowing the embedded shRNA to be processed by endogenous RNA-induced silencing complex. Additionally, this system uses the polymerase II promoter mCMV to control the expression of the shRNA, permitting the ability to observe *in vivo* systemic effects cause by the reduction of TS protein levels. Result from this study confirms that the mCMV promoter does have the capacity to control and RNAi sequence to suppress the expression of TS protein levels in vitro. However, does not have the ability to reduce protein levels enough to establish or enhance sensitivity to 5-FU displays by our growth inhibition assay results. Despite this system inability to enhance sensitivity *in vitro*, it is essential to investigate its potential of introduction into an *in vivo* cancer model. Indeed, results confirmed that transduced BMDCs expressing this vector system could be

introduced into lethally irradiated Balb/c mice without exhibiting any cytotoxic effect to the mice. Since *in vitro* studies were unsuccessfully in providing enhance sensitivity to therapy, we presume the same result would occurred *in vivo*. Conversely, we succeeded in identifying an RNAi vector system that exhibits the capacity to modulate BMDCs by lessen the expression of cellular TS for *in vivo* studies.

Thus, proceeding forward to enhance cellular response to tumor specific TS target therapy we decided to examine the functionality and aptitude of the OPN promoter. Prior studies by Denhardt and colleagues examined and investigated regions within the promoter that contain enhancers that are responsive to pro-inflammatory stimuli. Exploiting the inducible characteristic of the OPN promoter, we elect to use this promoter to establish tumor specific chemo-sensitivity. Nonetheless, before using this promoter to establish sensitivity it was necessary to determine if this promoter functionality would only be active within the TME; and indeed our results demonstrated that expression of mCherry protein driven by region -740 to +79 of the OPN promoter was only located in the tumor regions of intestinal tissues of $Apc^{Min/+}$ mice. Confirming activation of the OPN promoter only occurred within the tumor regions was essential for our strategy to achieve tumor specific chemo-sensitivity.

As important to determine the functionality of the OPN promoter, it was also necessary to determine its aptitude to drive the expression of the RNAi silencing sequence against TS. Thus, to investigate OPN promoter potential we elected to use the pZip-lenti-viral RNAi vector system, for which the OPN

promoter replaces the mCMV promoter. Our result illustrates OPN promoter ability to control the expression of a silencing sequence, and is able to establish significant reduction of cellular MusTS cellular unlike the mCMV promoter. In addition, we were able to establish enhance cellular sensitivity with the OPN promoter controlling the expression of TS silencing sequence, and so we acquired all the essential components to modulate BMDCs, such to investigate the ability to enhance efficacy of therapy within in the TME.

Although, we have the necessary components to establish tumor specific chemo-sensitivity our approach demonstrates inadequacies and the need for revisions in order to observe the optimal effects of this particularly therapy. For we successfully modulate BMDCs to express pZip-OPN vectors in a subcutaneous cancer mouse model, however the verdict of established tumor specific chemo-sensitivity to 5-FU needs further investigation and evidence. To begin we decide to use flow cytometry analysis to determine the difference of apoptotic indices for MDSCs, mast cells, Tregs, Tc cells, and CD45+ immune cells. However, the percentage of apoptotic cells for each cell types is rather high than expected for the PBS treated mice, thus comparing the effect of 5-FU was difficult to determine. A possible explanation for such high apoptotic percentages could be, due to the multiple step staining procedures need to identify each cell types apoptotic indices. In the samples, processing the integrity of the cell membrane may have been disturbed, causing higher percentages of apoptotic cell populations.

On the other hand, examining the total proliferative indices of cell types MDSCs, Tregs cells, mast cells, and CD45+ immune cells results show an increase in proliferation after subject to 5-FU in BMDCs expressing the OPN-shRNA vector. These results are alarming for expected results should display a decrease in proliferation within these cells type in comparison to the PBS treated mice. A possible explanation is that these results are illustrating residual response of 5-FU treatment, such that the system is attempting to recover from 5-FU effects by increase the proliferation.

The results from our flow analysis study did not provide any clear indication that we have accomplished tumor specific chemo-sensitivity, for our initially had approach flaws. For this, we decided to examine the apoptotic indices of MDSCs subgroups granulocytes and monocytes cells and mast cells by confocal analysis. For MDSCs are highly responsive to 5-FU therapy and mast cells exhibits minimal to no responsiveness to 5-FU therapy, and thus by examining these cells types we would be able to determine if we establish specific chemo-sensitivity and enhance the efficacy of 5-FU therapy within the TME. Our results demonstrate an increase in apoptotic indices for granulocytes, monocytes, and mast cells in 5-FU treated mice when compared to respective PBS treated mice. Most importantly, we observed higher apoptotic indices in mice reconstituted with BMDCs expressing the pZip-OPN-shRNA vector than with pZip-OPN vector control. This is promising data because we demonstrate the ability to enhance the efficacy of 5-FU within the TME of an *in vivo* cancer model. Unfortunately, with our confocal microscopy analysis we were unable to

distinguish which of the positive stained cells expressed the pZip vectors, for we were unable to determine the real signal of Zsgreen expression in the FITTC channel due to auto-fluorescent background signaling. Alternatively, we attempted to stain for Zsgreen with antibodies to quantify the number of cells triple positive (surface marker, apoptotic marker, and Zsgreen positive), however, there was only one antibody against Zsgreen on the market, and it did not successfully work for staining, for it known applications were for western blot and immunoprecipitation analysis. Such that we decide to theoretically, conclude our results from the animal studies by using data from both our flow cytometry and confocal microscopy studies. In theory, the same tissue samples used for both studies; therefore, the percentage of Zsgreen cells determined from the flow cytometry analysis is the same percentage of positive cells for the confocal analysis. Using these percentages, we were able to theoretically inference the percentage of Ly6, CD11b, or c-Kit positively stained cells that are also Zsgreen positive. From these results, again we were able to inference that mice reconstituted with BMDCs expressing the pZip-OPN-shRNA vector had a higher apoptotic indices than mice reconstituted with BMDCs expressing the vector control.

Even though, our confocal microscopy and theoretical results are promising, the results were not significant. Possible reasoning is that mice were reconstituted with twenty percent of modulated BMDCs. It is possible to enhance sensitivity by reconstituting mice with either a higher percentage or 100% of modulated BMDCs expressing the pZip-OPN-shRNA vector. Additionally, it is

necessary to determine if the Zsgreen expression correlates with shRNA expression, to accomplish this, transduced BMDCs needs to sorting into two populations Zsgreen positive and negative, such to measure and compare the differences of mRNA and/ or protein in the two populations. These results will provide a better depiction of the expression of the shRNA. Importantly, to examine the ability to enhance the effect of 5-FU therapy more acute studies are necessary at different time points post therapy to gain and assess the overall response of genetic modulate BMDCs to 5-FU therapy.

CHAPTER 4

THE EFFECTS OF MAST CELL INHIBITION ON TUMOR RESPONSE IN APC$^{MIN/+}$ MICE

[3]The Effects of Mast Cells Inhibition on Tumor Response to 5-FU in *Apc*$^{Min/+}$mice, Nikeya L. Tisdale, Celestia Davis, Maria Marjorette O. Peña. To be submitted.

4.1 ABSTRACT:

Mast cell precursors originate in the bone marrow and migrate through the blood into tissues where terminal differentiation and maturation occurs. They are essential for inflammatory stimuli, and when activated, release an array of cytokines and molecules. In response to treatment with 5-fluoruracil (5-FU), an inhibitor of the enzyme thymidylate synthase that is used in the clinical management of colorectal cancer, we found that mast cells are recruited into the tumor's beds in *Apc*$^{Min/+}$mice, where they may play a role in tumor recurrence post therapy. Because of this, we examined the possibility that pharmacological inhibition of mast cells might play a synergistic role when combined with 5-FU. The objective of this study is two-fold: 1) to determine if pharmacological inhibition of mast cells can interfere with tumor development, and 2) to determine if mast cell inhibition can enhance the antitumor effects of 5-FU or prolong tumor recurrence post therapy. *Apc*$^{Min/+}$ mice were subjected to a regimen of 5-FU or Cromolyn, a mast cell inhibitor, alone or in combination. Findings suggest that inhibition of mast cells can influence tumor development, but has little impact on tumor recurrence after therapy.

4.2 INTRODUCTION:

Tumors are complex organs consisting of a heterogeneous population of both cancer and host-derived non-cancer cells interactions. These non-neoplastic cells compose the tumor stroma and play a critical role in tumor initiation, progression, and response to therapy. Hence, effective anti-cancer

therapies should target not only the tumor cells but also the non-tumor cells, which may contribute to tumor resistance during therapy or its recurrence post therapy.

Tumor recurrence or resistance to TS inhibitors has been a major problem for many years. A previous study by Tucker et. al., systemically treated *Apc*$^{Min/+}$ mice with 5-FU. Results showed reduction in tumor burden, yet when treatment is ceased, tumor recovery is induced (Tucker, Davis et al. 2002). This result could potentially be influence by the associate BMDC such as mast cells that infiltrate or surround the tumor in a protective manner. In addition to this study unpublished data from our lab showed that a single dose of 5-FU (50mg/kg) administered to *Apc*$^{Min/+}$ mice reduced the number of tumor associated MDSC and regulatory T cells, but mast cells were still recruited to the tumor bed (Acharya, unpublished data). Implicating, mast cells potential to be effector cells that may influences tumor resistance and recurrence post 5-FU therapy.

Mast cells are effectors cells that play major role in inflammation, allergic reactions, and wound healing mechanism. Their precursors are release from the bone marrow compartment as immature cells, allowed to migrate into various tissues within the body to undergo maturation and differentiation. Maturation and differentiation is influence by the tissue environment for which infiltrated, resulting in a heterogeneity population, which can be promising for the development of the tumor, such that the TME can influence the maturation of the mast cells to assist in tumor progression. Mast cell pro-tumor responses are dependent on the type

of cancer and the stage of the tumor development (Groot Kormelink, Abudukelimu et al. 2009; Ribatti 2013; Oldford and Marshall 2014)

Several studies have examining mast cells pro-tumorigenic role in mouse models. Gounaris et al. have shown that mast cells infiltrate the microenvironment of gastrointestinal polyps in three mouse models, which include the *Apc*$^{Min/+}$ mouse, and that mast cells are require and essential for the initial stages of adenoma development (Gounaris, Erdman et al. 2007). Another, study showed that performing adoptive transplant of naive Tregs from healthy mice into APC$^{\Delta 468}$ mice reduces mastocytosis, resulting in a reduction of tumor burden (Gounaris, Blatner et al. 2009). A study demonstrates that deletion of mast cell 5-Lipoxgenase (5-LO), a pro-inflammatory mediator, proved to reduce tumor numbers, resulting in the mitigation of MDSCs in the polyps of APC$^{\Delta 468}$ mice (Cheon, Khazaie et al. 2011; Cheon, Strouch et al. 2012). These particular studies, show that mast cells are associated with tumor development and progression, and should be potential target when combating cancer.

One potential way to target the pro-tumorigenic functions of mast cells is to inhibit mast cells degranulation by administering a pharmacological drug, Cromolyn. Cromolyn is classified as a mast cell stabilizer that blocks mast cells degranulation by indirect inhibition of the influx of calcium by blocking chloride channels within the cells(Matthews, Neher et al. 1989; Norris and Alton 1996). The exact mechanism of inhibition is poor understood. However, several studies have administered Cromolyn to cancer mouse models and have observed promising effects. A study, by Souceket.al indicates the mast cells infiltrates and

assists with the expansion of Myc-induced pancreatic tumors. However, upon inhibition of mast cell degranulation with Cromolyn resulted in reduction of tumor islet expansion. (Soucek, Lawlor et al. 2007). Another, study subcanteously injected well-differentiated prostate cell line into the flank of mice, to allow tumor development. These mice were subjected to Cromolyn daily for three weeks, and treatment with Cromolyn showed reduction or elimination tumor growth (Pittoni, Tripodo et al. 2011). An additional study demonstrated that combining Cromolyn with Gemcitabine, a drug typically used to treat pancreatic cancer, enhance the effects of Gemcitabine to reduce tumor growth in pancreatic mouse models (Arumugam, Ramachandran et al. 2006).

Since, mast cells are essential in tumor formation we postulate that the inhibition of mast cell may reduce tumor counts as well as reduce the potential recurrence post therapy. Thus, ideally utilizing a pharmacological drug such as, Cromolyn that inhibits the degranulation of mast cells in combination with 5-FU therapy should be beneficial to treat colon cancer. We sought to determine if administration of Cromolyn post 5-FU therapy would hinder tumor development, and prolong the anti-tumor effects of 5-FU therapy. Results indicates that a synergistic effect does occur with the addition of Cromolyn post 5-FU therapy, however, additional studies are necessary to analyze the mechanistic effects to truly determines if this treatment has a prolong effect.

4.3 MATERIALS AND METHODS:

Treatment Schedules

Six week old *Apc*$^{Min/+}$ mice were subjected to systemic treatment with 5-FU (40 mg/kg) for five weeks with one week intervals between three treatment weeks. Mice treated with a combination of 5-FU and Cromolyn (10 mg/kg)

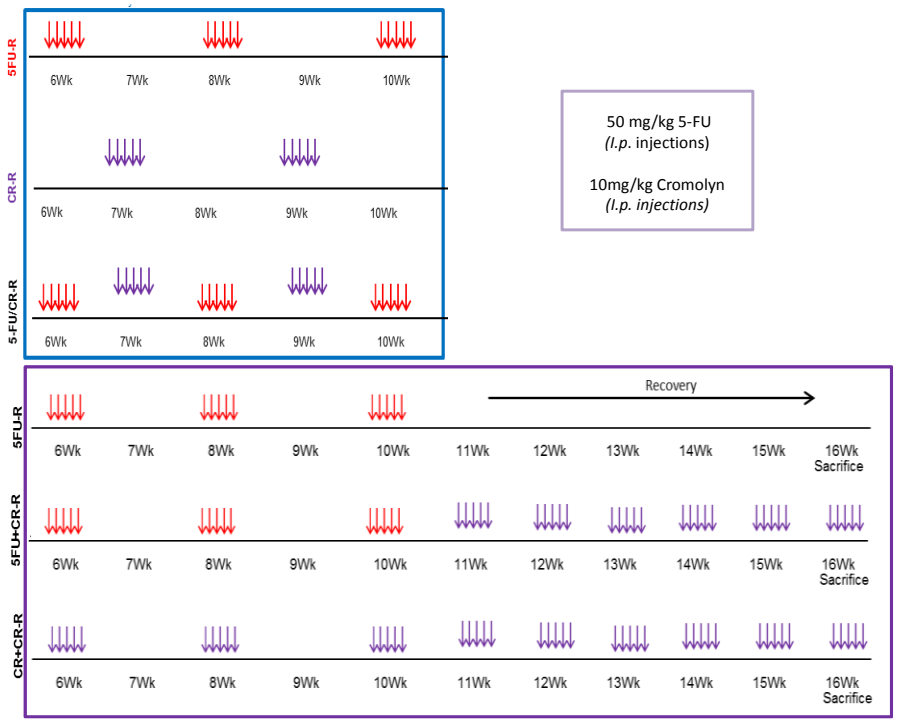


Figure 4.1: **Mast cells chemotherapy regimen**. Chemotherapy schedules for 5-FU, Cromolyn, and 5-FU+ Cromolyn administration for the non – tumor recovery groups (blue box), and for the tumor recovery groups (purple box). PBS (control group) is administrated during the duration of the chemotherapy schedules.

determined the effect of mast cell inhibition on tumor burden, response to therapy, and recurrence post therapy. Illustrated below (Figure 4.1), administering Cromolyn occurred in weeks 7 and 9, with 5-FU administered during weeks 6, 8, and 10. The Cromolyn treatment then resumed for the 6

weeks after the 5-FU therapy (Figure 4.1). Mice treated with PBS, Cromolyn, or 5-FU alone served as the controls.

Tissue collection, Tumor Scoring, and Granulocytes staining

Apc$^{Min/+}$ mice were humanely sacrificed at the end of the treatments either at 10 or at 16 weeks by cervical dislocation. Collecting the small intestines and colon require cleaning by flushing with PBS, cutting open longitudinally, and fixing in 4% paraformaldehyde overnight. This prepares the fixed tissues for tumor scoring or histological analysis. For tumor, scoring fixed tissue requires staining in 0.002% methylene blue in PBS pH 7.4 for visualization of the tumors with a dissecting microscope. Tumors were classified as being greater or less than 1mm in diameter with an ocular micrometer. For histological analysis fixed tissues was Swiss-rolled and embedded in paraffin cassettes and left to dry overnight. Slices from the embedded paraffin cassettes (3-5 micron) form sections, which are mounted on standard microscopy slides coated with poly-L-lysine. The Instrumentation Resource Facility (University of South Carolina School of Medicine) conducted the histological preparation of the fixed tissue. Histochemical staining of the tissue sections for mast cells was achieved by keeping with the manufacturer protocol, using naphthol-AS-D chloroacetate esterase (kit obtained from Sigma).

4.4 RESULTS:

4.4.1 Tumor response and granulocytes migration to the addition of Cromolyn during 5-FU therapy

To determine tumor response to the effect of adding Cromolyn during the weeks when 5-FU is not administered to *Apc*$^{Min/+}$ mice. These animals underwent

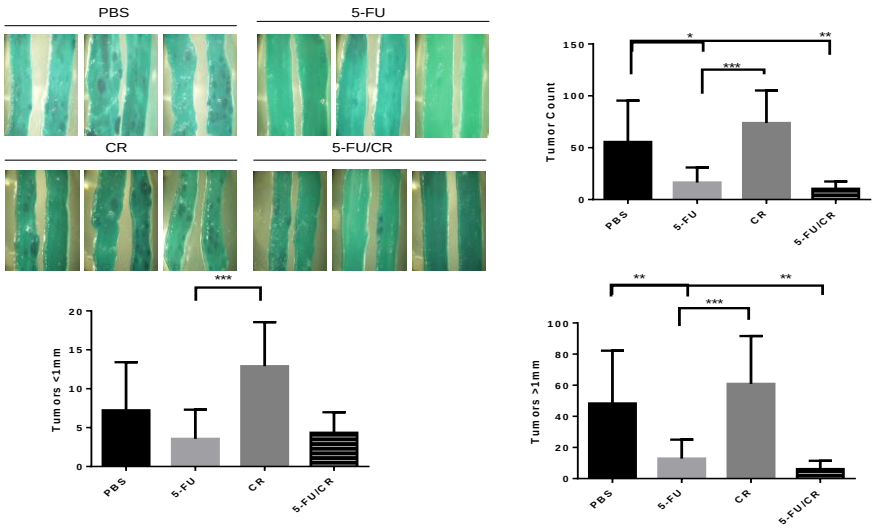


Figure 4.2: **Tumor analysis from *Apc*$^{Min/+}$mice subjected to therapy with no recovery.** a. .Intestinal sections stained with methylene blue from three mice in the treatment groups with no recovery from the chemotherapy regimen. b. Tumor burden in *Apc*$^{Min/+}$mice subjected to therapy with no recovery. Tumor burden in mice treated with 5-FU or 5-FU/CR indicated a significant decrease in tumor burden as compared to the PBS-treated group (p=0.015 and p= 0.007, respectively). Treatment with Cromolyn alone did not reduce tumor burden. c. Number of tumors that are less than 1 mm in size in mice treated with 5-FU, Cromolyn, or 5-FU and Cromolyn with no recovery. d. Number of tumors that are greater than 1 mm in size in mice treated with 5-FU, Cromolyn, or 5-FU and Cromolyn with recovery. Tumor size in mice treated with 5-FU and 5-FU/CR were significantly smaller when compared to those in mice treated with PBS (p=0.0094 and p=0.0020 respectively).

this alternative treatment schedule from six to ten weeks of age. PBS, 5-FU, and Cromolyn groups were used as control groups. Tumor burden for the intestines of these animals indicate that the addition of Cromolyn during the off weeks of 5-

FU treatment does significantly reduce tumor number compared to the PBS treated animals. In addition promising results show that tumor numbers in the 5-FU/CR group is slightly lower than the animals that were subject to 5-FU alone (Figure 4.2b). When scoring the tumors by size both 5-FU and 5-FU/CR groups indeed inhibit regression of tumor development, for both these groups had less

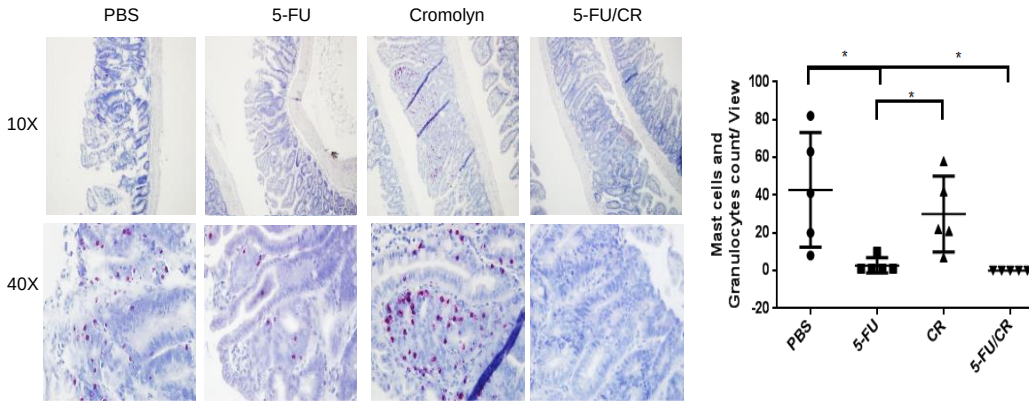


Figure 4.3: **Infiltration of mast cells and granulocytes in intestinal tissue from *Apc*$^{Min/+}$mice subjected to therapy with no recovery.** a. Localization of mast cells and granulocytes by histochemical staining of intestinal tissues from treated mice with no recovery using naphthol-AS-D chloroacetate esterase. Mast cells and granulocytes are highly abundant in the peripheral regions of tumors from mice treated with PBS, 5-FU, and Cromolyn. Mast cells are not observed in tumors from mice treated with a combination of 5-FU and Cromolyn. b. Quantification of mast cells and granulocytes in tumor regions of treated mice without recovery after chemotherapy. There is significant difference between treatment with 5-FU and 5-FU/CR (p=0.0192 and p=0.0135, respectively) as compared to treatment with PBS.

number of tumors measure greater than 1mm compared to the PBS animals, yet the 5-FU/CR treatment was slightly better than the 5-FU alone (Figure 4.2 d). These results suggest that a synergetic response is occurring by administer Cromolyn during the alternative weeks of 5-FU treatment. Conversely, in analyzing the tumor response in the animals treated with Cromolyn alone we

observe an increase in tumor burden as well as an increase of the tumors that are greater than 1mm when comparing to all of the other treatment groups (Figure 4.2). This data indicates the Cromolyn alone is not able to inhibit regression or the multiplicity of tumor development or growth in *Apc*$^{Min/+}$ mice. Upon histochemical analysis, infiltration of granulocytes and mast cells into the tumor occurs more frequently in the Cromolyn treated animals than the 5-FU or 5-FU/CR animals and appears to be similar to the PBS animal. This data suggest that 5-FU is either prohibiting the maturation of these cells for which then influencing these cells ability to infiltrate into the tumor or just influences the infiltration of these cells into the tumor for the environment is alter by the treatment, and therefore these cells are not recruited (Figure 4.3). The addition of Cromolyn during 5-FU treatment demonstrated promising results, however, 5-FU treatment alone as shown to be promising, however once treatment is ceased tumor recurrence is problematic. Thus, we examine tumor response to administering Cromolyn post 5-FU therapy.

4.4.2 Tumor response and granulocytes migration to the addition of Cromolyn post 5-FU therapy

To determine tumor response to the effect of adding Cromolyn post 5-FU therapy in *Apc*$^{Min/+}$ mice. These animals underwent this treatment therapy from six to fifteen weeks of age, for which the tenth up to the fifteenth week of age represent the period of treatment recovery. PBS, 5-FU, and Cromolyn groups were again used as control groups. Tumor burden for the intestines of these animals indicate that the addition of Cromolyn post 5-FU treatment does significantly reduces tumor number compared to both the PBS and 5-FU treated

animals (Figure 4.4b). Also from these result it is important to indicate that we did not observe any significant difference when comparing the PBS with 5-FU treated animals, indicating that rebound of tumor growth occurred during the period of recovery. Thus, the addition of Cromolyn post 5-FU therapy further

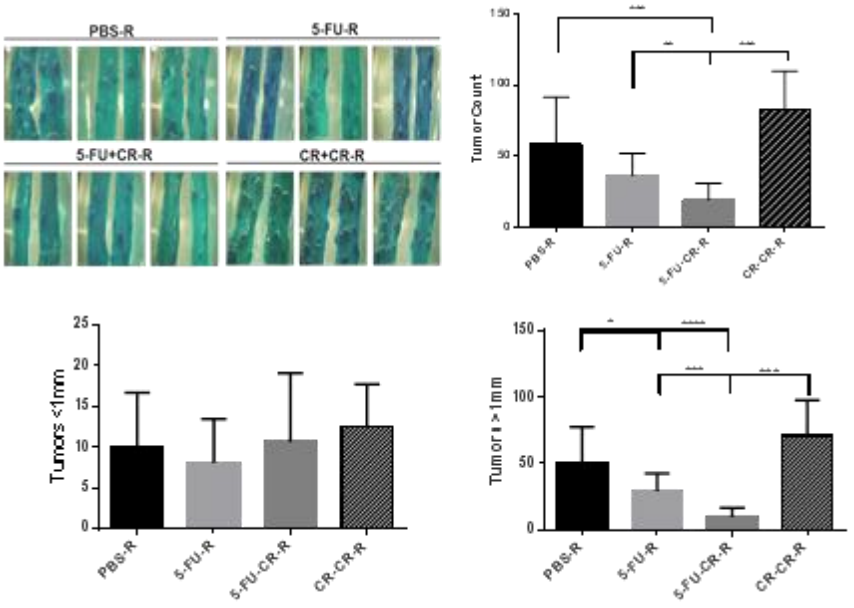


Figure 4.4: **Tumor analysis from *Apc*$^{Min/+}$mice subjected to therapy with recovery.** a.Intestinal sections stained with methylene blue from three mice in the treatment groups with recovery from the chemotherapy regimen. b. Tumor burden in *Apc*$^{Min/+}$mice subjected to therapy with recovery. Tumor burden in mice treated with 5-FU-CR-R caused a significant decrease in tumor burden as compared to the PBS-treated group (p=0.0009). There is a significant decrease in tumor burden in mice that were treated with 5-FU and Cromolyn with recovery as compared to mice treated with 5-FU alone and allowed to recover. The mice treated with Cromolyn alone did not show a decrease in tumor burden.c. Tumor counts for which the size is less than 1mm for each treatment groups for the recovery regimen. d. Tumor count sizes that is greater than 1mm for each treatment groups in the recovery regimen. Tumor sizes for groups 5-FU and 5-FU-CR-R (p=0.0469 and p=0.0001 respectively) were significantly smaller when compared to PBS tumor sizes.

suggests promising effects in inhibiting tumor growth. This implication is further validated when tumor scoring of size is analyzed and results show that there is a statistical reduction of the number of tumor, which are greater than 1mm in the

intestines of animals treated with 5-FU and Cromolyn during the recovery period in comparison to the PBS and 5-FU animals (Figure 4.4d). These results indicates that a synergetic response does occurring by administer Cromolyn post 5-FU treatment, by assisting in the inhibition of tumor regression.

Again in opposition, animals treated with Cromolyn alone we observe an increase in tumor burden as well as an increase of the tumors that are greater than 1mm when comparing to all of the other treatment groups (Figure 4.4). This data reiterates that Cromolyn alone is not able to inhibit regression or the

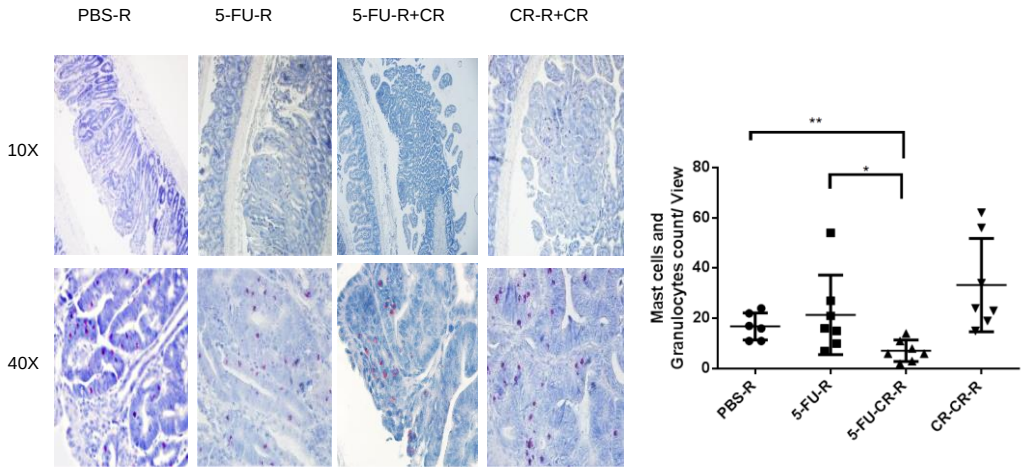


Figure 4.5: **Infiltration of mast cells and granulocytes in intestinal tissue from *Apc*$^{Min/+}$mice subjected to therapy with recovery.** a. Localization of mast cells and granulocytes by histochemical staining of intestinal tissues from treated mice with recovery from therapy using naphthol-AS-D chloroacetate esterase. Mast cells and granulocytes were highly abundant in the peripheral regions of the tumor. b. Quantification of mast cells and granulocytes in tumor regions of treated mice that were allowed to recover from chemotherapy. There is a significant difference in mice treated with the 5-FU-CR-R regimen as compared to mice treated with PBS (p=0.0041).

multiplicity of tumor development or growth in *Apc*$^{Min/+}$ mice. In addition, histochemical analysis, confirms more infiltration of granulocytes and mast cells into the tumor of the Cromolyn group than the animals treated with PBS, 5-FU or 5-FU/CR. The increase influx of granulocytes seen only in the Cromolyn treated animals suggests that Cromolyn is influencing either the maturation or function of these particular cells (Figure 4.5). This is important to understand so that we may better understand the synergetic response that is occurring when adding Cromolyn post 5-FU treatment in *Apc*$^{Min/+}$ mice.

4.4.3 Systemic response to the effects to Cromolyn treatment

To determine the influence of Cromolyn on granulocytes maturation and function, collection of serum from mice prior and after treatment is analyze to determine the systemic levels of various cytokines and chemokines by a multiplex or an ELISA assays. The multiplex analysis allowed evaluation of many cytokines and chemokines in one assay (data not shown); however, most relevant are the results from the analysis of the granulocyte colony-stimulating factor, which warned attention for it correlates with the previous observation of increase infiltration of granulocyte in the tumor region of those mice treated with only Cromolyn. Shown in figure 4.6a we observe higher systemic levels of G-CSF only in mice subjected to Cromolyn compared to the other treatment regimen. Implicating that Cromolyn is indirectly influence the maturation of bone marrow cells into granulocytes by elevating the systemic levels of G-CSF. Yet, in examining, the systemic histamine levels of each treatment group, results do not show any significant difference (Figure 4.6b). Histamine levels do not appear to

decrease from the addition of Cromolyn, suggesting either that mast cells are not sufficiently inhibit by Cromolyn effects, or that other immune cells are compensating for the lack of histamine released, due to mast cell inhibition by

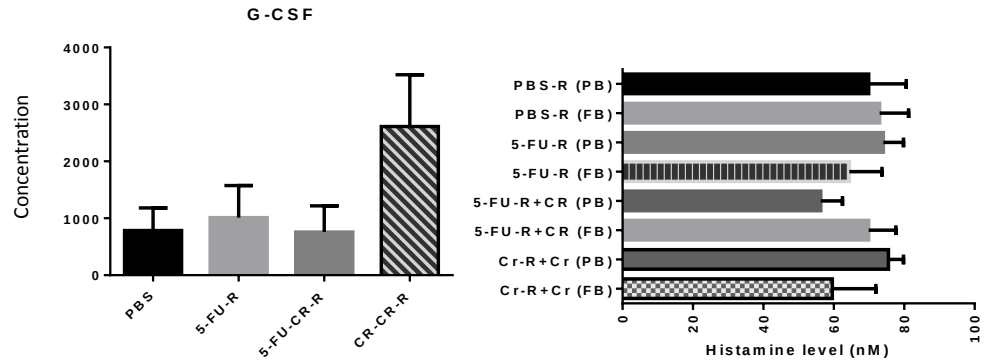


Figure 4.6: **Serum levels of G-CSF and histamine.** a. Serum levels of granulocyte colony stimulating factor were measure by multi-plex assay. Concentration of G-CSF is highly elevated in mice treated with CR-CR-R regimen. b. Serum levels of histamine in mice subjected to chemotherapy followed by recovery. Serum samples were obtained prior to treatment (pre-bleed, PB) and after treatment (final-bleed, FP). ELISA determined levels of histamine in serum samples.

Cromolyn. The latter of the two explanations seem more plausible, for clear Cromolyn is affecting other immune cells maturation and function as previous described.

4.5 DISCUSSION:

The data suggests that administration of Cromolyn during recovery lessen tumor multiplicity possibly by interfering with the regrowth of residual tumors for the tumor sizes were significantly smaller in mice subjected to Cromolyn post 5-FU therapy. However, even though these results are promising in sustaining regression of the tumor development, there are hesitations in consider Cromolyn

the appropriate drug choice for sustaining tumor regression. Solely evaluated animals treated with Cromolyn alone, results show a significant increase of tumors multiplicity and the inability of Cromolyn to inhibit the progression of tumor development. In actuality, Cromolyn appear to encourage tumor genesis in the *Apc*$^{Min/+}$ mice model, for the tumors drastically larger and highly vascularized. These results were alarming and contrary to previous studies that have indicated that Cromolyn mitigates tumor progression (Soucek, Lawlor et al. 2007; Pittoni, Tripodo et al. 2011). A plausible explanation for contradicting results is the differences in mouse models and the type of cancer examine to evaluate the effects of Cromolyn. Despite, the differences in results the data from this study is insightful, such that Cromolyn, is consider a mast cells stabilizer; and by affecting the function of mast cells we potentially influence the function of other immune cells, thus causing a negative effect. This increase progression of tumor development coincides with a study that demonstrates that the ablation of mast cells in *Apc*$^{Min/+}$ mice also results in the increase of tumor burden and size when compared to littermates (Sinnamon, Carter et al. 2008). In addition, this particular study observed that ablation of mast cells resulted in a significant decrease influx of tumor infiltrating eosinophils and a slight increase of neutrophils. Theses evidences can implicate that during tumor development mast cells exhibits protective properties, by means of influencing the function or migration of other immune cells into the tumor, to assist with the inhibition of tumor progression. Therefore, in accordance with the result observed by the animals treated only with Cromolyn, mast cells protective role of influencing other immune cells may

be disrupted, resulting in deficiency regulation of immune cells and the high infiltration of granulocytes cells within the tumor to compensate for the lack of mast cell activity. This high influx of granulocytes can potentially be the contributing factor to the expansion of tumor burden observed in the Cromolyn only treated animals.

Indeed, we did not observe any statistically difference in systemic levels of histamine for any of the treatment groups, thus unable to validate mast cell inhibition. Nonetheless, we have no inclination to suggest that Cromolyn failed to inhibit mast cells degranulation in this model, but incline to say that the inhibition effects were not observed. For we only examined the level of systemic histamine only at the beginning and end of any drug treatment. The longevity of Cromolyn's affect and the mechanism for which it inhibits mast cells is poorly understood. It is state to inhibit mast cells by indirectly inhibiting the influx of chloride ion across the cell membrane to assist with the activation of degranulation. The generalization of Cromolyn effects can indeed influence the function of other cell type if not all cell type. Several studies have observed Cromolyn's effect on other cell types, for instance, an *in-vitro* study showed that pre-incubation of human neutrophils, monocytes, and eosinophil with Cromolyn resulted in inhibits the activation of these cells in response to synthetic bacterial analogue (Kay, Walsh et al. 1987). In addition, Cromolyn has been shown to inhibit the migration of eosinophil (Bruijnzeel, Warringa et al. 1990). Another study shows Cromolyn ability to inhibit the function of chloride channels on epithelial intestinal cell line HT29 (Reinsprecht, Pecht et al. 1992). Thus, further studies need to be perform

to determine the effect of Cromolyn on cells in the microenvironment to assess its potential as a therapeutic agent that can be used synergistically with 5-FU to enhance anti-tumor effects of prolong tumor recurrence post therapy.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

The progression of cancer is a multi-step process, for which cells undergo several mutations, and the development of colorectal cancer is no exception. One of the many painstaking events of tumorigenesis has been underlining the involvement and the function of the tumor microenvironment. The TME comprised of non-neoplastic cells derived from the hematopoietic system; and ironically, some of these non-neoplastic cells exhibits characteristics, which promotes and assist with tumor development. This multifaceted interaction between cancer cells and the non-neoplastic cells within the TME influences both the progression of the tumor, and how the tumor responds to chemotherapy. Hence, the efficacies of chemotherapies are reliant on the responsiveness of both neoplastic and non-neoplastic cells.

For decades, the focus of chemotherapy drugs was design to target neoplastic cells, for tumors was perceived to be a composition of highly proliferative neoplastic cells. A prevalent drug used in the past and present to treat colorectal cancer and other cancers is 5-Fluorouracil. This drug has been effective for it inhibits the catalytic function of the enzyme Thymidylate Synthase.

TS is highly expressed in cells that are proliferating, and neoplastic cells are highly proliferative, thus the efficacy of 5-FU is promising in treating cancer. However, the pitfalls of using this drug are its systemic cytotoxic side effects, due to its lack of specificity; and the reoccurrence of cancer after treatment. Thus, to circumvent these shortcomings we developed and examined the effects of two therapeutic strategies that would target both neoplastic and non-neoplastic cells in the TME to enhance 5-FU effectiveness and reduce its cytotoxicity.

The first therapeutic strategy involved lessening the levels of TS in BMDCs that infiltrates and establishes the TME. The postulation of this therapeutic strategy evolved from previous studies that demonstrated reprogramming BMDCs to expressed E.coli TS, a resistant phenotype decreased the efficacy of 5-FU in tumors. The implications from these studies suggested that reprogramming the protein expression in cells influences response to chemotherapy. Conversely, reprogramming cells to express lesser levels of TS should enhance 5-FU efficacy. To accomplish reduction of TS protein, RNA interference technologies were used to inhibit the translation of TS mRNA, and results showed an increase of cellular sensitivity to 5-FU. However, troublesome results were observed in our first attempts to used genetic modified BMDCs. These troublesome results stress the importance of using an appropriate RNAi model system, especially if this particular therapy becomes applicable for treating cancer in humans.

Eventually we obtain an appropriate RNAi vector system, which permitted us to examine the effects of modulated BMDCs in the tumor of the subcutaneous

CT26 cancer mouse model. In addition, the identity of a non-cytotoxic RNAi vector system we confirm that the OPN promoter has inducible characteristics that are only functional in pro-inflammatory environments, such as the TME. Additionally, the OPN promoter has the aptitude to drive the expression of an RNAi sequence against TS to establish enhance sensitivity to 5-FU therapy. Although we obtain the essential components to establish tumor specific chemo-sensitivity our results were not significant, and therefore we are unable to confidently conclude that this therapeutic application is beneficial. Nevertheless, with additional studies describe in Chapter 4 are performed this therapeutic strategy could be utilized to modulate the expression of various proteins in BMDCs which infiltrates the tumor. Thus, essential our studies are just the beginning in providing insight to improve this therapeutic strategy. Future studies from this therapeutic strategy could involve reducing expression of VEGF, TGF-beta, HGF and other pro-tumorigenic proteins, or at best increase the expression of anti-tumorigenic proteins. After more scrutiny and investigation, the ability to modulate the protein expression of BMDCs that infiltrates the TME could disrupt pro-tumorigenic functionality of these cells, and provide several avenues of therapy.

On the other hand, incorporating additional pharmacological drugs which targets specific BMDCS with current therapies can also potential beneficial in reducing tumor growth and reducing reoccurrence after therapy. In our case, the administration of Cromolyn to inhibit the function of mast cell in conjunction with 5-FU therapy did indeed reduce tumor growth and reoccurrence. However, not

fully understand the mechanistic effect of Cromolyn we were unable to conclude the longevity of therapeutic application would be beneficial. Thus, with additional studies examining the effects of Cromolyn on other BMDCs will be beneficial in determine if this l drug is appropriate to use in combination with 5-FU to prevent reoccurrence of tumors. Additionally, the kinetics of Cromolyn effects within the tumor needs to be investigated to determine if prolong usage of this drug is beneficial in combination with 5-FU therapy.

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