PRO AND ANTI TUMORIGENIC EFFECT OF

PROTEINASES IN INTESTINAL TUMORIGENESIS

By

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PRO AND ANTI TUMORIGENIC EFFECT OF PROTEINASES IN INTESTINAL

TUMORIGENESIS

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Dissertation under the direction of Professor Lynn M. Matrisian

Matrix metalloproteinases (MMPs) are classically associated with late stage metastases, though previous work by our lab and others have expanded the role of MMPs to all stages of tumor development. Using a genetic model of intestinal tumorigenesis, the Min (*multiple intestinal neoplasia*) mouse, we previously demonstrated a role for MMPs, in particular MMP-7, in the development of intestinal adenomas. To further explore the role of proteinases in the development of intestinal neoplasia, I created a novel microarray to examine more than 500 proteases for differential expression in intestinal tumors. Relative microarray analysis found that MMPs-10, -13, and -14 have the highest fold change in expression in tumor compared to normal samples, while absolute microarray analysis indicated that MMPs-9, -12, -15 and -19 are present in tumor samples but not normal intestine. As a result of this screen, I generated Min mice genetically deficient for various proteinases that were differentially detected in Min adenomas (MMP-2, -9, -12, and -19). Genetic ablation of MMP-2, -12, or -19 did not affect tumor number or size, however, MMP-9 deficient Min mice developed 25% fewer tumors than littermate controls. Further, in the context of intestinal tumors, the major cellular source of MMP-9 is neutrophils thus suggesting a *pro-tumorigenic* role of inflammation in early tumorigenesis. Additionally, our screen detected several mast cell produced proteinases in tumor tissue that

were absent from normal intestine. To examine the effect of mast cell proteinases in tumorigenesis, I generated mast cell-deficient Min mice. Surprisingly, these mice developed 50% more tumors than mast cell Min mice, thus indicating that mast cells function in an *anti-tumorigenic* role early during intestinal tumorigenesis. Thus, these studies demonstrate that proteinases and infiltrating inflammatory cells function in both *pro-* and *anti-tumorigenic* roles during early intestinal tumor development.

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LIST OF ABBREVIATIONS

ACF	Aberrant Crypt Foci
APC	Adenomatous Polyposis Coli
AJCC	American Joint Committee on Cancer
BLAST	Basic Local Alignment Search Tool
BrDU	5-bromo-2-deoxyuridine
CIN	Chromosomal Instability
COX-2	Cyclooxygenase-2
DCC	Deleted in Colorectal Cancer
EST	Expressed Sequence Tag
EGF	Epidermal Growth Factor
ENSU	Ethylnitrosurea
ECM	Extracellular Matrix
FAP	Familial Adenomatous Polyposis
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK3	Glycogen Synthase Kinase-3
MBP	Major Basic Protein
MCP	Major Cationic Protein
MMP	Matrix Metalloproteinase
MMPI	Matrix Metalloproteinase Inhibitor
MCC	Mutated in Colorectal Cancer

NSAID	Non-steroidal Anti-Inflammatory Drug
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween 20
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription PCR
TIMP	Tissue Inhibitor of Metalloproteinase
TGFβ	Transforming Growth Factor β
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with 0.05% Tween 20
TNM	Tumor Node Metastasis
TUNEL	Terminal dUTP Nick End Labeling
UICC	Union Internationale Contre la Cancer

CHAPTER I

INTRODUCTION

<u>Colorectal Cancer</u>

General Characteristics, Staging, Pathology, and Genetics

Colorectal malignancies are the fourth most common type and the second most

common cause of cancer related deaths in the United States after cancers of the lung and

bronchus (American Cancer Society, 2007a). The disease occurs with equal frequency in

both men and women. In 2004 there were more than 150,000 new cases and

approximately 50,000 deaths due to colorectal cancer, and similar numbers are predicted

for 2007 (American Cancer Society, 2007a). Approximately 50% of the Western

population is predicted to develop colorectal tumors by 70 years of age with 10% of these

cases progressing to malignancy (Fahy and Bold, 1998). Worldwide there are predicted

to be more than 600,000 deaths due to colorectal cancer and more than 1,100,000 new

cases diagnosed in 2007 (American Cancer Society, 2007b). A variety of risk factors that

predispose a person's susceptibility to developing colorectal cancers have been identified

including family history, inflammatory bowel disease (IBD) , the presence of

adenomatous polyps and diet (Jacobs et al., 2007).

The two most commonly used staging systems used to describe the progression of

colorectal cancer are known as the Dukes' and AJCC/TNM systems (Benson, 2007). The

Astler-Coller modification of Duke's classification system groups colorectal cancers into

four stages based upon the depth of invasion and metastasis to local lymph nodes and

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distant organs (Astler and Coller, 1954). These stages are Dukes' A, which is defined as invasion into the underlying submucosa or muscularis propria but confinement to the bowel wall. Stage B designates tumors that have invaded deeper, through the serosa, but not spread to local lymph nodes. Dukes' stages C1 and C2 identify tumors that have metastasized to lymph nodes, and are differentiated in that stage C1 tumors, like stage A are confined to the bowel wall, while C2 tumors have spread through the serosa. Tumors that have spread to distant organs are designated as Dukes' stage D (Astler and Coller, 1954).

In contrast, the American Joint Committee on Cancer (AJCC) and Union International Contre le Cancer (UICC) advocate a classification scheme known as TNM6 (International Union against Cancer. et al., 2002). In the TNM6 system scores are calculated based upon three variables: the depth of invasion of the primary **T**umor (scored 0-4), the number of lymph **N**odes involved (scored 0-3), and the presence or absence of distant **M**etastasis (scored 0-1). Based upon the individual T, N and M scores, tumors are then grouped into stages identified by the roman numerals I-IV, with stage IV being the most advanced stage (Table 1) (International Union against Cancer. et al., 2002).

Patient survival strongly correlates with stage, with decreasing survival correlated with increasing stage (Benson, 2007). Patients diagnosed with Dukes' A (Stage I) colorectal cancer have a 5 year survival of greater than 90%; however, survival drops to 5% as the disease progresses and involves distant organ metastasis (Fry et al., 1989). A second important variable that correlates with survival rate is the degree of differentiation of the tumor. Tumors are classified as either well, moderately, or poorly differentiated

based upon histological criteria. The majority of colorectal cancers are "moderately

differentiated", and cancers that are graded as "poorly differentiated" are significantly

Table 1: Comparison of Dukes' Staging versus TNM6 grading schemes for colorecteal cancer. Astler-Coller modification of Dukes' stages (far left) is compared to the newer AJCC-TNM6 system, which measures the depth of invasion in the tumor (T), the number of positive lymph nodes (N), and presence or absence of distant metastases (M).

Stage	TNM	Description
0	Tis, N0, M0	The cancer is in the earliest stage. No invasion into the submucosa. Commonly referred to carcinoma in situ.
I	T1-2, N0, M0	Invasion through the muscularis mucosa into submucosa *or* into the muscularis propria. No spread to lymph nodes or distant sites.
IIA	T3, N0, M0	Invasion through the wall of the colon or rectum into the subserosa. No spread to lymph nodes or distant sites.
IIB	T4, N0, M0	Invasion through the wall of the colon or rectum into nearby tissues or organs. No spread to lymph nodes or distant sites.
IIIA	T1-2, N1, M0	Invasion through the muscularis mucosa into submucosa *or* into the muscularis propria, *and* has spread to 1-3 nearby lymph nodes, but not distant sites.
IIIB	T3-4, N1, M0	Invasion through the bowel wall or into nearby organs, *and* has spread to 1-3 nearby lymph nodes, but not distant sites.
IIIC	Any T, N2, M0	Any T stage, and has spread to 4 or more nearby lymph nodes, but not distant sites.
IV	Any T, Any N, M1	The cancer has spread to distant organs- commonly the liver, lung, peritoneum, or ovaries.

more likely to be associated with metastasis than moderately or well-differentiated

tumors (Fry et al., 1989). Colorectal cancers directly invade the bladder, prostate, vagina,

uterus, ureters, perineural muscle, pelvic bones, and sacral plexus (Willett et al., 1999).

Metastatic spread to distant organs most commonly involves the liver, which can be

detected in 10-30% of patients at time of diagnosis (Gatta et al., 2000). Less commonly,

metastases are found in the lungs, vertebrae, brain, and bone marrow (Kanthan et al., 1999).

Rather than one single disease, colorectal cancer includes several tumors of several different cellular origins. Most colorectal tumors are of epithelial origin, which includes adenocarcinoma, mucinous adenocarcinoma, signet-ring adenocarcinoma, squamous cell carcinoma, and adenosquamous carcinoma subtypes. However, several other tumor varieties can develop in the colon and rectum of non-epithelial origin including carcinoid tumors (neuroendocrine cells), lipomas (adipocytes), lymphomas (lymphoid cells), and leiomyosarcomas (smooth muscle) (Brunicardi et al., 2005). Adenocarcinoma of the colon, by definition a tumor of the glandular epithelium, is the most common variety of colorectal cancer, and accounts for 95% of all colorectal malignancies (Ponz de Leon and Di Gregorio, 2001).

The progression from normal colonic epithelium to invasive cancer involves several discreet morphological and genetic modifications. The earliest lesion identified in the development of colorectal tumors is the presence of aberrant crypt foci (ACF). ACF are small clusters of abnormal crypts that can exhibit a variety of histopathologic features typical of tumors including enlarged, crowded nuclei; stratification of epithelial cells; depletion of mucin; and varying degrees of dysplasia (Di Gregorio et al., 1997) that can be used to further refine ACF into three groups- dysplastic, non-dysplastic, and mixed (Cheng and Lai, 2003).

Adenomatous polyps, the smallest detectable lesion by routine colonoscopy, are defined as well demarcated, circumscribed lumps of epithelial dysplasia, and are the most common precursor lesion detected in the gastrointestinal tract (Haber, 2002). Polyps are

extremely common in the Western population, and have been reported in as much as 50% of individuals over 50 years of age (Ponz de Leon and Di Gregorio, 2001). Two general morphological categories of polyps exist: pedunculated, that is adenomas that possess a stalk; and sessile, which are flat adenomas that rise above the surface. Very small lesions (<1 cm) are occasionally referred to as "diminutive", though this designation is uncommon (Weston and Campbell, 1995). Polyps can be further subdivided into three major histological categories (villous, tubular, and tubulovillous) based upon their tissue architecture. However, distinguishing a tumor as one type is somewhat ambiguous as the spectrum of tumor morphology is more of a gradient than three discreet points (Morson and Day, 2003).

Several genetic changes occur during the progression from normal intestinal mucosa to invasive colorectal cancer including mutations to both oncogenes such as K-ras, and β-catenin (Bos et al., 1987; Morin et al., 1997); and tumor suppressor genes including APC (adenomatous polyposis coli), MCC (mutated in colorectal cancer), DCC (deleted in colorectal cancer), and p53 (Figure 1) (Fearon and Vogelstein, 1990; Groden et al., 1991; Hollstein et al., 1991; Kinzler et al., 1991). While there are likely numerous pathways that result in the development of colorectal cancer, a large majority (70-85%) of tumors develop via the "traditional pathway", also known as the chromosomal instability (CIN) pathway (Worthley et al., 2007). The traditional pathway involves a mutation or loss of APC, a mutation of K-ras, a loss of chromosome 18q (DCC), and finally deletion of chromosome 17p, which contains the tumor suppressor gene p53 (Grady, 2004). This pathway was generated by genetic profiling of both spontaneous and familial colorectal tumors at a variety of stages and compiling the results into a sequence

that reconciled the genetic and morphologic changes that occur during the development

of colorectal cancer (Fearon and Vogelstein, 1990).
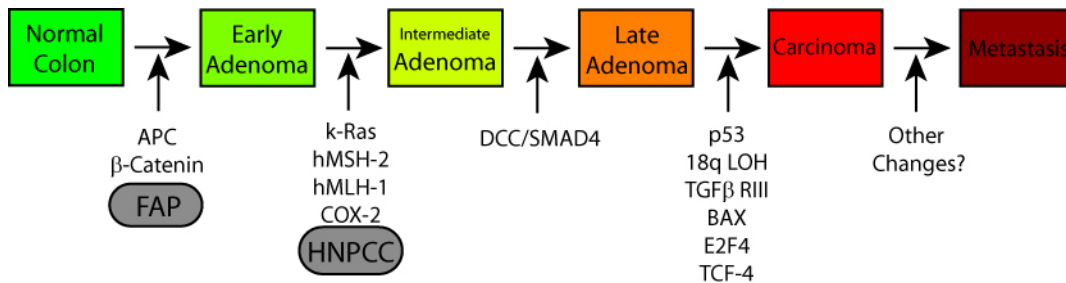
Figure 1: Genetic alterations that occur in the development of colorectal neoplasia.
Mutations in the tumor suppressor APC or β-catenin are the initiating event in the
development of colorectal cancer, and are carried in the germline of patients with the
hereditary cancer syndrome FAP. HNPCC, another hereditary cancer syndrome,
involves mutations in the mismatch repair genes MSH-2 and MLH-1. (Adapted from
Kinzler and Vogelstein, 1996.)

Spontaneous mutations in APC occur in nearly 70% of sporadic colorectal

adenomatous polyps (Powell et al., 1992), and germline mutations in APC are the

causative agent of the hereditary condition familial adenomatous polyposis (FAP)

(Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). Patients with FAP

develop hundreds to thousands of adenomatous polyps which invariably progress to

colorectal cancer (Rustgi, 2007). APC is a large 15 exon gene with several functional

domains and mutations often result in a truncated protein missing the carboxy terminus

(Rustgi, 2007).   APC has been shown be involved in a number of cellular processes

including cell cycle regulation, migration, microtubule assembly, cell adhesion, apoptosis

and cell fate determination via protein-protein interactions (Rustgi, 2007).

The most widely associated role for APC is as a master regulator of the *Wnt* pathway. The carboxy terminal region of APC along with axin and glycogen synthase kinase-3 (GSK3), is crucial for phosphorylation and ultimately degradation of β-catenin (Korinek et al., 1997; Morin et al., 1997; Su et al., 1993). In the absence of functional APC, β-catenin accumulates within the cytoplasm and eventually the nucleus where it promotes the transcription of *Wnt* pathway target genes through an interaction with a family of DNA binding proteins known as T-cell factor (Tcf) or lymphoid enhancing factor (Lef) (Behrens et al., 1996; Huber et al., 1996; Korinek et al., 1997). Tcf target genes shown to be activated by β-catenin include protooncogenes such as c-myc (He et al., 1998) and WISP-1 (Xu et al., 2000), matrix metalloproteinases (Crawford et al., 1999), and other genes known to contribute to tumor formation (Mann et al., 1999). Further, tumors that lack mutations in APC frequently have mutations in β-catenin, thus underscoring the importance of this pathway in the development of colorectal cancer.

In summary, the development of colorectal polyps is a frequently occurring disease that affects 50% of the population over 70 years of age. Fortunately, because of advances in early detection, many of these cases are detected and treated by routine colonoscopy before they progress to malignant lesions. However, our understanding of the cellular changes that occur during tumorigenesis and disease progression is still quite poor. In my work, I have sought to understand molecular events occurring in the tumor microenvironment of early stage tumors with the goal of identifying protective and pro-tumorigenic proteinases in particular.

The Min Mouse as a Model Organism of Intestinal Tumorigenesis

The *Min* (multiple intestinal neoplasia) mouse is a commonly used model for studying the development and treatment of colorectal cancer. Generated as part of a forward genetic screen using ethynitrosurea mutagenesis, these mice were identified after exhibiting severe adult-onset anemia that was the result of the development of dozens of tumors throughout both the small and large bowel (Moser et al., 1990). The mutation is heritable in an autosomal dominant manner, and subsequently, a single nonsense mutation (T→A transversion at base pair 2549) was found in the murine orthologue of the human *APC* gene (Su et al., 1992). This mutation results in a premature STOP codon at position 850, and as with human tumors, a truncated form of APC missing the carboxy terminus is produced (Su et al., 1992). Homozygosity for the *Min* allele is embryonic lethal due to a defect very early in embryogenesis (Tam and Loebel, 2007). Mice heterozygous for this allele develop numerous benign tumors through the intestinal tract, and females are predisposed to develop spontaneous mammary tumors. Further characterization of these tumors revealed that multiple epithelial lineages were involved, suggesting that lesions likely arise from initiated mutipotent stem cells (Moser et al., 1992).

Mice heterozygous for the *Min* allele develop many symptoms that resemble the human familial FAP condition; however, there are significant differences as well. While FAP tumors tend to be distributed throughout the colon, tumors in *Min* mice are much more commonly found in the small intestine, though colonic polyps do occur (Shoemaker et al., 1997a). Tumors range in size from 0.5-8mm in diameter and like human tumors are heterogeneous and can morphologically be either pedunculated or sessile (Moser et

al., 1990). Tumors develop from individual cells that have lost the second allele of APC, can be detected in animals as early as four weeks old (Yamada et al., 2002), and are frequently aneuploid (Rao et al., 2005). A key limitation to the *Min* model is that tumors that do form are almost always benign, rarely progressing to become locally invasive, and virtually never metastasize (Shoemaker et al., 1997a). Other APC mutant mice have been developed with varying tumorigenic profiles. Most notable are mice with mutations at codons 716 (*APCΔ716*) and 1638 (*APCΔ1638N*). In the case of *APCΔ1638N*, these mice have been reported to develop liver metastases (Kucherlapati et al., 2001), however, the life span of *APCΔ1638N* mutants is significantly longer than *Min* animals, and tumors are not detectable until 25 weeks of age (Kucherlapati et al., 2001). Moreover, the number of tumors that develop in each of these models is significantly fewer than in the *Min* mouse.

A number of genetic factors have been shown modulate tumor development in the Min system. The genetic background of the animal has been shown to tremendously influence tumor multiplicity and progression. To date, there have been 7 genetic loci identified as "<u>m</u>odifier <u>of</u> <u>min</u>" with strain specific tumor-resistant and sensitive alleles (Kwong et al., 2007). The best characterized modifier locus is *Mom1*. *Mom1* was initially described as a dominant acting allele that conferred resistance to tumor formation in the *Min* system, and was found in AKR/J, MA/MyJ and CAST/J strains, but not in C57Bl/6 (Dietrich et al., 1993). The allele mapped to a 4 cM region of chromosome 4, and was eventually identified as a secretory phospholipase, *Pla2g2a* (Cormier et al., 1997). Other modifier loci have been identified that both encode proteins, like *Mom1*, and are in non-coding regions, like *Mom7*. Further, many of these loci are highly

polymorphic with at least five variant alleles known to exist (Haines et al., 2005; Kwong et al., 2007).

Aside from the well established utility of the *Min* model in identifying the role of genetic modifiers of colorectal carcinogenesis, the model is commonly used to evaluate the efficacy of chemopreventative pharmacologic agents. An excellent example of this is the protective role of NSAIDs (non-steroidal anti-inflammatory drugs) in tumorigenesis. COX-2 (cyclooxygenase-2), an inducible enzyme that mediates the production of prostaglandins, is frequently over-expressed in a variety of human tumors. COX-2 produced prostaglandins have been shown to both stimulate proliferation and confer apoptosis resistance to tumor cells. Treatment of *Min* mice with NSAIDs that inhibit COX-2 activity dramatically inhibited tumor formation (Boolbol et al., 1996) and paved the way for human clinical trials which had similar results (Rostom et al., 2007).

A second example of the utility of pharmacologic agents in the *Min* system is in the case of MMPs (matrix metalloproteinases). MMP-7, normally produced by Paneth cells in the intestine, is frequently localized to tumor cells in colorectal lesions (McDonnell et al., 1991; Wilson and Matrisian, 1996). *Min* mice with MMP-7 genetically ablated develop ~60% fewer tumors than do littermate controls, implicating MMP-7 as pro-tumorigenic mediator of tumorigenesis (Wilson et al., 1997). Pharmacological inhibitors of MMPs, known as MMPIs (MMP Inhibitors), were also tested in the Min model. MMPIs with broad spectrum activity that includes MMP-7 inhibition resulted in a 48-70% inhibition of tumor formation (Goss et al., 1998; Wagenaar-Miller et al., 2003), indicating that the results of genetic modification can be recapitulated chemically in the Min model.

Despite some key differences, the molecular and pathologic parallels to human disease make the *Min* model an excellent tool for studying the development and treatment of early-stage colorectal tumorigenesis. Furthermore, genetic and pharmacologic studies in this model system have emphasized the importance of proteolytic enzymes, in particular MMP-7, in the development of intestinal neoplasms, thus making the MMPs an attractive target for further study.

<div align="center">

MMPs and Cancer

The Matrix Metalloproteinase Family

</div>

The matrix metalloproteinases (MMPs), a subfamily of extracellular zinc-dependant proteinases belonging to the metzincin subclan, MA(M), involved in numerous biological processes (Rawlings et al., 1998). The MMPs are Collectively, MMPs are able to degrade all structural components of the extra-cellular matrix (ECM) (Nagase et al., 2006), though a number of non-ECM bioactive substrates for MMPs have been discovered (Sternlicht and Werb, 2001). While the majority of MMP activity is extracellular, recent studies have indicated that in certain conditions MMP-1, -2 and -11 may act on intracellular proteins (Kwan et al., 2004; Limb et al., 2005; Luo et al., 2002). To date, 25 vertebrate, 23 human, and 22 murine MMPs have been identified (Sternlicht and Werb, 2001). Multiple structural motif similarities exist between family members including a conserved zinc binding motif (HEXXHXXGXXH) and a "Met turn" (usually ALMYP) both within the catalytic domain, and a "cysteine switch" motif (PRCGXPD) in the pro domain (Sternlicht and Werb, 2001).

Individual MMPs are referred to by a sequential numeric nomenclature system reserved for vertebrate MMPs, though many have common names that reflect substrates, expression, or cellular localization. Classically, human MMPs can be categorized into 6 groups based upon substrate preference and domain architecture: the matrilysins, the collagenases, the gelatinases, the stromelysins, the membrane-type MMPs, and "others"-MMPs that do not fit well into any of the other groups (Figure 2) (Nagase et al., 2006).

The minimal domain structure that distinguishes MMPs consists of three domains required for secretion (pre domain), enzyme latency (pro domain), and catalysis (catalytic domain) (Nagase and Woessner, 1999). The matrilysins are the only MMPs to have the minimal domain structure. With one exception, MMP-23, all other vertebrate MMPs contain a hemopexin/vitronectin-like domain that is juxtaposed to the catalytic domain through a short linker region. Rather than a hemopexin domain, MMP-23 contains a unique C-terminal cystein-array/immunoglobulin like domain.

MMP-7 (matrilysin, MEROPS M10.008) and MMP-26 (matrilysin-2, endometase, MEROPS M10.029) are the two members of the matrilysins. While humans have both members of this group, rodents lack MMP-26, which to date has only been detected in primate genomes (Huxley-Jones et al., 2007). MMP-7 acts on a wide variety of ECM substrates including non-fibrillar collagens, gelatins, fibronectins, and proteoglycans (Quantin et al., 1989; Sellers and Woessner, 1980; Woessner and Taplin, 1988). Additionally, several non-ECM substrates for MMP-7 have been identified including pro-α-defensin, Fas-ligand, pro-tumor necrosis factor, and E cadherin (Nagase et al., 2006). MMP-26 has the ability to proteolyze a number of ECM molecules as well, though, unlike most MMPs, is largely intracellulary stored (Nagase et al., 2006).
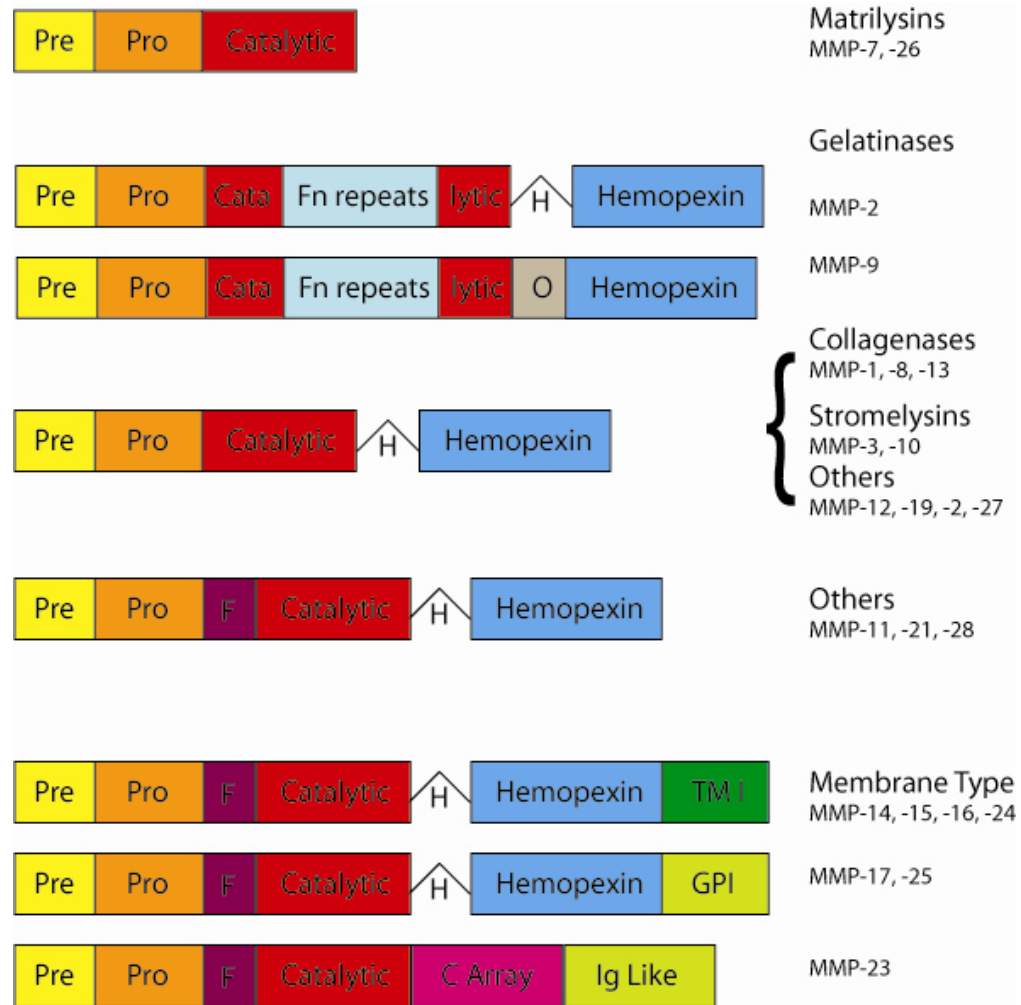
Figure 2: Domain architecture of the MMP family. MMP family members can be classified into five broad domains based upon substrate specificity and domain architecture. Abbreviations are as follows: Fn repeats (fibronectin repeats), H (hinge region), O (O-glycosylated domain), F (furin cleavage site), TM I (transmembrane domain Type I), GPI (GPI anchor), C Array ('Cysteine array'), Ig Like (Ig-like domain).

The collagenases include MMP-1 (interstitial collagenase, MEROPS M10.001), MMP-8 (neutrophil collagenase, MEROPS M10.002), MMP-13 (collagenase-3, MEROPS M10.013), and MMP-18 (*Xenopus* collagenase, MEROPS M10.018, not present in humans or mice). Like the vast majority of MMPs, collagenases contain a

heompexin domain, that when present, influences enzyme activation, substrate binding, membrane activation and has been shown to have some catalytic activity (Sternlicht and Werb, 2001). Collagenases are best known for their ability to degrade native, fibrillar collagen, though, like with all MMPs, non-matrix substrates have been found as well (Nagase et al., 2006). Mice and rats have two copies of the gene for MMP-1, designated as mcola and mcolb, though these genes are only rarely expressed, and MMP-13 acts as the major collagenase in these animals (Balbin et al., 2001).

The gelatinase group consists of two enzymes- MMP-2 (gelatinase A, 72kDa gelatinase, MEROPS M10.003) and MMP-9 (gelatinase B, 92kDa gelatinase, MEROPS M10.004). Gelatinases contain three fibronectin type II repeats within the catalytic domain that bind to and aid in the degradation of gelatin (Murphy et al., 1994). In addition to denatured collagen, the gelatinases have been shown to degrade type IV, V and XI collagen, laminin, and aggrecan; and, MMP-2, but not MMP-9, has been demonstrated to have some collagenolytic activity, but the *in vivo* relevance of this is not yet known (Nagase et al., 2006; Patterson et al., 2001).

There are three members of the stromelysin subgroup: MMP-3 (stromelysin-1, transin, MEROPS M10.005), MMP-10 (stromelysin-2, transin-2, MEROPS M10.006) and MMP-11 (stromelysin-3, MEROPS M10.007). The stromelysins and collagenases have similar domain architectures, but the stromelysins are unable to cleave insterstitial collagen (Nagase et al., 2006). MMP-3 and -10 are highly homologous proteins with similar structure and substrate specificity, but differ in their tissue expression. Stromelysins-1 and -2 degrade a wide variety of ECM substrates including laminin, fibronectin, entactin, collagen IV, and proteoglycans. However, MMP-11 is only

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distantly related to the other stromelysins, and has been demonstrated to have only weak activity towards ECM components, but has been shown to readily cleave serpins (a class of serine protease inhibitors) (Murphy et al., 1993; Pei et al., 1994). Evolutionary analysis of nucleotide sequence suggests that MMP-11 is more closely related to the membrane type MMPs than to the other stromelysins, though it is still classified as a stromelysin for historical purposes (Massova et al., 1998).

The membrane-type MMPs can be subdivided into two groups. There are four type I transmembrane MMPs: MMP-14 (MT1-MMP, MEROPS M10.014), MMP-15 (MT2-MMP, MEROPS M10.015), MMP-16 (MT3-MMP, MEROPS M10.016), and MMP-24 (MT5-MMP, MEROPS M10.023), and two glycosylphosphatidylinositol-anchored proteins: MMP-17 (MT4-MMP, MEROPS M10.017), and MMP-25 (MT6-MMP, MEROPS M10.024). Like MMP-11, all members of the membrane-type group contain a furin recognition motif at the C-terminus of the propeptide (Nagase et al., 2006). MT1-MMP has been shown to have collagenolytic activity towards collagens I, II and III (Ohuchi et al., 1997) and mutant mice lacking this enzyme display skeletal defects suggesting a critical role for MT1-MMP in osteogenesis (Holmbeck et al., 1999). A second important role for MT1-MMP is in cell surface activation of pro-MMP-2 (Sato et al., 1994), a capability probably also existing in MT2 and MT3-MMPs (Page-McCaw et al., 2007). Additionally, MMP-23 (cysteine array MMP, MEROPS M10.022), while not classically classified as a membrane-type, is proposed to be a type II membrane protein having a transmembrane domain at the N-terminus of the propeptide (Pei et al., 2000).

The remaining MMPs that do not fit well into any of the other categories, and are grouped together as "other MMPs". Three of the MMPs in this group (MMP-12, MMP-

20, and MMP-27) have structural and genomic homology to the stromelysins, but are not classified as such. MMP-12 (macrophage metalloelastase, MEROPS M10.009) has been shown to degrade elastin, fibronectin, proteoglycans and is primarily expressed by macrophages, but is also detected in chondrocytes and osteoclasts (Hou et al., 2004; Kerkela et al., 2001). MMP-19 (RASI, MEROPS M10.021), was first detected in the activated lymphocytes and plasma from patients with rheumatoid arthritis, and has a very wide expression pattern including most organs and proliferating keratinocytes (Sadowski et al., 2003). MMP-20 (enamelysin, MEROPS M10.019) has an extremely restricted expression pattern and is found in newly formed tooth enamel and during periodontal disease (Ryu et al., 1999). MMP-21 (*Xenopus* MMP, XMMP, MEROPS M10.026), is a poorly characterized MMP with a wide expression pattern, and is commonly expressed by basal and squamous cell carcinomas (Ahokas et al., 2003). MMP-27 (chicken MMP, CMMP, MEROPS M10.027) was originally detected in chicken embryo fibroblasts where it was given the designation MMP-22. However, in mammals it is only known from genomic sequences and to date has not been characterized (Yang and Kurkinen, 1998). The last member of the "other" group, MMP-28 (epilysin, MEROPS M10.030), is thought to function in wound repair and is detected in a wide variety of tissues including cartilage, heart, lung, placenta, testis, and the gastrointestinal tract (Saarialho-Kere et al., 2002). Like MMPs-11 and -17, MMPs-21, -23 and -28 contain furin recognition sequences adjacent to the catalytic domain suggesting that they are likely to be intracellularly activated and secreted as activate enzymes (Nagase et al., 2006).

Lastly, a specialized class of protein inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) are frequently associated with the MMPs. There are 4

TIMP family members, and TIMPs reversibly inhibit MMP activity and demonstrate some inhibitory selectivity towards particular MMP family members. In addition, TIMPs have also been shown to also mediate activation of pro-MMPs by acting as a scaffolding partner and bringing the pro-MMP is close juxtaposition to an activating proteinase (Sternlicht and Werb, 2001).

Regulation of MMP Activity

The expression and activation of MMPs is tightly regulated at multiple levels to ensure that active MMPs are present only in appropriate conditions. This strict control affects every stage of the MMP's lifecycle including modulators of transcription, translation, secretion, activation, and catalytic activity of mature enzymes. Transcriptional control of MMPs can be up- or downregulated by factors including several growth factors (TGFβ, EGF), ECM proteins, phorbol esters (12-*O*-tetradecanoyl-phorbol-13-acetate [TPA]), cell stress, and changes in cell shape (Parks and Mecham, 1998). Many of these stimuli function by inducing the expression or activity of the proto-oncogenes *c-fos* and *c-jun*, which bind AP-1 sites within the promoter region of several MMPs (Sternlicht and Werb, 2001). However, while some stimuli coordinately regulate some MMP genes, other stimuli can have differential effects. In humans, transforming growth factor-β (TGFβ) signaling increases transcription of MMP-13, but downregulates expression of MMPs-1 and -3 (Uria et al., 1998). Additionally, the same stimulus may have different effects on expression depending on the cellular target. Cultured human keratinocytes express MMP-10 when treated with TPA, but fibroblasts react to the same stimulus by expressing MMP-3 (Windsor et al., 1993). Thus, MMP transcription is

dependent upon the context of not only appropriate stimulus, but of cellular origin as well.

While atypical for MMPs, mechanisms do exist that affect transcribed MMP sequences prior to translation and effectively modulate activity and amount of mature protein. One way that MMP activity is modulated is by the production of splice variants. There are several splice variants of MMP-16, normally a cell surface associated protein, and one splice variant results in the production of a soluble and proteolytically active isomer (Matsumoto et al., 1997). MMPs also may be regulated by modulating the stability of mature mRNA, prior to translation. Stabilizing mRNA results in more proteins produced per mRNA molecule, thus effectively increasing the concentration of the target gene. For example, phorbol esters and epidermal growth factor (EGF) have been shown to stabilize transcripts of MMP-1 and MMP-3 (Delany et al., 1995). Conversely, TGFβ has been shown to destabilize MMP-13 transcripts, resulting in fewer molecules of MMP-13 made per transcript (Delany et al., 1995).

Freshly synthesized MMPs contain two domains that are absent in active enzymes, a pre- and a pro- domain. Prior to secretion of pro-MMPs, a hydrophobic stretch of amino acids known as the 'pre' domain is removed. This pre-domain is important for targeting MMPs for extracellular secretion. While most MMPs are secreted constitutively once they are translated, instances exist of where pro-MMPs are stored in secretory granules until the cell receives an appropriate stimulus. Circulating neutrophils, a class of leukocytes, synthesize and store MMP-8 and MMP-9 in tertiary granules, which are only released following neutrophil activation by specific pro-inflammatory mediators (Hasty et al., 1990). A second example of this is MMP-12, which is produced

and then stored by macrophages until an appropriate stimulus (protein kinase C activation as a result of PAR-1 stimulation) leads to secretion (Raza et al., 2000). Furthermore, evidence exists that MMP-26 may also be stored intracellularly, but the molecular mechanisms behind this are unknown (Marchenko et al., 2004).

Most MMPs are synthesized and secreted as inactive zymogens (proMMPs) that must first be activated to become enzymatically capable of acting on substrates. A mechanism commonly referred to as "stepwise activation" is used to cleave the pro domain and produce an active MMP. Stepwise activation refers to a two-step mechanism whereby a proteinase susceptible bait region is removed from the pro region by non-specific proteinases resulting in a conformational change. This conformational change allows other MMPs, or in some circumstances the MMP intermediate itself, to completely remove the remainder of the propeptide, and produce a mature, active protein (Nagase et al., 1990). MMP-2, however, is an interesting exception to this mechanism, and is activated at the cell surface through a multistep pathway that involves MMP-14 (or MMP-15) and TIMP-2. In this tri-molecular mechanism, activated MMP-14 on the cell surface binds to and is inhibited by free TIMP-2. The C-terminal domain of the bound TIMP-2 acts as a receptor for the hemopexin domain of proMMP-2, which is then cleaved by a second molecule of MMP-14, and activated by a second molecule of MMP-2 (Deryugina et al., 2001). This system has a secondary control in that low levels of TIMP-2 favor activation of MMP-2, while high levels of TIMP-2 saturate free MMP-14 and thus prevent MMP-2 activation.

Finally, once active, MMPs are sensitive to inhibition by protein inhibitors- the TIMPs. To date, four TIMP members have been identified: TIMP-1, TIMP-2, TIMP-3,

and TIMP-4 (Sternlicht and Werb, 2001). TIMP family members share a 37-51%

sequence homology, and as a group reversibly inhibit all MMP family members in a 1:1

stoichiometry (Clendeninn and Appelt, 2001). TIMPs contain 12 highly conserved

cysteine residues that form six intrachain disulfide bridges to produce a six-loop, two-

domain structure (Williamson et al., 1990). Additionally, there are non-specific

proteinase inhibitors such as the serum constituent α_2-macroglobulin. Like MMPs, the

expression pattern of TIMPs varies considerably between tissues, and individual TIMPs

differ in their ability to bind and inhibit MMPs (Woessner and Nagase, 2000). Thus,

multiple control mechanisms exist that allow for tight control of MMP activity at several

levels (Figure 3). These mechanisms frequently overlap; resulting in distinct expression

patterns and generating feedback loops that result in tightly regulated MMP expression,

activation, and activity.

MMPs in Tumorigenesis and Cancer Progression

Cancer is the result of a progressive series of genetic and physiologic events that

disrupt the normal tissue homeostasis. In 2000, Hanahan and Weinberg described

"Hallmarks of Cancer", six events that are essential in the transformation from normal

cells to a malignant neoplasm. These events are: (i) immortalization, (ii) resistance to

apoptosis, (iii) self sufficiency with respect to growth signals, (iv) insensitivity to anti-

proliferative signals, (v) sustained angiogenesis, and (vi) the ability to invade local and

distant tissues (Hanahan and Weinberg, 2000). By virtue of a degrading a wide variety of

ECM substrates and localizing to the invasive edge, MMPs have been historically

associated with only the latest of these hallmarks - local invasion and metastasis.
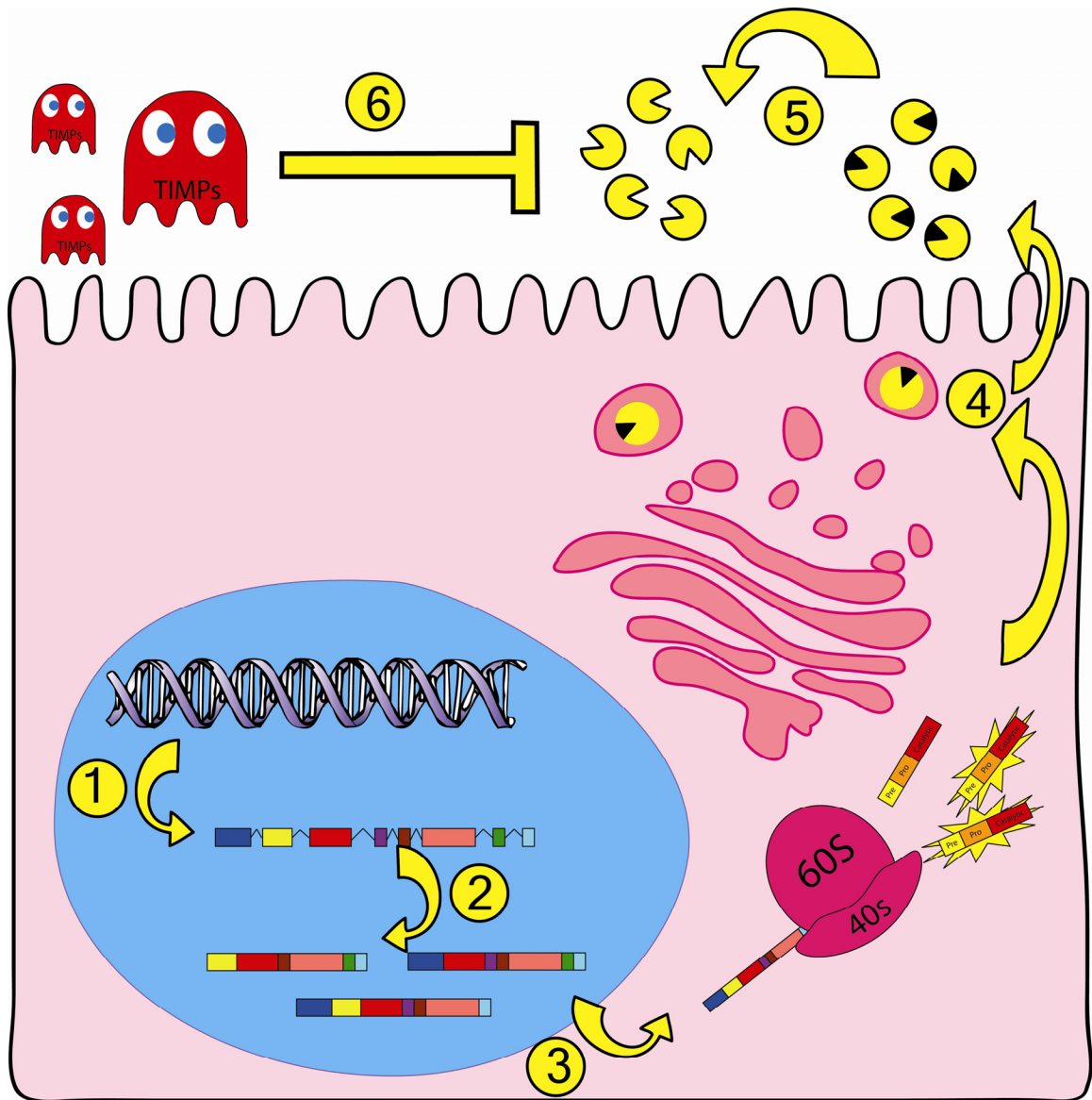
Figure 3: Regulation of MMPs occurs at multiple levels. MMPs activity is tightly regulated at the RNA level through control of (1) transcription, (2) post-transcriptional splicing, and (3) RNA stabilization. At the protein level MMP activity is modulated by (4) storage in vesicles until an appropriate signal mediates controlled release, (5) activation of latent pro-enzymes to a catalytically active form, and (6) inhibition by a class of endogenous proteins known as TIMPs.

However, several lines of experimental evidence have demonstrated roles for MMPs at all stages of tumor development in both pro- and anti- tumorigenic roles (Lopez-Otin and Matrisian, 2007; Martin and Matrisian, 2007; Noel et al., 2008).Aberrant MMP activity has been shown to mediate the earliest stages of tumorigenesis. MMP-3, a stromally expressed MMP, is commonly over-expressed in breast tumors. Exposure of a normal mammary epithelial cell line, Scp2, to MMP-3 causes cells to undergo epithelial to mesenchymal transition (EMT), presumably through the cleavage of E-cadherin, and a loss of cell-cell adhesions (Radisky et al., 2005). Further, MMP-3 treated cells express a splice variant of Rac1, which causes intracellular concentration reactive oxygen species (ROS) to increase.   High intracellular ROS concentrations directly cause oxidative damage of DNA and subsequently genomic instability, as well as the promotion of EMT by stimulating the expression of the transcription factor Snail (Radisky et al., 2005; Sternlicht et al., 1999).

MMP overxpression has been shown to produce tumors that are refactory to pro-apoptotic stimuli, a process that is thought to work through Darwinian selection for resistant clonal populations. Fas ligand (FasL) is a type II membrane protein expressed by several inflammatory cell lineages and upon binding to its receptor, Fas, rapidly induces a signal cascade that ultimately results in target cell apoptosis. Using a mouse model of prostate involution, a process dependent upon FasL-Fas interactions, mice that lack MMP-7 have a 67% decrease in apoptotic index, when compared to wild type controls (Powell et al., 1999). MMP-7 has been shown to generate a soluble form of FasL that is functionally competent, and rapidly induces apoptosis of cells in culture upon acute exposure. However, chronic exposure to this soluble form selects for a population

of cells that are refractory to Fas mediated apoptosis (Fingleton et al., 2001; Vargo-Gogola et al., 2002).

MMPs are capable of stimulating tumor cell proliferation in a number of ways. Perlecan and decorin, two components of the ECM, bind and sequester fibroblast growth factor (FGF) and TGF-β, respectively, both of which can be liberated by MMP activity, and result in cellular proliferation (Imai et al., 1997; Whitelock et al., 1996). Similarly, MMPs are capable of degrading inhibitory binding proteins, resulting in the release of active growth factors. Insulin-like growth factor binding proteins (IGF-BPs) bind to and sequester active IGFs, limiting their bioavailability. By direct proteolytic cleavage, MMP-7 degrades IGF-BP3, and releases active IGF-II which promotes cellular proliferation (Miyamoto et al., 2007). MMPs activity directly can convert inactive growth factors to an active form, as seen by MMP-2 and MMP-9 processing of inactive TGF-β to an active form (Yu and Stamenkovic, 2000). Thus, by degrading inhibitory binding proteins, or by directly liberating or activating latent growth factors, MMPs have been shown to modulate proliferation of tumor cells.

Angiogenesis, the process by which tumors become vascularized, is both positively and negatively regulated by MMPs. Vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis, exists in an inactive complexed state bound to connective tissue growth factor (CTGF). This complex is attacked by MMP-14, CTGF is degraded, releasing active VEGF and stimulating angiogenesis(Hashimoto et al., 2002). Further, VEGF is cleaved by MMPs-3 and MMP-9 to generate a truncated protein incapable of binding heparin sulfates, and thus promoting an irregular pattern of neovascularization (Lee et al., 2005). Additionally, since VEGF binds strongly to matrix

proteins due to heparin binding domains, degradation of ECM by MMPs is thought to be another mechanism by which MMPs can increase levels of bioactive VEGF (Bergers et al., 2000). Conversely, MMP activity can inhibit angiogenesis. MMPs--3, -12, -19, and -25 cleave cell-surface urokinase-type plasminogen activator receptor (uPAR), resulting in less cellular urokinase plasminogen activator (uPA) binding, and consequently, diminished formation of angiogenic structures (Andolfo et al., 2002; Koolwijk et al., 2001). A number of studies have shown that anti-angiogenic peptides such as angiostatin or tumstatin can be generated by MMP cleavage of plasmin or collagen IV, respectively. In this regard, MMP-12 mediated generation of angiostatin is considered to be highly relevant to its apparent anti-tumor role (Acuff et al., 2006; Cornelius et al., 1998; Houghton et al., 2006). Similarly, MMP-9 appears to suppress tumor growth in some models through generating of angiostatin or tumstatin (Chen et al., 2005; Hamano et al., 2003).

Several studies have recently detailed other apparently protective, anti-tumorigenic roles for MMPs. Mice lacking MMP-8, an enzyme predominantly expressed by neutrophils, develop significantly more papillomas than littermate controls when treated with DMBA+TPA, a common chemical carcinogenesis model of skin tumorigenesis. This effect was rescued by bone-marrow transplantation, demonstrating that neutrophil-derived MMP-8 is sufficient to restore the protective effect of this MMP (Balbin et al., 2003). Similarly, mice deficient for MMP-3 develop tumors sooner than littermate controls, and this increased growth rate was coupled with an elevated rate of proliferation, though apoptosis was unaffected (McCawley et al., 2004). In both of these

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studies, MMP-mediated alteration of chemokines was suggested as the mechanism behind the effects seen.

In conclusion, in recent years the role of MMPs in tumor development has expanded dramatically. Historically, MMPs have been viewed as pro-tumorigenic, and in particular, associated with pro-metastatic spread, although current evidence demonstrates that MMPs are key players during all stages of tumor development. Several MMPs are differentially over-expressed in human colon cancer. To date, MMPs-1, -2, -3, -7, -8, -9, -10, -11, -12, -14, -21, and -25 have been found to be more abundant in colon tumors compared to normal colonic tissue. Conversely, MMP-19 and -28, while normally present in colon tissue, are less abundant in colonic neoplasms, however, the consequence of the dysregulation of these MMPs is unclear (Ahokas et al., 2002; Bister et al., 2004; Sun et al., 2007; Wagenaar-Miller et al., 2004). Because of the expanded role of MMPs to include all stages of tumor development, and the protective, anti-tumorigenic role observed for several MMPs, I aimed to generate a comprehensive profile of MMPs expressed in a mouse model of colorectal tumor development. Furthermore, genetic ablation of several MMPs in the *Min* system allowed me to investigate functions of specific pro- and anti-tumorigenic MMPs that can be used to develop more specific MMPIs.

CHAPTER II

MATERIALS AND METHODS

Mice Breeding and Genotyping

Preparation of Genomic DNA

Genomic DNA for genotype analysis was prepared from the distal tip of the tail

by incubating a 3mm section of tail in 100μl of a lysis buffer composed of 25 mM NaOH,

0.2 mM EDTA. After incubation in the lysis buffer for 25 minutes at 95° C, 100μl of 40

mM Tris-HCl solution was added to neutralize the alkali, and the preparation was briefly

mixed by vortexing.

Min Mice

Min mice (C57Bl/6J-APC$^{Min/+}$) were purchased from the Jackson Laboratory (Bar

Harbor, ME) to establish a breeding colony with wild-type C57Bl/6J mice from the same

source. All mice were maintained on a high-fat 5015 diet (LabDiet, Purina, St. Louis,

MO) which has been shown to enhance tumorigenesis in the Min model system (van

Kranen et al., 1998). Pups were weaned at 3 weeks of age and genotyped by PCR

analysis. A 340 base pair product was amplified using the primers 5'- TTC TGA GAA

AGA CAG AAG TTA-3' and 5'-TTC CAC TTT GGC ATA AGG C-3'. PCR reactions

contained 50 pmol each primer (Invitrogen), 1μl NaOH isolated genomic DNA, and

HotstarTaq master mix (Qiagen, Valencia, CA) consisting of Taq polymerase, reaction

buffer, and dNTPs. Reactions were performed on a RoboCycler Gradient 96 (Strategene,

La Jolla, CA) using the following program: 15 min at 94° C (1 cycle); 30 sec at 94° C, 1

min at 55° C, 1 min at 72° C (35 cycles); 2 min at 72° C (1 cycle). The product from this

PCR reaction was run out on a 1.5% agarose 1X TBE gel containing 0.001% (v/v)

ethidium bromide and visualized under UV light.

Generation of MMP-2$^{-/-}$ Min Mice

Min mice lacking MMP-2 were generated by crossing female mice deficient for

MMP-2 (Itoh et al., 1997) with male mice carrying the Min allele. Resulting female

offspring heterozygous for MMP-2, were crossed with male mice heterozygous for

MMP-2 and carrying the Min mutation. Four week old mice were genotyped for MMP-2

by PCR analysis using genomic DNA isolated from the tail as previously described and

the following primers: MMP-2 KO FW: 5'-TAG AAT TCC TGC AGC CCG-3', MMP-

2 KO RV: 5'-CAA ACT ACA ACC AGC TGC TC-3' (knockout product 243bp); MMP-

2 WT FW: 5'-CAA CGA TGG AGG CAC GAG TG-3', MMP-2 WT RV: 5'-GCC GGG

GAA CTT GAT CAT GG-3' (wild type product 535bp). PCR reactions contained 50

pmol each primer (Invitrogen), 1µl NaOH isolated genomic DNA, and HotstarTaq master

mix (Qiagen, Valencia, CA) consisting of Taq polymerase, reaction buffer, and dNTPs.

Reactions were performed on a RoboCycler Gradient 96 (Strategene, La Jolla, CA) using

the following program: 15 min at 94° C (1 cycle); 1 min at 94° C, 45 sec at 52° C, 30 sec

at 72° C (33 cycles); 7 min at 72° C (1 cycle). Resulting products were run out on a 1.5%

separated on a 1.5% agarose gel by electrophoresis and visualized with ethidium

bromide.

Generation of MMP-2$^{-/-}$;MMP-7$^{-/-}$Min Mice

Min mice deficient for both MMP-2 and MMP-7 were generated by crossing female mice deficient for MMP-2 with male mice deficient for MMP-7. The resulting female offspring, heterozygous for both MMPs (MMP-2$^{-/+}$;MMP-7$^{-/+}$), were crossed back to a male MMP-7 deficient animal generating animals heterozygous for MMP-2, but lacking MMP-7 (MMP-2$^{-/+}$;MMP-7$^{-/-}$). Non-littermate F$_2$ animals were then crossed to generate mice lacking both MMP-2 and MMP-7 (MMP-2$^{-/-}$;MMP-7$^{-/-}$). Double knock out female animals were crossed with a male mouse heterozygous for the Min allele, but wild type for both MMP loci to generate a single male MMP-2$^{-/+}$;MMP-7$^{-/+}$;APC$^{Min/+}$ founder, from which four discrete lines were selectively generated- mice lacking MMP-2 (MMP-2$^{-/-}$;APC$^{Min/+}$), MMP-7 (MMP-7$^{-/-}$;APC$^{Min/+}$), double deficient (MMP2$^{-/-}$;MMP-7$^{-/-}$;APC$^{Min/+}$) and wild type controls (APC$^{Min/+}$) (Figure 4).

Generation of MMP-9$^{-/-}$ Min Mice

MMP-9 deficient Min mice were generated by crossing female mice deficient for MMP-9 (Vu et al., 1998) with male mice carrying the Min allele. Resulting female MMP-9 heterozygous pups, were crossed with male pups carrying the Min allele and heterozygous for MMP-9. Four week old mice were genotyped for MMP-9 by PCR analysis using genomic DNA isolated from the tail as previously described and the following primers: MMP-9 KO FW: 5'-CTC AGA AGA ACT CGT CAA GA-3',
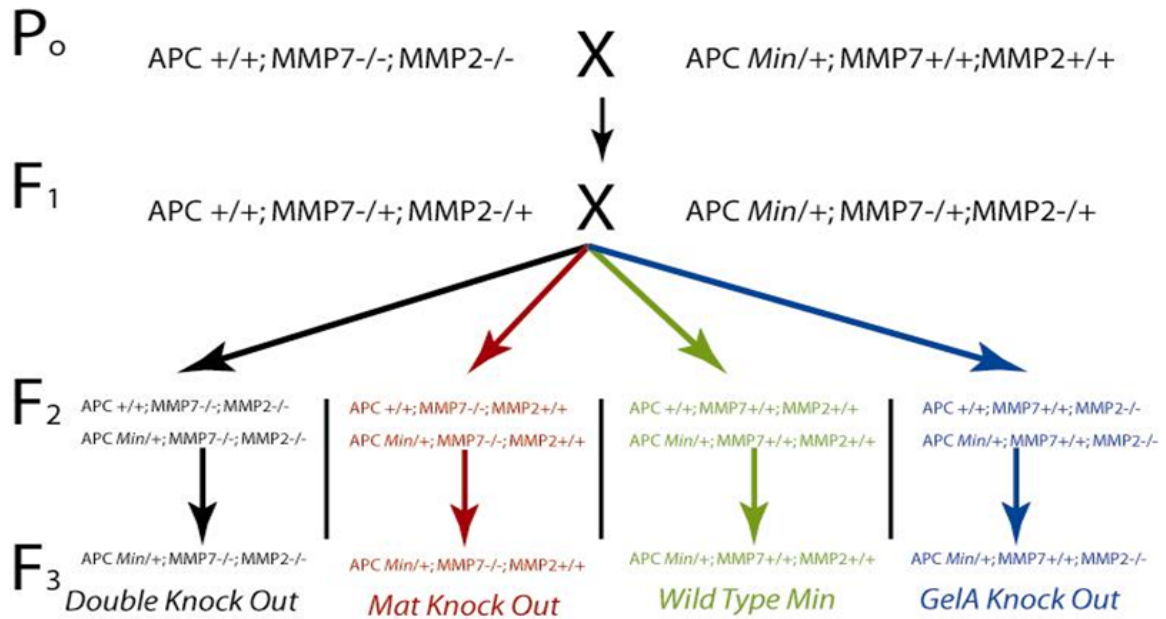
Figure 4: Summary of the breeding strategy used to generate MMP-2; MMP-7 double deficient Min mice. To minimize genetic variation between lines, a single $APC^{Min/+}$; MMP-7$^{-/+}$; MMP-2$^{-/+}$ male founder was used to selectively generate four separate mouse lineages to examine the effect of ablating MMP-7 and MMP-2 in the Min model.

MMP-9 KO RV: 5'-GGA TTG CAC GCA GGT TCT CC-3'; MMP-9 WT FW: 5'-GCA TAC TTG TAC CGC TAT GG-3', MMP-9 WT RV: 5'-TAA CCG GAG GTG CAA ACT GG-3' were used to generate a 600bp product indicative of the knockout allele, and a 300bp product indicative of the wild type allele. PCR reactions contained 50 pmol each primer (Invitrogen), 1µl NaOH isolated genomic DNA, and HotstarTaq master mix (Qiagen, Valencia, CA) consisting of Taq polymerase, reaction buffer, and dNTPs. Reactions were performed on a RoboCycler Gradient 96 (Strategene, La Jolla, CA) using the following program: 15 min at 94° C (1 cycle); 1 min at 94° C, 1 min at 58° C, 1 min at 72° C (35 cycles); 5 min at 72° C (1 cycle). Resulting products were run out on a 1.5% agarose gel and visualized using ethidium bromide.

Generation of MMP-12$^{-/-}$ Min Mice

MMP-12 deficient Min mice were generated by crossing female mice deficient for MMP-12 (Shipley et al., 1996) with male mice carrying the Min allele. Resulting female progeny heterozygous for the MMP-12 allele, were crossed with male siblings carrying the Min allele and heterozygous for MMP-12. Four week old mice were genotyped for MMP-12 by PCR analysis using genomic DNA isolated from the tail as previously described and the following primers: MMP-12 KO FW: 5'-GAC CAC CAA GCG AAA CAT-3', MMP-12 KO RV: 5'- CAA GAA GGC GAT AGA AGG -3'; MMP-12 WT FW: 5'-GCA TAC TTG TAC CGC TAT GG-3', MMP-12 WT RV: 5'-TAA CCG GAG GTG CAA ACT GG-3' were used to generate a 150bp product indicative of the knockout allele, and a 230bp product indicative of the wild type allele. PCR reactions contained 50 pmol each primer (Invitrogen), 1μl NaOH isolated genomic DNA, and HotstarTaq master mix (Qiagen, Valencia, CA) consisting of Taq polymerase, reaction buffer, and dNTPs. Reactions were performed on a RoboCycler Gradient 96 (Strategene, La Jolla, CA) using the following program: 15 min at 94° C (1 cycle); 30 sec at 94° C, 30 sec at 55° C, 20 sec at 72° C (35 cycles); 5 min at 72° C (1 cycle). Resulting products were run out on a 2% agarose gel and visualized with ethidium bromide.

Generation of MMP-19$^{-/-}$ Min Mice

MMP-19 deficient Min mice were generated by crossing female mice deficient for MMP-19 (Pendas et al., 2004) with male Min mice. Resulting female MMP-19

heterozygous pups were crossed with male pups heterozygous for MMP-19 and carrying

the Min allele. Four week old mice were genotyped for MMP-19 by PCR analysis using

genomic DNA isolated from the tail as previously described and the following primers:

MMP-19 KO FW: 5'-TCT TGA TTC CCA CTT TGT GGT TC-3', MMP-19 KO RV:

5'-GTC CGG CTC ATC CTC GAC TAT TT-3'; MMP-19 WT FW: 5'-TTT GGT AGG

GCA GGA GTT AAT GG-3', MMP-19 WT RV: 5'-GGT AGG TTG GAG GGC AAG

AGG AA-3' were used to generate a 1500bp product indicative of the knockout allele,

and a 300bp product indicative of the wild type allele. PCR reactions contained 50 pmol

each primer (Invitrogen), 1µl NaOH isolated genomic DNA, and HotstarTaq master mix

(Qiagen, Valencia, CA) consisting of Taq polymerase, reaction buffer, and dNTPs.

Reactions were performed on a RoboCycler Gradient 96 (Strategene, La Jolla, CA) using

the following program: 15 min at 95° C (1 cycle); 30 sec at 95° C, 30 sec at 55° C, 30

sec at 72° C (38 cycles); 10 min at 72° C (1 cycle). Resulting products were run out on a

1.5% agarose gel and visualized with ethidium bromide.

Generation of c-kit$^{W-sh/W-sh}$ Min Mice

Male and female Min (C57Bl/6J-APC$^{Min/+}$), Sash (C57Bl/6J-c-kit$^{W-sh/W-sh}$) and

wild type littermates (C57Bl/6J) were bred in our laboratory from breeder mice obtained

from The Jackson Laboratory (Bar Harbor, ME). Min-Sash (C57Bl/6/J APC$^{min/+}$;c-kit$^{W-sh/W-sh}$) mice were generated by crossing a male Min mouse with female Sash (C57Bl/6/J -

c-kit$^{W-sh/W-sh}$) mice. The resulting mice, heterozygous for the *W-sh* allele, were crossed to

each other, carrying the *Min* allele along the paternal lineage. Mice were genotyped for

the Min allele by PCR and for the Sash mutation based upon coat color (Grimbaldeston et al., 2005).

Tissue Collection, Processing and Tumor Measurements

Tissue Collection and Tumor Measurements

Intestines were excised from freshly sacrificed animals, flushed with PBS, filled with 10% phosphate buffered formalin and fixed overnight at 4ºC. The following day, intestines were rinsed and immersed in 70% ethanol for long term storage. Tumor incidence and location were recorded with the aid of a dissecting microscope by two counters, one of which is blinded to the genotype of the animals. Tumor diameter at the widest point was measured to the nearest 100μm using digital calipers and a dissecting microscope.

Tumors designated for histological analysis were excised with minimal surrounding uninvolved tissue and paraffin embedded on end using a histoprocessor (Tissue-Tek Vacuum Infiltration Processor) according to the manufacturer's instructions. 5 μm thick sections were cut and mounted on Superfrost Plus glass slides (Fisher Scientific) for histological or immunohistochemical analyses.

RNA Isolation, Microarray and Real-Time PCR Analysis

RNA Isolation

Pools of small intestinal adenomas were harvested from the ilea of six 15-week old Min mice. To prevent contamination of control tissue with microscopic tumors, normal ileal tissue was harvested from non-Min littermates for use as control tissue. Samples obtained from individual mice were processed and analyzed independently.

Tissues were placed into RNAlater solution (Qiagen, Valencia, CA) and stored

overnight at 4°C. The following day, total RNA was isolated from and purified using an

RNeasy mini kit (Qiagen). Briefly, 5 adenomas were pooled such that they had a total

mass of 150mg. For control samples, 150mg of normal ilea with Peyer's patches

removed was used. Tissue was rinsed in DEPC treated PBS to remove residual

RNAlater, then immersed in 2ml Trizol reagent (Invitrogen, Carlsbad, CA) and

homogenized using a rotor/stator homogenizer. Trizol prepared RNA was further

purified using a Qiagen RNeasy Mini kit with on column RNase-free DNase I digestion

(Qiagen).

RNA concentration and integrity was measured by the Vanderbilt Microarray

Shared Resource (VMSR) using a NanoDrop ND-1000 Spectrophotometer (NanoDrop

Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies,

Santa Clara, CA), respectively. Samples showing a RNA integrity number (RIN) of less

than 7.0 were excluded from further analysis.

cDNA Microarray Construction

A custom cDNA microarray containing probes capable of identifying species

specific human and murine MMPs and TIMPs was constructed by cloning divergent

regions of orthologous genes. Briefly, divergent regions were identified by aligning

orthologues and highly homologous genes as previously identified using the

CLUSTALW multi-sequence alignment tool (Massova et al., 1998; Thompson et al.,

1994). Divergent regions of low homology were visually identified from CLUSTAL W

alignments and targeted for probe construction. To ensure that divergent regions were

specific for the gene of interest, nucleotide sequences were submitted to the BLAST

(**B**asic **L**ocal **A**lignment **S**earch **T**ool) search algorithm and compared against both the

human and murine genomic sequences, and considered unique if no other transcripts

were detected with an e score of less than $1x10^{-30}$ (Altschul et al., 1990).

Once identified, targeted sequences were cloned by obtaining EST (Expressed

Sequence Tag) clones (Research Genetics, Huntsville, AL) of the region of interest to be

used as a template for PCR amplification. However, in several instances, EST coverage

of unique regions was poor and genomic DNA or RT-PCR generated cDNA products

were used as templates (Table 2**)**. Additionally, several orthologous MMPs exhibited

extremely high homology between human and murine sequences such that creating

probes capable of differentiating between sequences was not possible. Thus, gene

profiling of MMPs-16, -19, -24, -25, -27 and -28 are shared common probes for human

and murine and cannot differentiate between species.

Probes were generated by PCR amplification of targeted regions from publicly

available EST clones. PCR reactions were performed by using EST positive bacteria as a

template and a re-annealing temperature gradient of 47-58°C on a Robocycler Gradient

96 thermocycler (Stratagene). Specifically, 5μl of bacteria containing the EST of interest

was used as a template for a 50ul PCR reaction containing 20pmol each primer using a

Promega 2X PCR Taq kit (Promega). Eight identical reactions were setup for each

probe, reactions were first heated to 95ºC for 5 minutes to lyse the EST containing

bacteria, reactions were then amplified for 35 cycles: 30 seconds melt at 95ºC, 1 minute

reannealing across a 47-58ºC gradient, and 1 minute extension at 72ºC. Resulting

Table 2: Summarization of primers and templates used for generation of cDNA probes. . Abbreviations **H:** Human, **M:** Mouse, **EST:** Expressed Sequence Tag, **cDNA:** coding DNA.

Gene	Species	Source	Forward Primer	Reverse Primer
MMP-1	H	Subclone	5'-TTT CTT TCA TGG AAC AAG AC-3'	5'-TTT TGT ACC CAC CAT TTG TG-3'
	M	Genomic	5'-AAA AAC GTG AAT GGC AAG GA-3'	5'-TGT GTT TTG GTC CAA CGA GG-3'
MMP-8	H	EST	5'-GTT TAT AAT CCT GTA GGT CAG-3'	5'-AAA CTG CTG AGA ATT ACC TA-3'
MMP-11	M	EST	5'-TGG GGG AAC TGG AGT GTC CTT GCT G-3'	5'-AGC AGT GCT GCG AAA AGG GCT TCA G-3'
MMP-12	M	cDNA	5'-TGA AGG GTG CTT GCT GGT TTT-3'	5'-TTA CAG ATA AAC CAG TTG GCC TCT G-3'
MMP-14	H	cDNA	5'-CGC AGC CTC CTT GCT TCT CT-3'	5'-GGG GCT GGA CAG ACA CAA AG-3'
	M	EST	5'-GGC TGC TCT CAC CCG TCC TG-3'	5'-TTG CCT TCA AGT GCA GAG CC-3'
MMP-15	H	EST	5'-TGT GGT TCT GAG ATG GCT CCC AGG G-3'	5'-ACC AGG GGG CCC AGA CAG TCT CC-3'
	M	EST	5'-ATC AGG TAG CAC ACC GCA GC-3'	5'-TGT GTG TGG CTA GGA GGG CA-3'
MMP-16	H	Genomic	5'-CCC AGT AAA ATT TCA GGA TT-3'	5'-GAA GTT CAA TTG TGG GTT TT-3'
MMP-17	H	EST	5'-TGT TGG TCT GGA ACG CGT CAC AGG G-3'	5'-GCT GGG CAG GCT CAG GTG GCA AG-3'
	M	EST	5'-GGA TCC CCA GTA CTC AGC AGG ACT TG-3'	5'-GCC CTC GAG AAC TCT CTT ACT TGT GAA AC-3'
MMP-19	H/M	EST	5'-CGA TAT TCC CAG GCC CTC ATG-3'	5'-AGA CAC TCG GAA CAA GGG GC-3'
MMP-20	H	cDNA		
	M	cDNA	5'-GTC GTG TGT CAG AGA CAC TC-3'	5'-CAC ACA CAT GAG TGC ACA TA-3'
MMP-23	H	EST	5'-CAG CCC TGA GCC CCA CAG CA-3'	5'-AGC GCA GCA ACG TCT CCC TG-3'
	M	EST	5'-GGC ACG AGG CCT ACG TCG CG-3'	5'-ACA TTT CCC TGA GCT GCA TT-3'
MMP-24	H/M	EST	5'-GCC AAG CTT ACT CCC CCT CAT TAA TGT TG-3'	5'-GCC CTC GAG AAA AGG TAG TTT GGG TTA GG-3'
MMP-25	H/M	EST	5'-GCG CTT CCC CAA GAA CAG CA-3'	5'-TGT TCG GTC TGG ATG GCT CC-3'
MMP-26	H	cDNA	5'-GCC AAG CTT CAG ACA TCA AGG TTT CTT TC-3'	5'-GCC TCG AGA TTA TGG AGC TCT GAT TCC C-3'
MMP-27	H/M	EST	5'-GCC AAG CTT AGA CAT GTC TTC TCC AAG TC-3'	5'-GCC CTC GAG ATC ATA AGA GTT TAA GCT TG-3'
MMP-28	H/M	EST	5'-CGC TGA CAT CCG GCT CAC CTT CTT C-3'	5'-TGG GAG CTG GAC GGC ACT GA-3'
TIMP-1	M	Subclone	5'-CTA AAA GGA TTC AAG GCT GT-3'	5'-AAA ACT CTT TGC TGA GCA GG-3'
TIMP-3	H	EST	5'-CAG GAG ACA CTA CCC TTC CA-3'	5'-ATC TAA GAA GCC TCT ACC CC-3'
TIMP-4	H	EST	5'-TTT TTG GTT TCA TTC CTG CC-3'	5'-AAG AAG TGC CAA GTG GAC AG-3'
	M	EST	5'-GTC CTT TGA CCA TCA CCA CCT G-3'	5'-TTG GGA GAC AGG AAG AGG GG-3'

products were gel purified using low melting point agarose (SeaKem) and ligated into pCR2.1-TOPO using a Topoisomerase I mediated ligation according to manufacturer's directions (TOPO-TA cloning kit, Invitrogen). DNA was prepared using a Qiagen MaxiPrep column, and 20µg of purified plasmid DNA was provided to the Vanderbilt DNA Sequencing Core for sequencing to ensure that the constructed probe was free of PCR artifacts. Probe sequences were then realigned with their target sequences using CLUSTALW for annotation purposes. Positive clones were supplied to the Vanderbilt Microarray Shared Resource (VMSR), amplified using M13 primer sites that flank the probe sequence and spotted onto glass microarray slides.

MMP-1

Human: A 314-base pair fragment corresponding to bases 1382-1695 of human MMP-1 (NM_002421) was subcloned by PCR into pCR2.1-TOPO-TOPO (Invitrogen) from a plasmid containing a sub-clone of human MMP-1 using the following primers: FW: 5'-TTT CTT TCA TGG AAC AAG AC-3' and RV:5'-TTT TGT ACC CAC CAT TTG TG-3'.

Murine: A 205-base pair fragment corresponding to bases 128-332 of murine MMP-1b (mcolb, NM_032007), but common to both mcola and mcolb was cloned into pCR2.1-TOPO using murine genomic DNA as a template. The primers used for this reaction were FW: 5'-AAA AAC GTG AAT GGC AAG GA-3' and RV:5'-TGT GTT TTG GTC CAA CGA GG-3'.

MMP-2

Human: An 884-base pair fragment corresponding to bases 1201-2084 of human MMP-2 (NM_004530.1) was previously generated by inserting an PvuII-BamHI fragment of human MMP-2 into pBluescript KS II+ (Rodgers et al., 1994).

Murine: A 1089-base pair fragment corresponding to bases 890-1987 of murine MMP-2 (NM_008610.1) was created by inserting an RT-PCR product murine post partum uterus origin into pGEM3Z. RT-PCR was performed using primers FW:5'-GGT GGC AAT GCT GAT GGA CA-3' and RV:5'-TTG GTT CTC CAG CTT CAG GT-3' (Reponen et al., 1992).

MMP-3

Human: A 555-base pair fragment corresponding to bases 1227-1781 of human MMP-3 (NM_002422.2) was previously generated by inserting a ClaI-AccI fragment of full length human MMP-3 into pGEM7Z (McDonnell et al., 1991).

Murine: A 480-base pair fragment corresponding to bases 904-1383 of murine MMP-3 (NM_010809.1) was previously generated by inserting a KpnI-XhoII fragment of murine MMP-3 into pGEM4 (Witty et al., 1995).

MMP-7

Human: A 818-base pair fragment corresponding to bases 20-837 of human MMP-7 (NM_002423.2) was previously generated by inserting a XhoI-XbaI fragment of full length human MMP-7 into pGEM7Z (McDonnell et al., 1991).

Murine: A 479-base pair fragment corresponding to bases 406-884 of murine MMP-7 (NM_010810.1) was previously generated by RACE PCR from RNA of post partum uterus origin as previously described (Witty et al., 1995).

MMP-8

Human: A 501-base pair fragment corresponding to bases 1532-2032 of human MMP-8 (NM_002424.1) was previously generated by PCR amplification (Heppner et al., 1996).

Murine: A 260-base pair fragment corresponding to bases 177-436 of murine MMP-8 (NM_008611.2) was cloned into pCR2.1-TOPO using an EST (AI265065) as a template. The primers used for this reaction were FW: 5'- GTT TAT AAT CCT GTA GGT CAG-3' and RV:5'- AAA CTG CTG AGA ATT ACC TA-3'.

MMP-9

Human: A 798-base pair fragment corresponding to bases 1537-2334 of human MMP-9 (NM_004994.1) was cloned by generating a EcoRV-HindIII fragment using a full length cDNA template (Heppner et al., 1996).

Murine: An 835-base pair fragment of murine MMP-9 (NM_013599.1) was previously cloned by isolating an EcoRV-NdeI fragment from full length murine MMP-9 cDNA (Rudolph-Owens, unpublished).

MMP-10

Human: A 528-base pair fragment corresponding to bases 1047-1574 of human MMP-10 (NM_002425.1) was cloned by generating a EcoRV-NdeI fragment into pGEM5Z using a full length cDNA template (McDonnell et al., 1991).

Murine: A707-base pair fragment corresponding to bases 632-1338 of murine MMP-10 (NM_019471) was created by restriction digest of full length murine MMP-10 cDNA with PstI and SacI. The resulting fragment was ligated into pGEM4 (Witty et al., 1995).

MMP-11

Human: A 324-base pair fragment corresponding to bases 1819-2160 of human MMP-11 (NM_005940.2) was generated by PCR using an EST (BG104963) as a template and primers FW:5'- TGG GGG AAC TGG AGT GTC CTT GCT G-3' and RV:5'- AGC AGT GCT GCG AAA AGG GCT TCA G-3'. The resulting product was inserted into pCR2.1-TOPO.

Murine: A 1042-base pair fragment corresponding to bases 998-2039 of murine MMP-11 (NM_008606.1) was created by digesting a cDNA for full length murine MMP-11 with restriction enzymes PvuII and PstI, and the resulting fragment was inserted into pGEM5Z (Rudolph-Owen et al., 1997).

MMP-12

Human: A 424-base pair fragment corresponding to bases 1-423 of human MMP-12 (NM_002426.1) was generated by digesting a cDNA for full length human MMP-12

with restriction enzymes HindIII and BamHI, and the resulting fragment was inserted into pGEM7Z (Curci et al., 1998).

Murine: A 242-base pair fragment corresponding to bases 1401-1642 of murine MMP-12 (NM_008605.1) was produced by PCR amplification using a full length cDNA for murine MMP-12, and primers FW:5'- TGA AGG GTG CTT GCT GGT TTT-3' and RV:5'- TTA CAG ATA AAC CAG TTG GCC TCT G-3'. The resulting amplicon was inserted into pCR2.1-TOPO.

MMP-13

Human: A 690-base pair fragment corresponding to bases 103-792 of human MMP-13 (NM_002427.2) was inserted into pGEM-T for use as a *in situ* hybridization probe (Vaalamo et al., 1997).

Murine: An 838-base pair fragment corresponding to bases 1624-2461 of murine MMP-13 (NM_008607.1) was amplified by PCR and inserted into pGEM7Z (Rudolph-Owen et al., 1997).

MMP-14

Human: A 180-base pair fragment corresponding to bases 2091-2270 of human MMP-14 (NM_004995.2) was cloned from a full length cDNA by PCR using the primers FW:5'-CGC AGC CTC CTT GCT TCT CT-3' and RV:5'-TTG CCT TCA AGT GCA GAG CC-3' and inserted into pCR2.1-TOPO.

Murine: A 216-base pair fragment corresponding to bases 1999-2214 of murine MMP-14 (NM_008608.1) was cloned from an EST (accession BE332566) using the PCR

primers FW:5'-GGC TGC TCT CAC CCG TCC TG-3' and RV:5'-TTG CCT TCA AGT

GCA GAG CC-3' and inserted into pCR2.1-TOPO.

MMP-15

Human: A 366-base pair fragment corresponding to bases 2097-2462 of human

MMP-15 (NM_002428.1) was generated by PCR amplifying a corresponding region of

an EST (BG327005) using the primers FW:5'-TGT GGT TCT GAG ATG GCT CCC

AGG G-3' and RV:5'-ACC AGG GGG GCC AGA CAG TCT CC-3' and inserted into

pCR2.1-TOPO.

Murine: A 105-base pair fragment corresponding to bases 2002-2106 of murine

MMP-15 (NM_008609.1) was PCR amplified using an EST (BF537603) as a template

and primers FW:5'-ATC AGG TAG CAC ACC GCA GC-3' and RV:5'-TGT GTG TGG

CTA GGA GGG CA-3'. The resulting amplicon was ligated into pCR2.1-TOPO.

MMP-16

Human and Murine: The loci encoding human and murine MMP-16 (are highly

homologous and EST coverage is poor. Accordingly, a single probe to a highly

conserved region common to both orthologues was constructed for this MMP. Primers

FW:5'-CCC AGT AAA ATT TCA GGA TT-3' and RV:5'-GAA GTT CAA TTG TGG

GTT TT-3' were used to amplify a 234-base pair fragment using human genomic DNA

(Promega) as a template. The resulting product was inserted into pCR2.1-TOPO.

MMP-17

Human: A 325-base pair fragment corresponding to bases 2076-2400 of human MMP-17 (NM_016155.2) was cloned from an EST (AI829838) using PCR primers FW:5'-TGT TGG TCT GGA ACG CGT CAC AGG G-3' and RV:5'-GCT GGG CAG GCT CAG GTG GCA AG-3' and inserted into pCR2.1-TOPO.

Murine: A 372-base pair probe for murine MMP-17 (NM_011846.3) corresponding to bases 1897-2268 was constructed using an EST (BF471718) as a template for PCR amplification with the primers FW:5'-GGA TCC CCA GTA CTC AGC AGG ACT TG-3' and RV:5'-GCC CTC GAG AAC TCT CTT ACT TGT GAA AC-3'. The resulting amplicon was inserted into pCR2.1-TOPO.

MMP-19

Human and Murine: Human and murine MMP-19 exhibit a high degree of homology, and no ESTs specific for murine MMP-19 are commercially available. Because of this, a single probe was constructed that would recognize both human and murine MMP-19 transcripts. To create this probe, a 312-base pair region of a human EST (AA481345) was cloned by PCR amplification. Primers FW:5'-CGA TAT TCC CAG GCC CTC ATG-3' and RV:5'-AGA CAC TCG GAA CAA GGG GC-3' were used to generate an amplicon which was then inserted into pCR2.1-TOPO.

MMP-20

Human: A 512-base pair fragment corresponding to bases 926-1437 of human MMP-20 (NM_004771.2) was cloned from full length cDNA (J. Caterina) using PCR

primers FW:5'-TGA AAT CTA GTT CTT GGA TTG GTT G-3' and RV:5'-TCT AAT

GTG GAT TGA AAT TC-3' and inserted into pCR2.1-TOPO.

Murine: A 254-base pair fragment of murine MMP-20 (NM_013903.1)

corresponding to bases 1539-1792 was cloned from a full length cDNA provided by C.

Overall using primers FW:5'-GTC GTG TGT CAG AGA CAC TC-3' and RV:5'- CAC

ACA CAT GAG TGC ACA TA-3' for PCR. The resulting amplicon was inserted into

pCR2.1-TOPO.

MMP-21

Human and Mouse: MMP-21 is a poorly characterized MMP with highly

restricted expression currently only detected during narrow developmental windows and

during certain malignancies (Ahokas et al., 2002). Furthermore, searching for suitable

EST clones to use as template for generating a probe for either human or murine MMP-

21 (NM_147191.1 and AY124569.1, respectively) revealed no suitable ESTs that are

commercially available. Thus, the decision was made to omit MMP-21 from the array.

MMP-23

Human: A 201-base pair probe corresponding to bases 11-211 of human MMP-

23A (AJ005256.1) was generated by PCR amplification using primers FW:5'- CAG CCC

TGA GCC CCA CAG CA-3' and RV:5'- AGC GCA GCA ACG TCT CCC TG-3' and

an EST (BI834812) as a template. The resulting fragment was ligated into pCR2.1-

TOPO.

Murine: A 207-base pair fragment of murine MMP-23 (NM_011985.1) corresponding to bases 159-365 was cloned from an EST (BF549980.1) using PCR primers FW:5'- GGC ACG AGG CCT ACG TCG CG-3' and RV:5'- ACA TTT CCC TGA GCT GCA TT-3', the resulting aplicon was gel purified and ligated into pCR2.1-TOPO.

MMP-24

Human: Because of a high degree of homology between human and murine MMP-24, a shared probe was generated.

Murine: A 348-base pair fragment of murine MMP-24 (NM_10808.3) corresponding to bases 2854-3201 was cloned from an EST (AB021226) using PCR primers FW:5'- GCC AAG CTT ACT CCC CCT CAT TAA TGT TG-3' and RV:5'- GCC CTC GAG AAA AGG TAG TTT GGG TTA GG-3' and the resulting fragment was ligated into pPCR-Script (Stratagene).

MMP-25

Human and Murine: Murine MMP-25 currently only exists as a theoretical sequence based upon genomic sequencing, to date full length cDNA has not been published. Further, human and murine MMP-25 (NM_022468.3 and NM_001033339.1, respectively) exhibit nearly 100% homology when aligned using CLUSTALW or FASTA. Thus, a decision was made to generate a shared probe.

To do this, a 263-base pair fragment was cloned from a human EST (NM_022468.3) using the PCR primers FW:5'- GCG CTT CCC CAA GAA CAG CA-3'

and RV:5'- TGT TCG GTC TGG ATG GCT CC-3'. The cloned fragment was gel purified and inserted into pCR2.1-TOPO.

MMP-26

Human: A 238-base pair fragment of human MMP-26 (AF248646.1) corresponding to bases 449-686 was cloned from a full length cDNA provided by Q. A. Sang using the PCR primers FW:5'- GCC AAG CTT CAG ACA TCA AGG TTT CTT TC-3' and RV:5'- GCC TCG AGA TTA TGG AGC TCT GAT TCC C-3', and inserted into pCR2.1-TOPO.

Murine: MMP-26 has only been detected in primate genomes to date, thus no probe was generated.

MMP-27

Human and Murine: As with MMP-25, murine MMP-27 only exists as a theoretical sequence based upon genomic sequencing, an human and murine MMP-27 (NM_022122.1 and NM_001030289.1, respectively) exhibit nearly 100% homology when compared by CLUSTALW alignment. Thus, a decision was made to generate a shared probe.

A 152-base pair probe corresponding to bases 1478-1629 of human MMP-27 was produced by PCR amplification using an EST (AI436025.1) as a template and FW:5'- GCC AAG CTT AGA CAT GTC TTC TCC AAG TC-3' and RV:5'- GCC CTC GAG ATC ATA AGA GTT TAA GCT TG-3' as primers. The resulting amplicon was propogated by insertion into pCR2.1-TOPO.

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MMP-28

Human and Murine: Human and murine MMP-28 (NM_024302.2 and

NM_080453.2, respectively) exhibit an extremely high degree of homology thus a single

probe that recognized both sequences was generated. A 356-base pair fragment of human

MMP-28 corresponding to bases 809-1164 was generated by PCR amplification using an

EST (BI914743) as a template and the primers FW:5'- CGC TGA CAT CCG GCT CAC

CTT CTT C-3' and RV:5'- TGG GAG CTG GAC GGC CAC TGA G-3', the resulting

product was gel purified and ligated into pCR2.1-TOPO.

TIMP-1

Human: A 650-base pair fragment of human TIMP-1 (NM_003254.1)

corresponding to bases 68-717 was previously generated

Murine: A 212-base pair fragment of murine TIMP-1 (NM_011593.1)

corresponding to bases 211-422 was generated by PCR amplification using a previously

described subclone as a template (Rudolph-Owen et al., 1997) and FW:5'- CTA AAA

GGA TTC AAG GCT GT-3' and RV:5'- AAA ACT CTT TGC TGA GCA GG-3' as

primers. The resulting fragment was ligated into pCR2.1.

TIMP-2

Human: A 446-base pair fragment of human TIMP-2 (S48568.1) corresponding

to bases 508-953 was previously generated and inserted into pGEM3Z by Ken Newell.

Murine: A 364-base pair fragment of murine TIMP-2 (M82858.1) corresponding to bases 294-657 was previously generated by digesting a full length cDNA with PstI and KpnI and ligating the resulting fragment into pGEM3Z (Rudolph-Owen et al., 1997).

TIMP-3

Human: A 294-base pair probe corresponding to bases 4031-4324 of human TIMP-3 (U14394.1) was produced by PCR amplification using an EST (BG621406) as a template and FW:5'- CAG GAG ACA CTA CCC TTC CA-3' and RV:5'- ATC TAA GAA GCC TCT ACC CC-3' as primers. The resulting product was ligated into pCR2.1-TOPO.

Murine: A 318-base pair fragment of murine TIMP-3 (NM_011595.1) corresponding to bases 578-895 was previously generated by digesting full length TIMP-3 cDNA with EcoRI and PstI, the resulting fragment was gel purified and ligated into pBlueScript KSII(+) (Rudolph-Owen et al., 1997).

TIMP-4

Human: A 331-base pair fragment of human TIMP-4 (NM_003256.1) corresponding to bases 843-1173 was generated by PCR amplification using an EST (AW293304) as a template and FW:5'- TTT TTG GTT TCA TTC CTG CC-3' and RV:5'- AAG AAG TGC CAA GTG GAC AG-3' as primers. The aplicon produced by this reaction was gel purified and ligated into pCR2.1-TOPO.

Murine: A196-base pair fragment corresponding to bases 735-930 of murine TIMP4 (AF282730.1) was generated by PCR amplification of an EST (AW047381)

using the primers FW:5'- GTC CTT TGA CCA TCA CCA CCT G-3' and RV:5'- TTG GGA GAC AGG GAA GAG GG-3', resulting in a fragment that was then ligated into pCR2.1-TOPO.

Affymetrix Hu/Mu ProtIn Array Construction

In conjuction with the Protease Consortium, a novel microarray capable of distinguishing between closely related homologous and orthologous protease transcripts was created on the Affymetrix platform. The constructed array, known as the Hu/Mu ProtIn Array, or alternatively as Protease Chip (Affymetrix product "Protease520066F"), contains 972 custom probesets that cover all identified human and murine proteinases, proteinase inhibitors, and interacting proteins (Schwartz et al., 2007).

Initially, default Affymetrix probe sets were used for construction of the array, however, not all probes were species specific. To fix this, probes set sequences provided by Affymetrix were compared to orthologous and highly homologous sequences by BLAST-N analysis with weighting for "short, nearly exact matches" (Altschul et al., 1990). Unique and highly homologous regions prone to cross talk were identified and probes were then redesigned and by Affymetrix to avoid homologous regions.

Microarray analysis was performed using the Hu/Mu ProtIn array, product #Protease520066F (Affymetrix, Santa Clara, CA) (Schwartz et al., 2007). cRNA synthesis, labeling, fragmentation and microarray hybridization, scanning, and analysis of differentially expressed transcripts was performed by the VMSR.

Real Time PCR Analysis

Real-time PCR analysis of mast cell protease genes (mcpts) and MMPs was performed on RNA samples used for microarray analysis to further characterize mast cell protease expression. cDNA was synthesized from 1μg total RNA using an iScript cDNA reverse transcriptase kit, following the manufacturer's instructions (Bio-Rad, Hercules, CA). The resulting cDNA products were diluted 1:5 using sterile distilled water prior to real-time PCR analysis. Real-time PCR reactions were performed using IQ Real-Time Supermix with SYBR Green (Bio-Rad) on a Bio-Rad iQ5 thermocycler. Reactions were run in triplicate for murine mcpt and MMP gene family members using previously described PCR primers (Martinez et al., 2005; Yamada et al., 2003) with a standard 2-step amplification program. Fold change was determined by comparing the mean C_t levels between tumor and control samples, after adjusting for reference gene expression (GAPDH), significance was determined by using a two-sample t-test as previously described (Bowen et al., 2006).

Histochemical and Immunohistochemical Analyses

Chloroacetate Reaction (CAE) to Demonstrate Mast Cells

Five-micron paraffin embedded, formalin-fixed sections were dewaxed and brought to water through graded alcohols. To demonstrate mast cells in tumor and normal tissue the chloroacetate reaction (Leder, 1979) was performed using Fast Blue BB (Sigma, St. Louis, MO), counterstained using Alum-Kernechtrot (Humason, 1979) and mounted in permount (Sigma). Positive cells were counted per unit area as measured by NIH ImageJ software.

Immunohistochemistry

Five-micron paraffin embedded, formalin-fixed sections were dewaxed and brought to water through graded alcohols. Sections were treated with 0.6% hydrogen peroxide in methanol to destroy endogenous peroxidase prior to antigen retrieval. Antigen retrieval was accomplished by heat denaturation by microwaving sections for 10 minutes in a 10mM sodium citrate solution, except where noted. Non-specific binding was inhibited by incubation in a blocking solution (10mM Tris-HCl pH7.4, 0.1M $MgCl_2$, 0.5% Tween20, 1% BSA, 5% Serum) for 1hr at room temperature. Primary antibodies were diluted in blocking solution and applied at 4 C° overnight. Appropriate IgG controls were used on adjacent sections to evaluate background staining. Sections were washed with TBST (150mM NaCl, 10mM Tris, 0.05% Tween 20) and incubated with appropriate biotinylated secondary antibody for 1hr at room temperature. Positive cells were visualized with an avidin-biotin peroxidase complex (Vectastain Avidin-Biotin Complex kit, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine tetrahydrochloride substrate (Sigma). Nuclei were counterstained with Mayer's hematoxylin (Sigma), washed in TBS, dehydrated through alcohols, cleared in xylenes and mounted.

Immunohistochemical Visualization of Proliferation and Apoptosis

Proliferating cells were detected by immunohistochemical staining for phospho-Histone H3 (Ser10) (Upstate, Lake Placid, NY). Secions were prepared as described using heat denaturation. Primary antibody was applied at a 1:250 dilution overnight at 4°

C overnight, and detected using a goat anti-rabbit secondary (Vector Laboratories, Burlingame, CA).

Alternatively, in BrdU treated animals, proliferation was measured by staining for BrdU incorporation. Briefly, mice were given a single injection of 75mg/kg BrdU in PBS one hour before sacrifice. Incorporated BrdU was then detected with a monoclonal antibody (Accurate Chemical, Westbury, NY) using trypsin denaturation according to the manufacturer's directions. Monoclonal primary antibody was applied at a 1:400 dilution at 4° C overnight, and a (mouse immunoabsorbed) rabbit anti-rat secondary antibody was used at 1:500.

Apoptotic nuclei were visualized by immunohistochemical staining of cleaved caspase-3(Asp175) (Cell Signaling Technology, Danvers, MA) using citrate antigen retrieval. A 1:400 dilution of a polyclonal primary antibody was applied overnight, and a 1:500 dilution of biotinylated goat anti-rabbit IgG was used for visualization using DAB as a substrate. Alternatively, TUNEL staining according to manufacturer's instructions (Millipore, Billerica, MA) on adjacent sections for comparison between methods.

Immunohistochemical Demonstration of Leukocyte Populations

Eosinophils were detected using a monoclonal rat antibody specific to murine major basic protein (MBP), kindly provided by James J. Lee, Ph.D. (Mayo Clinic Arizona, Scottsdale, AZ). Antigen was retrieved using Dako antigen retrieval solution (Dako, Glostrup, Denmark) and 3-in-1 proteinase solution (Zymed, Carlsbad, CA).

Primary antibody was applied at 1:500 dilution overnight at 4° C, and a biotin conjugated rabbit anti-rat (mouse immunoabsorbed) was used to detect positive cells.

Neutrophils were stained using a monoclonal anti-neutrophil antibody (AbD Serotec, Oxford, UK) at 1:100 dilution, and a rabbit anti-rat secondary antibody. T cells were visualized using polyclonal antibodies recognizing CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1500, and a rabbit anti-goat secondary (Vector Laboratories). Macrophages were demonstrated by staining for F4/80 antigen using a monoclonal antibody and proteinase K antigen retrieval (AbD Serotec) at 1:100 dilution overnight at 4° C and a rabbit anti-rat secondary.

MMP-9 and von Willebrand Factor Immunohistochemistry

Paraffin embedded, formalin-fixed sections were dewaxed and rehydrated through a series of graded alcohols. Sections were treated for 30 minutes with 0.6% hydrogen peroxide in methanol to destroy endogenous peroxidase prior to antigen retrieval. Antigen was retrieved either by microwaving sections for 10 minutes in 10mM sodium citrate buffer (MMP-9), or treatment with a [40ug/ml] solution of Proteinase K for 30 minutes at room temperature (vWF). Non-specific binding was inhibited by incubation in a blocking solution (10mM Tris-HCl pH7.4, 0.1M $MgCl_2$, 0.5% Tween20, 1% BSA, 5% Serum) for 1hr at room temperature. Rabbit polyclonal anti-mouse MMP-9 (used at 1:250 dilution) (Abcam, Cambridge, MA) or rabbit anti-human vWF antibodies (1:1000 dilution) (Dako), were diluted in blocking solution and applied at 4° C overnight. Appropriate IgG controls were used on adjacent sections to evaluate background staining. Sections were washed with TBS (150mM NaCl, 10mM Tris) and incubated with

appropriate biotinylated secondary antibody for 1hr at room temperature. Positive cells

were visualized with an avidin-biotin peroxidase complex (Vectastain Avidin-Biotin

Complex kit, Vector Laboratories, Burlingame, CA) and 3,3'-diaminobenzidine

tetrahydrochloride substrate (Sigma). Nuclei were counterstained with Mayer's

hematoxylin (Sigma), washed in TBS, dehydrated through alcohols, cleared in xylenes

and mounted.

Immunohistochemical Quantification

Metamorph software (Molecular Devices, Downingtown, PA) was used to

measure vWF staining intensity. Three sections from five each MMP-9 null and wild

type littermate mice were analyzed for positive staining area per total area and reported as

percent. Groups were compared using a two-tailed t test.

Immunofluorescent Colocalization of MMP-9 and Leukocyte Markers

Five micron, paraffin embedded, formalin fixed sections were dewaxed and rehydrated

through alcohols. Sections were microwaved in a 10mM sodium citrate solution to

retrieve antigen for 10 minutes and allowed to cool. Non-specific staining was prevented

by treating sections with a blocking solution for 1 hour at room temperature. Sections

were simultaneously treated with a rabbit polyclonal antibody to detect MMP-9 and an

antibody to detect leukocytes for 6 hours at room temperature. Neutrophils were stained

using a monoclonal rat anti-neutrophil antibody (AbD Serotec, Oxford, UK). B cells

were visualized using a rat monoclonal antibody recognizing CD45R/B220 (AbD

Serotec). Macrophages were demonstrated by staining for F4/80 antigen using a rat

monoclonal antibody (AbD Serotec). Sections were washed in PBS, and incubated with

fluorescently labeled secondary antibodies (Molecular Probes, Carlsbad, CA) and DAPI

to visualize nuclei then mounted in aqueous mounting media (Biomeda, Foster City, CA).

CHAPTER III

RESULTS

<u>MMPs in Mouse Models of Colon Cancer</u>

MMPs are widely expressed by a variety of neoplasms. Previously, we have

investigated the expression of various MMP family members in the development of

intestinal adenomas using different mouse models of intestinal tumorigenesis. Strikingly,

genetic ablation of MMP-7, an epithelially expressed MMP-7, reduced tumor multiplicity

by 58% in the Min mouse model. Furthermore, analysis of tumors from these animals by

in situ hybridization revealed that like human tumors, murine tumors express MMPs-2, -

3, -7, -10, and -13 (Wilson et al., 1997). While only a subset of tumors normally

expressed MMP-2, upon genetic ablation of MMP-7, all tumors that formed were found

to express MMP-2 (Table 3). However, since our initial screen, several additional MMP

family members have been identified. Many of these MMPs have been detected in

tumors. We sought to generate a comprehensive profile of MMPs expressed by normal

murine small intestine and intestinal adenomas that we could then use to identify targets

for further study.

Microarray Profiling of Intestinal Adenomas

The development of microarray technology has allowed an investigator to

compare the expression of thousands of genes simultaneously. Microarray based analysis

uses thousands of unique cDNA or oligonucleotide probe sequences that are spotted onto

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Table 3: Expression analysis of MMPs expressed in Min tumors as previously determined by *in situ* hybridization. (Wilson et al., 1997)

MMP	No. of positive tumors per total no. of tumors (%)			
	Min/+		Min/+; mmp7-/-	
MMP-2	13/21	(62)	16/16	(100)
MMP-3	11/21	(52)	9/16	(56)
MMP-7	22/25	(88)	--/--	(--)
MMP-10	17/26	(65)	12/15	(80)
MMP-11	0/21	(0)	0/15	(0)
MMP-13	14/26	(53)	7/15	(46)

microscope slides. Depending on the commercial platform used, purified mRNA is then converted into fluorescently labeled cDNA (spotted arrays) or cRNA (Affymetrix based arrays). For traditional spotted arrays, samples to be compared are labeled with different fluorophores, hybridized to the same slide, and the difference in hybridization between samples is detected by laser scanning. Alternatively, oligonucleotide based arrays such as the Affymetrix platform, require each sample to be hybridized to an individual array, and hybridization between arrays is compared (Figure 5) (Bucca et al., 2004; Staal et al., 2003).

To generate a profile of which MMPs are differentially expressed in intestinal tumors, we developed two separate microarrays capable of distinguishing between highly homologous sequences. Initially, I subcloned fragments of all human and mouse MMPs and TIMPs capable of distinguishing between orthologous sequences for the construction of a cDNA array. Additionally, in conjunction with the Protease Consortium, I aided in
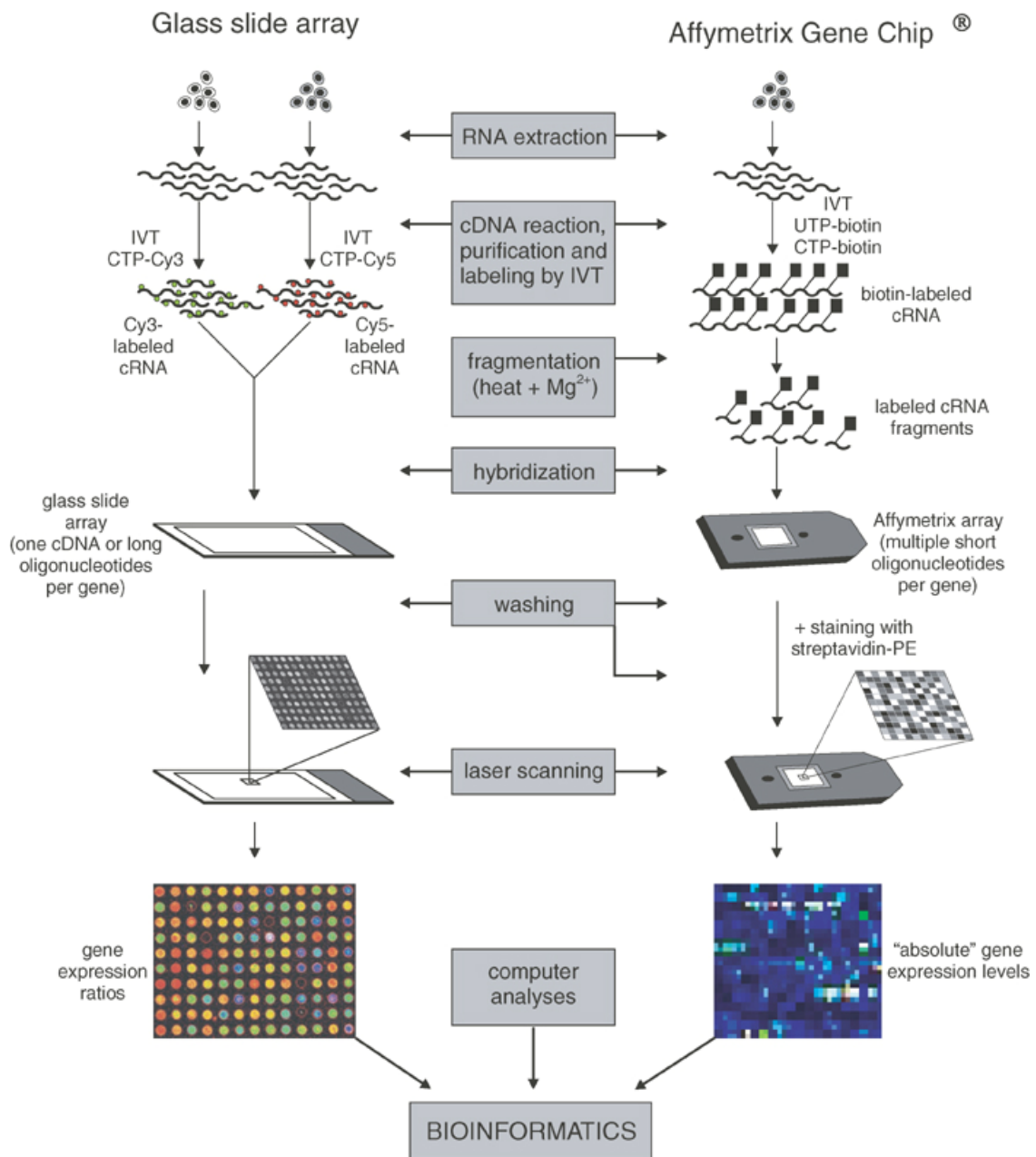
Figure 5: Comparison of traditional glass slide cDNA microarrays to the Affymetrix Gene Chip platform. Isolated RNA is labeled by *in vitro* transcription with either fluorescent dyes (Glass slide arrays) or biotin (Affymetrix) labeled nucleotides prior to hybridization to an array containing thousands of genes. Hybridization intensity is determined by laser scanning. For traditional array analysis the ratio between sample intensity is compared, whereas with Affymetrix arrays hybridization intensity between samples is compared using for individual Gene Chips per sample (Staal et al., 2003).

the construction of a novel dual-species Affymetrix array also capable of distinguishing closely related orthologues (Schwartz et al., 2007). Tissue was harvested from normal small intestine and tumors from the ilea of Min mice and stored overnight in RNAlater, a mild fixative that has been demonstrated to stabilize RNA and improve yield. Total RNA was isolated from tissue by using a rotor-stator homogenizer and RNeasy mini kit (Qiagen). RNA concentration and integrity were assessed using UV spectrophotometry, and samples with a 260/280 absorbance ration of 1.95-2.05 were submitted to the Vanderbilt Microarray Shared Resource (VMSR) for cDNA transcription, labeling, hybridization, and microarray scanning. Two adenoma samples were compared on our cDNA array to a common normal sample, and an intensity ratio was determined by ArrayAssist Lite software (Table 4). Alternatively, multiple samples were compared using a novel Affymetrix array containing probes for all currently known human and murine proteinases.

Because of the high degree of divergence observed between samples as assayed on the cDNA array, as well as the enhanced ability of the Affymetrix array to distinguish signal versus noise and the availability of a greatly expanded probe set, we opted to continue our studies using the Affymetrix platform rather than spotted arrays. Additional samples were isolated and assayed using the Affymetrix Hu/Mu ProtIn array using both absolute and relative analysis. Relative analysis identified MMP-10, -13, and -14 as significantly more abundantly expressed in tumor tissue than normal intestine, a finding also reported by others (Martinez et al., 2005). Additionally, comparing absolute analysis calls between groups revealed MMP-9 and -12 as present in tumor tissue, but

Table 4: Expression analysis of MMP family members in tumor tissue compared to normal ileum. Total RNA was isolated from ileal tumors and normal small intestine of Min mice as described in the Materials and Methods. Microarray analysis was performed using a custom created cDNA microarray by the VMSR. Numbers indicate fold change of hybridization intensity, with positive numbers indicative of increased expression in tumor samples and negative numbers indicative of less abundant transcript in tumor.

Gene	Mouse A	Mouse B
MMP-1	0.99	2.32
MMP-2	5.75	-0.19
MMP-3	3.17	-0.56
MMP-7	3.59	1.11
MMP-8	-3.15	-2.00
MMP-9	3.63	1.56
MMP-10	5.38	2.46
MMP-11	2.94	6.29
MMP-12	7.91	10.20
MMP-13	4.36	3.95
MMP-14	1.79	7.35
MMP-15	1.76	1.00
MMP-16	-3.00	4.00
MMP-17	3.81	2.32
MMP-19	2.37	1.00
MMP-20	-11.22	0.67
MMP-23	1.10	-1.27
MMP-24	1.46	0.71
MMP-25	1.61	1.55
MMP-27	1.31	-41.67
MMP-28	1.26	1.68
TIMP-1	48.50	-1.38
TIMP-2	0.95	4.39
TIMP-3	1.87	3.57
TIMP-4	3.27	-3.13

Table 5: Affymetrix microarray analysis of MMP family members from normal ilea and intestinal tumors. Four samples of normal ileal tissue were compared to six pools of adenomas isolated from Min mice and compared using a novel Affymetrix microarray by relative and absolute analysis. Relative analysis revealed that transcripts for MMP-10, -13, and -14 are more abundant in tumor tissue than normal intestine. Absolute analysis identified MMP-9 and -12 as expressed in tumor, but not in normal intestine.

GENE	RATIO	NORMAL				TUMOR					
		1	2	3	4	1	2	3	4	5	6
MMP-1a	1.050	A	A	A	A	A	A	A	A	A	A
MMP-1b	0.979	A	A	A	A	A	A	A	A	A	A
MMP-2	0.966	P	P	P	P	P	P	P	P	P	P
MMP-3	1.424	A	A	A	A	A	A	A	A	A	A
MMP-7	1.464	P	P	P	P	P	P	P	P	P	P
MMP-8	1.072	A	A	A	A	P	A	A	A	A	A
MMP-9	1.133	P	A	A	A	P	P	P	P	P	M
MMP-10	**3.160**	P	P	P	P	P	P	P	P	P	P
MMP-11	0.883	A	A	A	A	A	A	A	A	A	A
MMP-12	1.569	A	A	A	A	P	M	M	P	P	A
MMP-13	**2.549**	P	P	P	P	P	P	P	P	P	P
MMP-14	**2.000**	P	P	P	P	P	P	P	P	P	P
MMP-15	1.505	A	M	A	M	P	P	P	P	P	P
MMP-16	0.973	A	A	A	A	A	A	A	A	M	A
MMP-17	0.847	P	P	P	P	P	P	P	P	P	P
MMP-19	1.064	A	A	M	M	P	A	A	A	P	A
MMP-20	0.901	A	M	A	A	A	A	A	A	A	A
MMP-21	0.966	A	M	A	A	A	A	A	A	A	P
MMP-23	0.953	M	M	M	P	P	M	P	M	P	P
MMP-24	0.953	A	A	A	A	A	A	A	A	A	A
MMP-25	1.000	A	A	A	A	A	A	A	A	A	A
MMP-28	0.959	A	A	A	A	A	A	A	A	A	A
TIMP-1	1.778	A	A	A	A	P	P	P	P	P	P
TIMP-2	0.829	P	P	P	P	P	P	P	P	P	P
TIMP-3	0.785	P	P	P	P	P	P	P	P	P	P
TIMP-4	0.946	A	A	A	A	A	A	A	A	A	A

absent from normal intestine (Table 5). Generally, the expression profile generated by Affymetrix analyses was more similar to the cDNA profile generated for Mouse B, though methodological differences between the two techniques minimize the utility of such a comparison.

To confirm our microarray findings we used real-time PCR analysis, which allows relative quantification of the abundance of target genes across samples. Real-time PCR was performed on all samples submitted for Affymetrix microarray analysis, and relative abundance of transcripts for MMPs-10, -12, and -13 was determined in comparison to GAPDH, a housekeeping gene with relatively stable expression across cells. Primers for MMP-9 and -14 did not produce reliable results. Using an algorithm built into the PCR machine software package (BioRad iQ 4) levels of transcripts were quantified. Thus, microarray results were validated by Real Time PCR, confirming that MMPs-10, -12, and -13 are differentially expressed in tumor samples (Figure 6). These results have identified MMPs-9, -10, -12, -13, -14, and -15 as MMPs that are expressed differentially by intestinal adenomas.

Analysis of Heterogeneity of MMP Expression in Min Tumors

Previously, it has been shown that ablation of specific MMPs results in alterations of the expression of other MMP family members (Rodgers et al., 1994; Rudolph-Owen et al., 1997). Because of the variation in MMP expression between tumors and apparent expressional compensation of MMP-2 previously observed (Wilson et al., 1997), I hypothesized that individual tumors may express different subsets of MMPs, and in mice
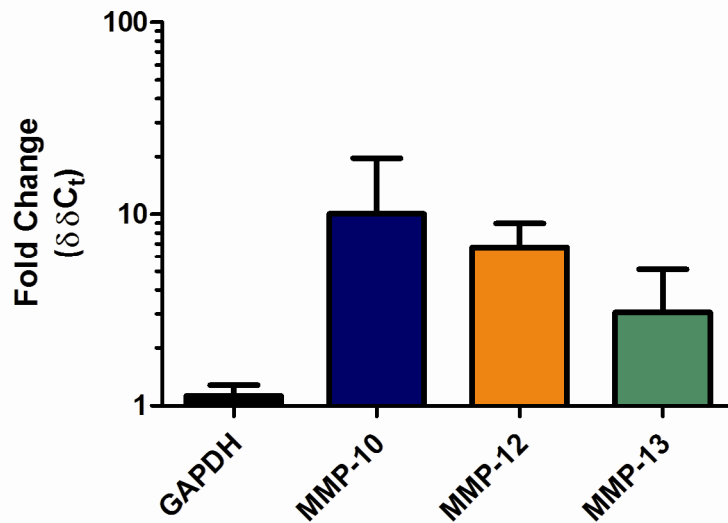
Figure 6: **Relative expression analysis of selected MMPs in intestinal adenoma tissue compared to normal intestine.** Real time PCR was used to validate microarray analysis. Abundance of transcripts for selected MMPs was compared to GAPDH, and results are reported as a ratio compared to normal tissue. PCR to detect MMP-9 and -14, two other genes found to be differentially expressed was also performed, but did not produce measurable product.

with particular MMPs genetically ablated, that presence of other MMP family members

is able to compensate for this and would be detectable by relative expression analysis.

The construction of the Affymetrix protease array provided a unique opportunity

to assay both the absolute presence of transcript as well as quantification of "present"

transcripts. To test my hypothesis, I isolated individual tumors from wild type, MMP-7

deficient, and MMP-2; MMP-7 double deficient animals. Relative analysis across

samples did not detect any significant variations within groups, and across groups the

only difference detected was a decrease in the abundance of transcripts for MMP-7 and

MMP-2 in animals with those MMPs genetically ablated. Absolute analysis of samples

revealed that in contrast to the hypothesis, tumors were very homogeneous in their

expression of MMPs (Table 6). The only difference detected between groups by absolute analysis was that "present" signal for MMP-8 was detected in all tumors from all mice lacking MMP-7, but in only one out of six tumors assayed from wild-type Min mouse. MMP-8 is largely produced by neutrophils, and the presence of this transcript

Table 6: Tumors from wild-type and mice lacking MMP-7 or MMP-2 and MMP-7 do not exhibit variability in their expression of MMPs. To examine the possibility that MMP ablation resulted in transcriptional compensation of other MMP family members, microarray analysis was used to assay the expression of all MMPs in mouse tumors. No significant variation within sets was observed, though all tumors examined from mice lacking MMP-7 expressed transcript encoding MMP-8, which was detected in only one tumor from wild type Min.

Gene	Normal				Tumor							7KO			2/7 KO
	1	2	3	4	1	2	3	4	5	6	7	1	2	3	1
MMP1a	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
MMP1b	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
MMP2	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
MMP3	A	A	A	A	P	A	A	A	A	A	P	M	P	A	P
MMP7	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
MMP8	A	A	A	A	P	A	A	A	A	A	A	P	P	M	P
MMP9	P	A	A	A	P	P	P	P	P	M	P	P	P	P	P
MMP10	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
MMP11	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
MMP12	A	A	A	A	P	M	M	P	P	A	P	P	P	P	P
MMP13	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
MMP14	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
MMP15	A	M	A	M	P	P	P	P	P	P	P	P	A	M	P
MMP16	A	A	A	A	A	A	A	A	M	A	A	A	A	A	A
MMP17	P	P	P	P	P	P	P	P	P	P	A	P	P	A	P
MMP19	A	A	M	M	P	A	A	A	P	A	A	A	M	P	A
MMP20	A	M	A	A	A	A	A	A	A	A	A	A	A	A	A
MMP21	A	M	A	A	A	A	A	A	A	P	A	A	A	M	A
MMP23	M	M	M	P	P	M	P	M	P	P	P	P	P	P	P
MMP24	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
MMP28	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
TIMP1	A	A	A	A	P	P	P	P	P	P	P	P	P	P	P
TIMP2	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
TIMP3	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
TIMP4	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

may indicate enhanced neutrophil infiltration into tumors from mice lacking MMP-7. Expression analysis using real time PCR analysis produced similar results (data not shown). From these findings we can conclude that transcriptional compensation does not occur in the context of the intestinal adenomas that form in Min mice.

Data presented here have expanded the spectrum of MMP family members shown to be expressed in intestinal adenomas. Profiling of tumors using a novel Affymetrix microarray has largely mirrored our previously generated profile generated by *in situ* hybridaztion, with a few additions. MMP-3, detected in roughly half of tumors by *in situ* hybridization was not regularly detected by absolute microarray analysis; however, this may be due to methodological assumptions used by this method. Absolute analysis uses a proprietary algorithm to compare the ratio of binding to "perfect match" probes to "mismatch" probes, which other than a single base substitution at position 13 are sequentially identical (Staal et al., 2003). False absent calls are favored by this approach as high background and sequentially homologous genes tend to increase the mismatch signal.

MMP-7, another gene we previously detected as differentially expressed in tumor was not found to be differentially expressed using either absolute or relative analysis. However, our previous data looked at expression of only epithelial cells rather than whole tumor. Paneth cells normally produce this MMP, so it is not surprising that absolute analysis did not detect a difference. While the relative transcript abundance was increased in tumor, this gives no detail to the cellular origin of the transcript, and if this experiment were to be repeated using laser captured epithelial cells only, I would expect a difference to be obvious.

An interesting finding of these experiments is the modulation of expression of MMP-9 and -12. Both of these MMPs are largely produced by inflammatory cells (Noel et al., 2008), thus enhanced expression observed in tumor tissue may simply be a reflection of leukocyte infiltration into the tumor, a concept further explored later in this chapter. Enhanced expression of MMPs-10 and -13 in intestinal adenomas of the Min mouse has previously been reported (Martinez et al., 2005; Wilson et al., 1997). Both of these MMPs have previously been shown to be produced by stromal cells within the tumor, though the functional consequence of this expression is unknown. Based upon previous data and the results from my microarray screen, I chose to further examine the role of MMPs-2, -9, -10, -12, -13, and -19 in tumorigenesis using the Min mouse model.

<u>The Role of MMP-2 in Intestinal Tumorigenesis</u>

Previously, we have demonstrated that genetic ablation of MMP-7 reduces tumor multiplicity by 58% in the Min mouse. Additionally, while only a subset of wildtype Min tumors normally express MMP-2, upon genetic ablation of MMP-7 the reduced number of tumors that develop all express MMP-2, suggesting that MMP-2 may functionally compensate for MMP-7 in the development of intestinal tumors. To examine this possibility we used selective breeding to develop a MMP-2 deficient Min mouse.

Female MMP-2 deficient mice were crossed with male Min mice to generate mice heterozygous for MMP-2. Two heterozygotes were crossed carrying the Min allele along the parental lineage in order to generate littermate mice that are either deficient, heterozygous or wild-type for MMP-2. Mouse genotype at the MMP-2 and APC loci
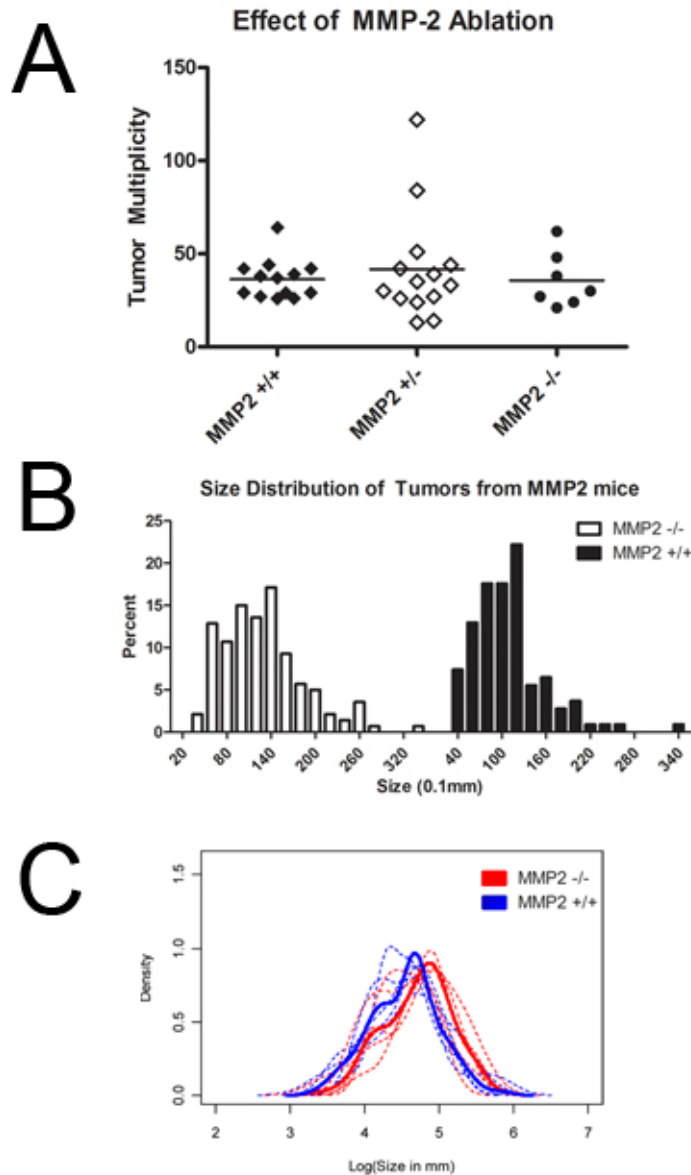
Figure 7: MMP-2 ablation does not affect tumor multiplicity or size in the Min model. (A) Tumor multiplicity in MMP-2 deficient Min mice (35.7 tumors/mouse) was similar to that of wild-type (36.3 tumors/mouse) and heterozygous (41.7 tumors/mouse) littermates. Difference is not statistically significant, Mann-Whitney *t* test. (B) Tumors from MMP-2 deficient mice are of similar size distribution to tumors from wild type littermates. Least squares estimate of average tumor diameter found that the typical tumor from a MMP-2 deficient mouse was 1.1mm, while wild-type littermates was 0.95mm. Difference is not statistically significant, mixed model ANOVA analysis. (C) Overlay of trace from panel B. Dashed lines represent individual mice, solid lines represent average tumor distribution.

were analyzed by PCR analysis. Mice with MMP-2 genetically ablated developed a similar number of tumors to wild-type and heterozygous littermate controls (Figure 7A). Further, ablation of MMP-2 did not affect either spatial distribution of tumors within the intestine, nor size (Figure 7B-C).

Previous data suggest that while MMP-7 is a key mediator of tumorigenesis, MMP-2 may be able to functionally compensate for ablation of MMP-7. To examine this possibility we developed MMP-2; MMP-7 double deficient mice. In contrast to previously published results, MMP-7 deficient Min mice did not develop statistically fewer tumors than wild-type Min littermates. However, unlike previous studies, separate lineages derived from a common progenitor were compared rather than littermates, which could generate divergent lines within the sample pool. Although a conservative breeding strategy was utilized in an attempt to minimize divergence, I cannot exclude this as an explanation for the variability observed. Additionally, at the time when this pilot study was conducted a syndrome of unknown etiology arose within several lineages of the mouse colony that manifested in hydronephrotic kidneys, extraordinary variability in tumor burden, increased juvenile mortality, and frequent gastrointestinal inflammation as determined by gross necropsy. Mice displaying any of these symptoms were excluded from analysis, which severely limited our sample pool of wild-type Min and MMP-7 deficient Min mice. Regardless, tumor multiplicity was similar in mice lacking both

Effect of MMP-2 and MMP-7 Ablation in the Min mouse
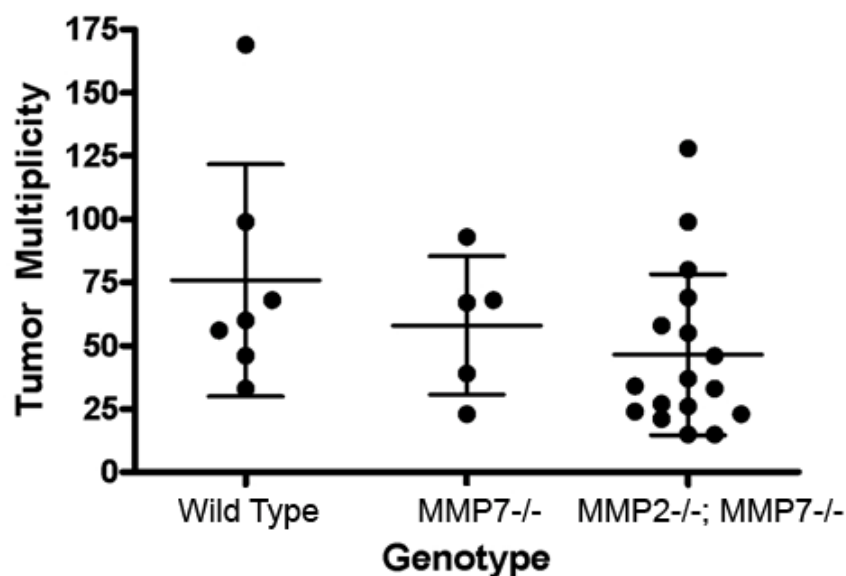
Figure 8: Effect of ablating both MMP-2 and MMP-7 in the Min model. Because of the potential for MMP-2 and -7 to functionally compensate for each other, we created mice lacking both of these MMPs. No further reduction in tumor multiplicity was observed when compared to MMP-7 deficient animals suggesting that MMP-2 does not significantly contribute to adenoma formation in the Min model.

MMP-2 and MMP-7 when compared to wild-type or MMP-7 deficient littermates (Figure 8), and was similar to historical data comparing MMP-7 deficient Min mice. Taken together, all these data suggest that MMP-2 does not significantly contribute to the development of intestinal adenomas in the Min model.

The Role of MMP-12 in Intestinal Tumorigenesis

MMP-12 was one of two MMPs differentially detected by absolute microarray analysis as absent in normal intestinal tissue, but present in adenomas. High levels of

macrophage derived MMP-12 is commonly associated with an improved prognosis in colorectal tumors (Asano et al., 2007; Zucker and Vacirca, 2004). Because of these changes, we postulated that MMP-12 functionally acts to inhibit tumor development, and that genetic ablation of this gene would result in a significant enhancement of tumor multiplicity, size and progression.

Male Min mice and female MMP-12 deficient mice on the C57BL/6 background were purchased from the Jackson Lab and crossed to generate pups heterozygous for MMP-12. Wild type and heterozygous control Min mice developed an average of 35.2 ± 14.9 and 54.3 ± 21.6 tumors per mouse, respectively. In comparison, Min mice lacking MMP-12 developed an average of 44.0 ± 23.9 tumors per mouse, not a statistically significant difference (Figure 9A). Similarly, the average tumor diameter was not significantly different between from wild-type control (1.1mm) and MMP-12 deficient (1.2mm) animals (Figure 9B-C).

Taken together, these data suggest that while MMP-12 expression has been shown to predict a good prognosis, genetic ablation does not affect early stage tumorigenesis. However, while MMP-12 may be functionally protective during later stages of tumor progression, we could not assess such using the current model system. Alternatively, high macrophage density within colorectal tumors has also been shown to be an indicator of positive prognosis (Forssell et al., 2007). While macrophages are abundant throughout human tumors, they tend to localize peritumorally in Min adenomas, and MMP-12 may simply be acting as a marker for macrophages in the context of human intestinal adenomas.

Figure 9. MMP-12 ablation does not affect tumor multiplicity or size in the Min model. (A) Tumor multiplicity in MMP-12 deficient Min mice (44.0 tumors/mouse) was similar to that of wild-type (35.2 tumors/mouse) and heterozygous (54.3 tumors/mouse) littermates. Difference is not statistically significant, Mann-Whitney t test. (B) Tumors from MMP-12 deficient mice are of similar size distribution to tumors from wild type littermates. Least squares estimate of average tumor diameter found that the typical tumor from a MMP-12 deficient mouse was 1.2mm, while wild-type littermates was 1.1mm. Difference is not statistically significant, mixed model ANOVA analysis. (C) Overlay of trace from panel B. Dashed lines represent individual mice, solid lines represent average tumor distribution.

The Role of MMP-19 in Intestinal Tumorigenesis

MMP-19 has been detected on the surface of activated peripheral blood mononuclear cells, T_{H1} lymphocytes (Sedlacek et al., 1998), and is normally expressed throughout the intestine in both enterocytes (Mueller et al., 2000) and occasional stromal fibroblasts and macrophages, though expression by intestinal epithelial cells is decreased upon transformation suggesting a role in enterocyte shedding (Bister et al., 2004). Microarray analysis detected only a weak presence of transcripts for MMP-19 in normal tissue, yet in two tumor samples transcripts were found. Because of these seemingly disparate findings, we examined the effect of MMP-19 ablation in the Min model. We hypothesized that if MMP-19 was functioning in a protective role, then ablation of MMP-19 would enhance tumorigenesis, with deficient animals developing more tumors and tumors that are larger in size. Male Min mice and female MMP-19 deficient mice on the C57BL/6 background were obtained from J. Caterina and crossed to generate pups heterozygous for MMP-19. Min mice lacking MMP-19 developed an average of 41.0 ±19.8 tumors per mouse, whereas wild type and heterozygous control Min mice developed an average of 49.7±28.9 and 35.1±10.8 tumors per mouse, respectively (Figure 10A). Similarly, the average tumor diameter was not significantly different between wild-type control (1.0mm) and MMP-19 deficient (1.0mm) animals (Figure 10B-C).

Taken together, these data suggest that MMP-19 does not significantly contribute to intestinal tumorigenesis in the Min model despite the apparent expression difference. Given that MMP-19 has been detected on the surface of various lymphocytes, it is possible that the difference observed in transcript abundance is due to differential T_{H1} infiltration.
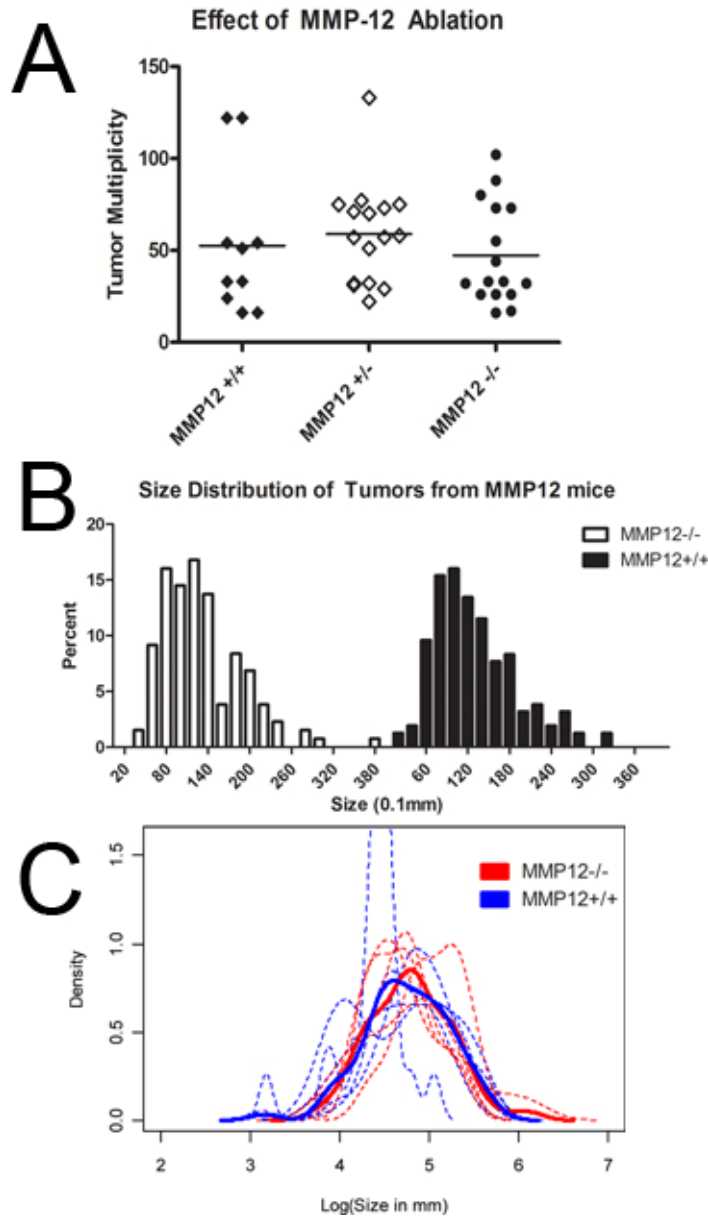
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Figure 10: MMP-19 ablation does not affect tumor multiplicity or size in the Min model. (A) Tumor multiplicity in MMP-19 deficient Min mice (41.0 tumors/mouse) was similar to that of wild-type (49.7 tumors/mouse) and heterozygous (35.1 tumors/mouse) littermates. Difference is not statistically significant, Mann-Whitney t test. (B) Least squares estimate of average tumor diameter found that the typical tumor from both MMP-19 deficient and wild-type littermates was 1.0mm. Difference is not statistically significant, mixed model ANOVA analysis. (C) Overlay of trace from panel B. Dashed lines represent individual mice, solid lines represent average tumor distribution.

The Role of MMP-9 in Intestinal Tumorigenesis

Transcripts encoding the proteinase MMP-9, like MMP-12, were absent from normal tissue, but abundantly expressed in tumor. MMP-9 is produced by several stromal cell populations including neutrophils, mast cells, macrophages, fibroblasts, as well as by the tumor epithelial cells directly (Noel et al., 2008). Previous studies using mice with MMP-9 genetically ablated have shown that MMP-9 deficient animals develop fewer tumors than do littermate controls, but those tumors that do develop are more aggressive and of a more advanced phenotype (Coussens et al., 2000). While an excellent model of early stage tumorigenesis, the Min model is limited in that tumors that develop rarely invade and never metastasize. Because of the seeming dual role of MMP-9 in both promoting tumorigenesis but inhibiting tumor progression, we hypothesized that MMP-9 ablation in the Min mouse would decrease tumor multiplicity, but that those tumors that formed may be larger or more progressed as determined by the presence of local invasion or distant metastasis.

MMP-9 deficient Min mice were developed by crossing male Min mice to MMP-9 deficient females, obtained from Z. Werb. Mice lacking MMP-9 developed 26.5±17.7 tumors on average. In comparison, wild-type littermate Min mice developed 35.0±26.1, and heterozygotes 32.9±16.2 (Figure 11A). This decrease indicates a 25% reduction in tumor multiplicity in MMP-9 deficient animals.

Tumors that arose in MMP-9 deficient animals were further characterized for phenotypic differences. Average tumor diameter was similar between MMP-9 deficient and wild-type Min littermates (Figure 11B-C), suggesting that MMP-9 ablation affected tumor
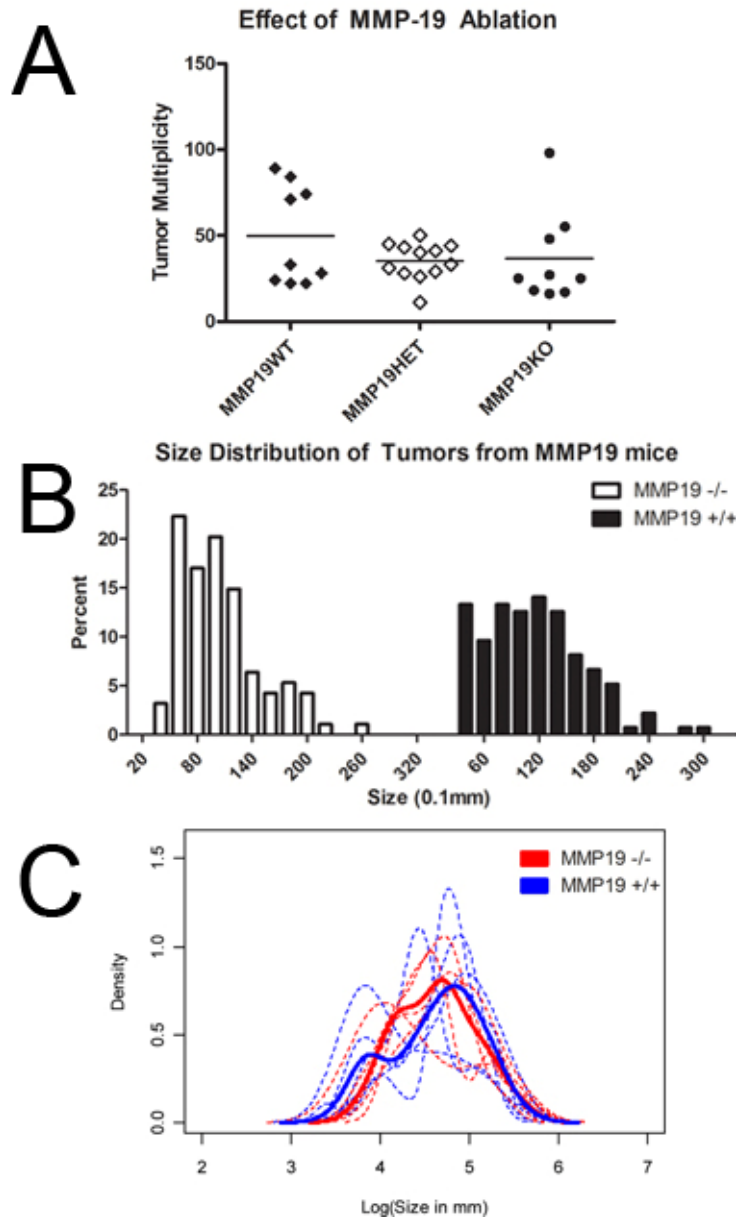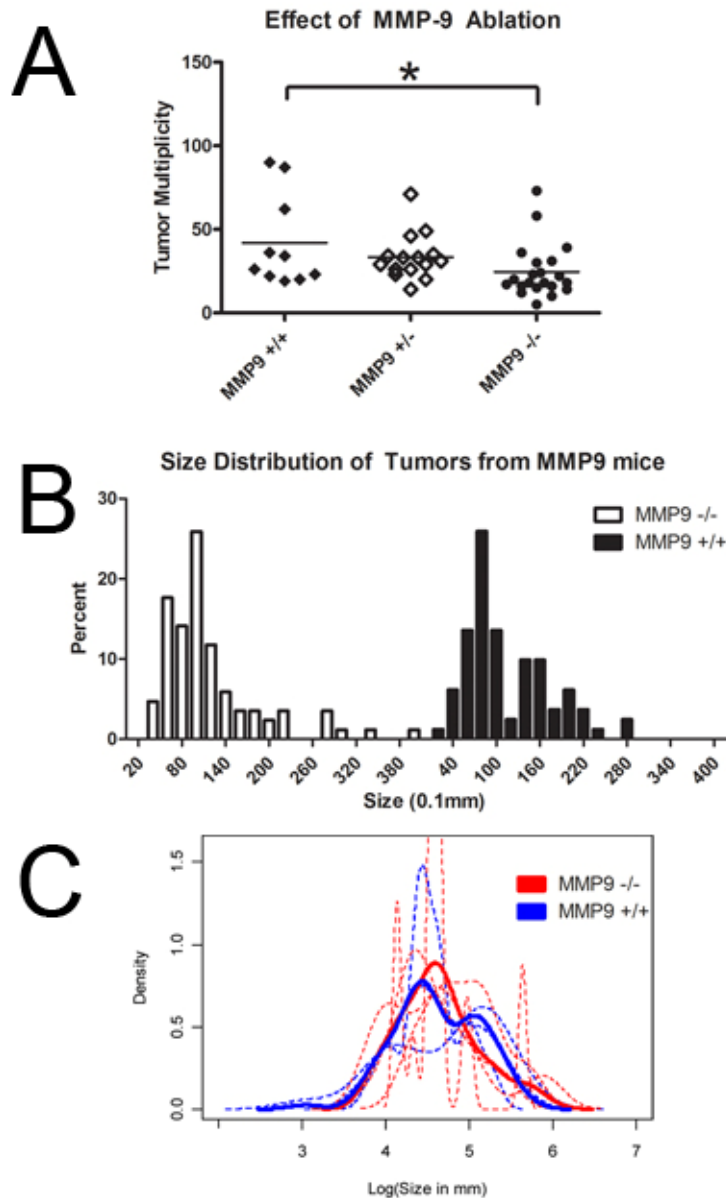
Figure 11: MMP-9 ablation does not affect tumor multiplicity or size in the Min model. (A) Tumor multiplicity in MMP-9 deficient Min mice (26.5 tumors/mouse) was significantly reduced in comparison to wild-type (35.0 tumors/mouse) and heterozygous (32.9 tumors/mouse) littermates. p=0.0440, Difference is statistically significant, Mann-Whitney *t* test. (B) Tumors from MMP-9 deficient mice are of similar size distribution to tumors from wild type littermates. Least squares estimate of average tumor diameter found that the typical tumor from a MMP-9 deficient mouse was 1.0mm, while wild-type littermates was 1.0mm. Difference is not statistically significant, mixed model ANOVA analysis. (C) Overlay of trace from panel B. Dashed lines represent individual mice, solid lines represent average tumor distribution.

incidence, but not tumor growth. MMP-9 expression has been observed in several cell types, including tumor epithelial cells. To identify the cellular source of MMP-9 in these tumors, immunohistochemical staining was employed. MMP-9 staining was not observed in any epithelial cells, but was abundantly detected throughout stromal and intravascular cells, suggesting that intratumoral MMP-9 was of leukocyte origin (Figure 12). To further examine this, I performed immunofluorescent co-localization using markers for different leukocyte populations. All intratumoral MMP-9 positive cells co-stained with an antibody that specifically recognized neutrophils. A single MMP-9 positive macrophage was observed at the tumor periphery, and none were detected intratumorally (Figure 13). No MMP-9 positive B lymphocytes were detected. Because MMP-9 staining localized to neutrophils, we examined intratumoral neutrophil abundance in both wild-type and MMP-9 deficient Min mice. Neutrophils were equally abundant in tumors from both groups (Figure 14).

Neutrophil derived MMP-9 has been shown to be a critical mediator of angiogenesis (Ardi et al., 2007; Nozawa et al., 2006). To determine if MMP-9 deficiency resulted in less vascularized tumors, we employed immunohistochemical staining to detect total (von Willebrand Factor) and angiogenic (CD-31) vasculature in tumors from MMP-9 deficient animals and littermate controls. Initial experiments indicated that tumors from wild type mice on average had twice as much total vasculature as measured by immunohistochemistry for vWF, a marker of total vasculature; however
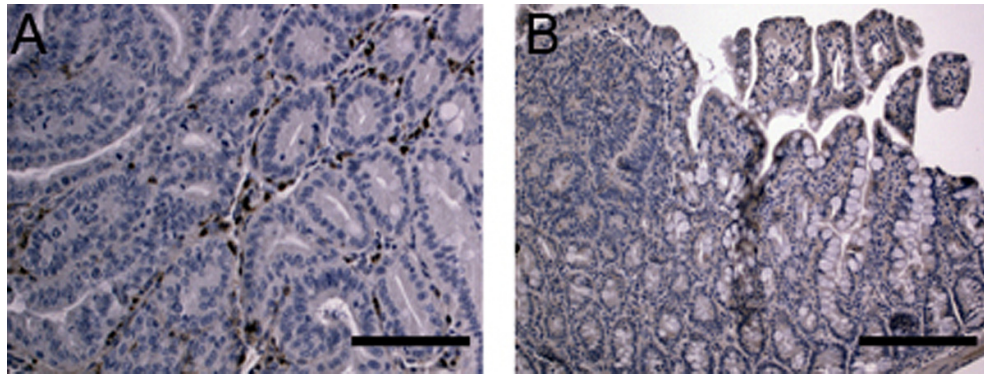
Figure 12: MMP-9 localization by immunohistochemical staining. A: A monoclonal antibody raised to react with murine MMP-9 demonstrated abundant stromal cells were positive for MMP-9 (brown staining, hematoxylin counterstain). Scale bar indicates 40μm. **B:** Serotype control antibody produced no positive staining. Scale bar indicates 40μm.
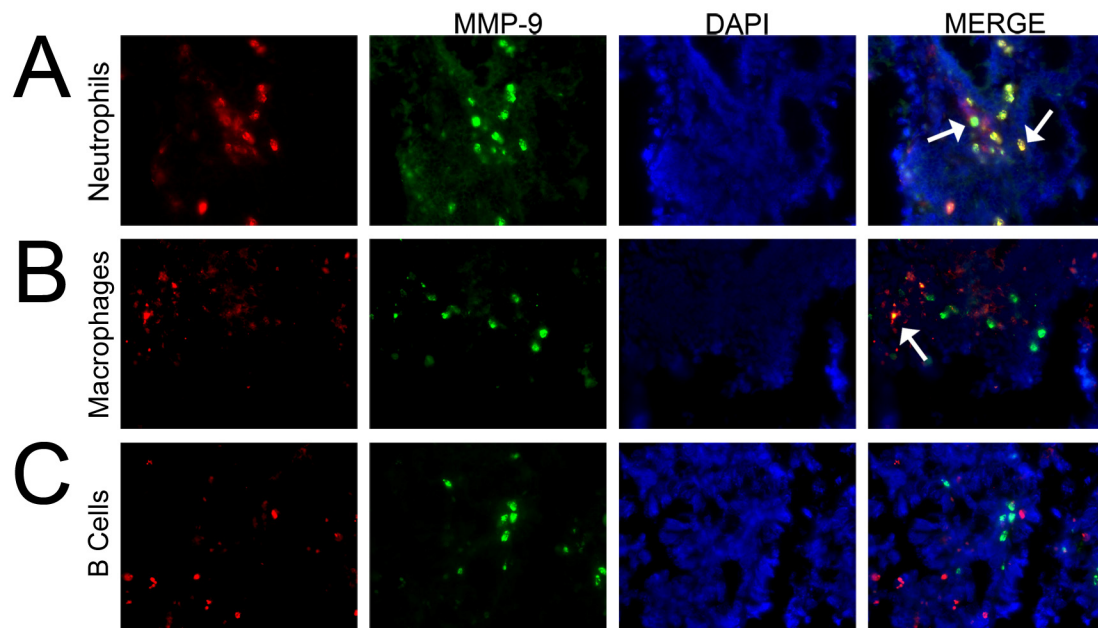
Figure 13: Imunofluorescent co-staining of MMP-9 and Leukocyte Markers. To further determine the cell populations producing intratumoral MMP-9, fluorescent co-staining was used to visualize **(A)** neutrophils (anti-neutrophil), **(B)** macrophages (F4/80) and **(C)** B lymphocytes (B220). All intratumoral MMP-9 was of neutrophil origin, while one MMP-9 positive macrophage was observed at peri-tumorally. No MMP-9 positive B cells were observed.
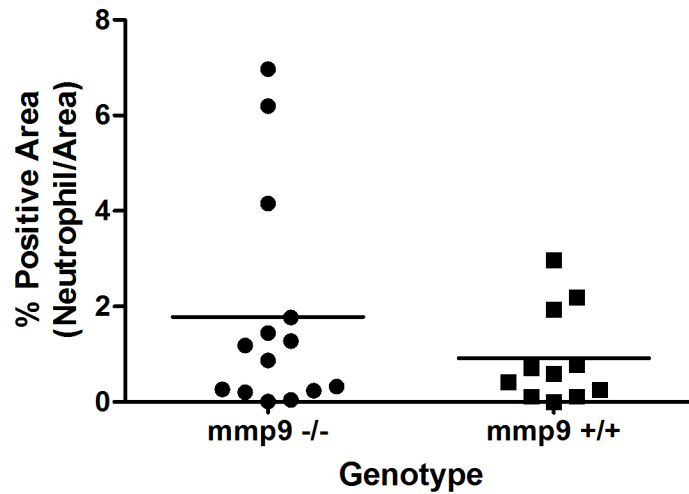
Figure 14: Neutrophil density is similar in tumors from wild type and MMP-9 deficient tumors. Immunohistochemical staining for neutrophils was performed on tumors from 5 each MMP-9 deficient mice and wild type littermates. Percent positive area was calculated using MetaMorph software by first masking an area of interest, and then quantifying positive pixels per total pixels.

this finding was not supported in follow up experiments using larger data sets. Repeated

Taken together, these studies indicate that like epithelial produced MMP-7, neutrophil

derived MMP-9 functions in a *pro-tumorigenic* capacity early in the development of

intestinal tumors.

CHAPTER IV

AN ANTI-TUMORIGENIC ROLE FOR MAST CELLS

Introduction

Mast cells are a group of bone marrow derived granulocytes most commonly associated with allergy, though recent studies show them to be involved in a wide range of physiological and pathological processes (Leslie, 2007). Classically, mast cells are divided into two sub-populations based upon their histochemical properties and anatomical distribution (Hallgren and Gurish, 2007). Connective-tissue mast cells (CTMCs) are found throughout the skin, peritoneum and intestinal submucosa, while mucosal mast cells (MMCs) are found throughout the epithelial surface of the lung and intestinal mucosa (Miller and Pemberton, 2002). In addition to anatomical distribution, these two broad classes of mast cells also differ in their expression of tryptases and chymases (Caughey, 2007). Generally, MMC tend to express both chymases and tryptases, while CTMC express only tryptases, though recent evidence suggests that mast cell heterogeneity is broader than just the two classically defined groups (Caughey, 2007).

Mast cell activation, typically by IgE receptor cross-linkage or Toll-like receptor (TLR) binding (Metz et al., 2007), induces mast cells to secrete various pro-inflammatory mediators (Bischoff and Kramer, 2007). These mediators include cytokines and small molecules involved in leukocyte recruitment and activation, vasodilation, angiogenesis, and mitogenesis (Frankenstein et al., 2006; Theoharides et al., 2007). Conversely, in

some circumstances, mast cells have been demonstrated to limit inflammation, and many mast cell secreted mediators have both pro- and anti-inflammatory aspects indicating a central role for mast cells in modulating inflammation (Metz et al., 2007). Increased abundance of mast cells has been observed in various human tumors, and is frequently associated with a good prognosis (Hedstrom et al., 2007; Rajput et al., 2007).

Results of microarray profiling of intestinal adenomas, revealed a striking increase in the abundance of mast cell expressed transcripts as reported here. Because of the well established role of inflammation in the development and progression of colorectal neoplasms (Clevers, 2004), we generated a mast cell-deficient Min mouse to examine the role of mast cells in the early stages of intestinal tumorigenesis.

Results

Mast cells are present in intestinal adenomas of Min mice

As part of a broader screen to determine which proteinases were differentially expressed in Min adenomas compared to normal murine small intestine, we used the Hu/Mu ProtIn microarray from Affymetrix that contains probe sets for all known human and murine proteinases (Schwartz et al., 2007). Numerous mast cell related transcripts were more abundant in tumor tissue than in normal distal small intestine (Table 7). Real-time PCR analysis of murine mast cell protease (mcpt) transcripts was performed to validate microarray findings, and mcpt-1, -2, -5, -6 and -7 transcripts were found to be significantly more abundant in adenomas than normal tissue (Figure 15).

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Table 7: List of 31 genes differentially expressed in intestinal adenomas of Min mice as compared to normal intestinal tissue. Microarray profiling intestinal adenomas using a novel Affymetrix Hu/Mu ProtIn chip revealed 31 genes that significantly differentially expressed as revealed by relative microarray analysis. Several mast cell related transcripts were identified from this screen as indicated by gray shading. Positive Log_2 ratio indicates genes more abundant in tumor, while negative rations indicate genes more abundant in normal tissue.

Log_2 Ratio	Gene Symbol	Gene Description
Genes MORE Abundant in Tumor Tissue		
4.57	**Mcpt2**	**Mast cell protease 2**
4.18	**Mcpt1**	**Mast cell protease 1**
4.14	Cfi	Complement factor i
3.84	**Mcpt1**	**Mast cell protease 1**
3.22	Ctse	Cathepsin E
2.61	**Mcptl**	**Mast cell protease-like**
2.35	Plat	Plasminogen activator, tissue
2.11	**Cpa3**	**Carboxypeptidase A3**
1.90	Serpina3n	SerpinA3N
1.78	Anxa3	Annexin A3
1.66	Mmp10	MMP-10
1.47	Casp6	Caspase 6
1.43	Pnliprp1	Pancreatic lipase related protein 1
1.35	Mmp13	MMP-13
1.30	Serpinb5	SerpinB5
1.19	Capn2	Calpain 2
1.19	Serpine2	SerpinE2
1.15	Hp	Haptoglobin
1.13	Serpinb5	SerpinB5
1.09	Prss11	Protease, serine, 11
1.01	**Mcpt5**	**Mast cell protease 5**
1.00	Mmp14	MMP-14
1.00	Mtf	Tissue factor (mtf) mRNA
Genes LESS Abundant in Tumor Tissue		
-1.24	Usp2	Ubiquitin specific protease 2
-1.27	Anpep	Alanylaminopeptidase
-1.27	Adamdec1	ADAM-like, decysin 1
-1.38	Disp	Distal intestinal serine protease
-1.62	Dpp4	Dipeptidylpeptidase 4
-1.82	And	Adipsin (Complement factor D)
-2.32	Mep1a	Meptirn 1 alpha
-2.56	Serpina1b	SerpinA1b
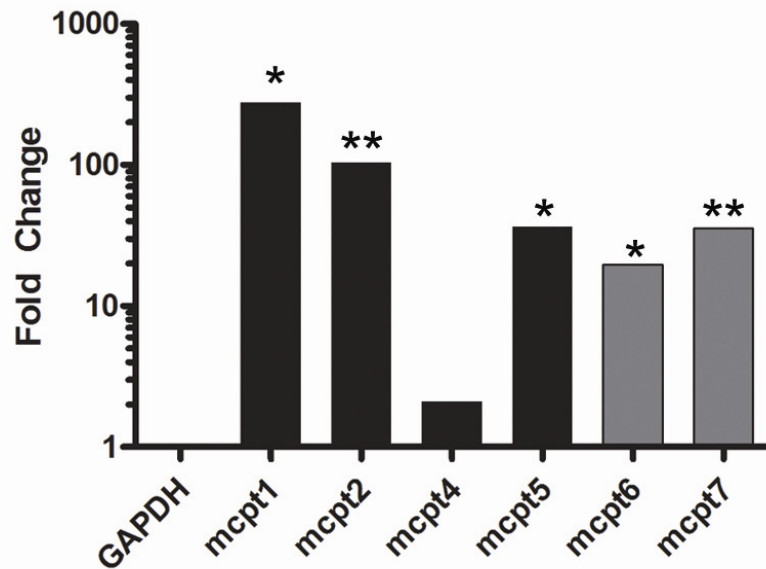
Figure 15: Relative expression analysis of mast cell proteases in intestinal adenoma tissue compared to normal intestine. Real time PCR was used to validate microarray results indicating an increase in the abundance of several mast cell proteinase transcripts in intestinal adenomas. Abundance of transcripts for mcpt genes was compared to GAPDH, and results are reported as a ratio compared to normal tissue. Black bars represent chymase family members, and gray bars represent tryptase family members. Reactions were run for six tumor samples and four normal controls in triplicate. Fold change was determined in comparison to change in GAPDH. *, $P<0.05$; **, $P<0.005$.

To examine the distribution of mast cells within tumors, chloroacetate staining was performed to demonstrate chymase, a marker of mucosal mast cells (Leder, 1979). Mast cells were only occasionally present in normal intestinal tissue, and when present, were restricted to the base of the villus. However, in tissue isolated from tumors, mast cells were found throughout the tumor and surrounding stroma (Figure 16A-D). As previously observed in a chemical-induced model of colorectal tumorigenesis (Wedemeyer and Galli, 2005), mast cell density was enhanced in tumor tissue compared to normal small intestine (Figure 16E). Combined, these observations demonstrate that

mast cell distribution is changed and abundance is increased in both benign and

malignant tumor tissue.
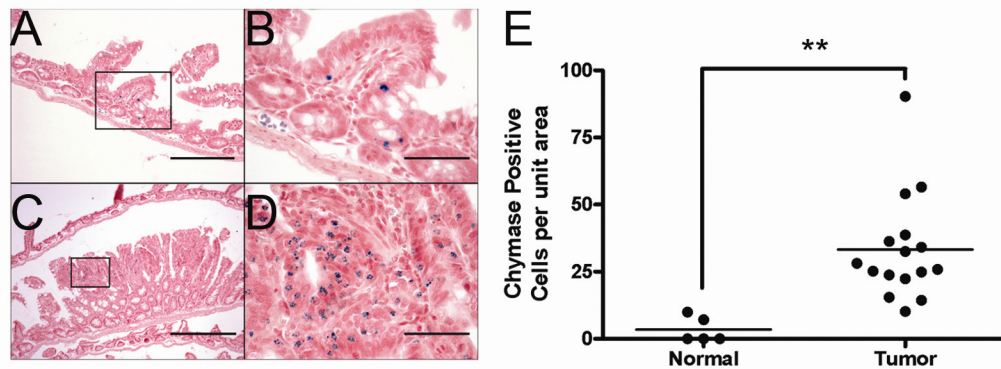
Figure 16: Mast cells are more abundant in adenomas from Min mice than normal murine small intestine. A-D: Histochemical staining for chymase. Leder's esterase reaction was employed to visualize mast cells (dark blue stain) in normal small intestine at low (*A*, *scale bar 150μm*) and high power (*B, scale bar indicates 50μm)* and tumor in low (*C, scale bar 320μm)* and high power (*D, scale bar indicates 50μm).* Tissue was counterstained with Kernetrocht's nuclear red. **E:** Abundance of chymase positive cells was measured in normal and tumor tissue. Significantly more chymase positive cells were found in tumor tissue than normal controls. Multiple sections of individual tumors and normal small intestine were counted from four each Min mice and wild-type littermate controls. Each point represents an individual measurement; *hash mark,* mean. ******, *P<0.005*, difference is statistically significant, Student's two-tailed *t* test.

Mast cell ablation results in increased tumor multiplicity and size

Previously, epidemiological studies have linked high numbers of infiltrating mast

cells into tumors with a positive prognosis (Hedstrom et al., 2007; Rajput et al., 2007);

hence the hypothesis was that the increase in mast cell density was evidence of a host

response to the tumor. To test this possibility we used a genetic approach.

To examine the role of mast cells in intestinal tumorigenesis, we generated Min-Sash (APC$^{Min/+}$;c-kit$^{W-sh/W-sh}$) mice. Mice homozygous for the W-sh allele at the c-kit locus lack mucosal mast cells, though unlike the phenotypically similar c-kit W/W-v mutants, Sash mice are not sterile, nor anemic, have normal levels of intestinal TCRγδ T cells (Metz and Maurer, 2007), and are easily genotyped based upon their coat color (Figure 17).
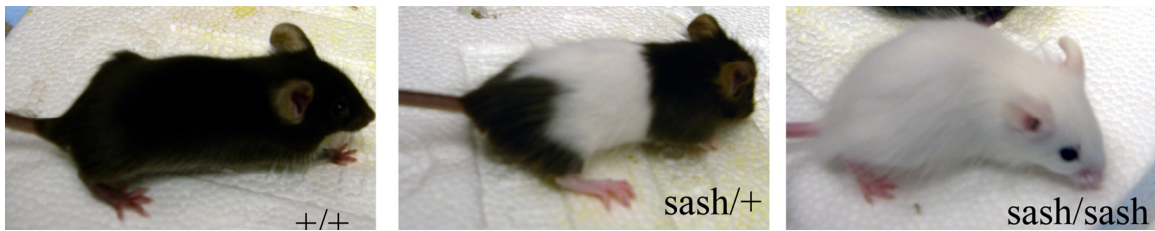
Figure 17: Sash mutants are easily genotyped based upon coat color. Sash mice, a c-kit mutant, are named for the characteristic white "sash" seen in heterozygous mice on the C57Bl/6 background. Wild type mice have a normal coat color, while mice homozygous for the *W-sh* allele are white due to a defect in melanocyte survival.

Mice were raised on a high-fat diet, which has been shown to enhance intestinal tumorigenesis (van Kranen et al., 1998), and were euthanized at 17 weeks of age. On average, Min-Sash mice developed 52.8 tumors while Min littermates developed 38, representative of a 36% increase on average of tumor multiplicity when mast cells are absent (Figure 18A). Additionally, tumor diameter was 33% larger in Min-Sash mice
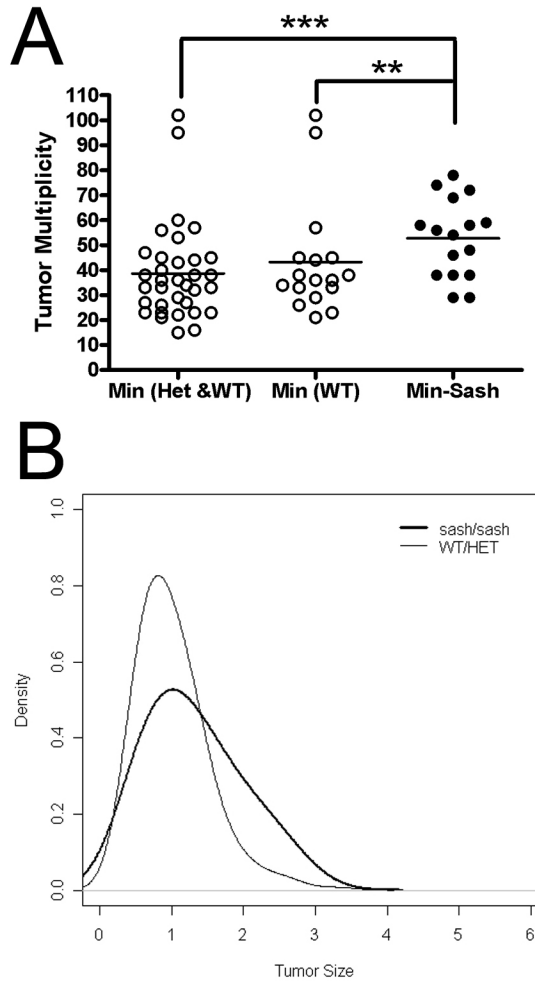
Figure 18: Tumor multiplicity in the small intestine in mice generated from an APC$^{Min/+}$-c-kit$^{W-sh/W-sh}$ cross. A: Min-Sash (APC$^{Min/+}$;c-kit$^{W-sh/W-sh}$) mice develop significantly more tumors than Min littermates at 17 weeks of age. A total of 50 Min mice, 16 homozygous for the W-sh allele, 17 heterozygous for the W-sh allele, and 17 wild-type for the W-sh allele mice were counted for tumors; *hash mark*, mean. **,$P<0.005$; ***, $P<0.0005$, differences are statistically significant, Poisson regression. **B:** Size distribution of min adenomas from wild-type (gray) and Min-Sash (black) mice. Tumors were measured with digital calipers from four Min-Sash mice (n=209) and five Min (n=213) littermate controls. Tumors from a total of 10 mice, 4 Min-Sash (homozygous for the W-sh allele), 1 heterozygous for the W-sh allele and 5 wild-type were measured. Min-Sash mice develop significantly larger tumors than wild-type littermates, $P<0.05$, difference is statistically significant, repeated measures ANOVA test.

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than in Min littermates (Figure 18B). These findings suggest a protective role for mast

cells in intestinal tumorigenesis.

Mast cell deficiency impairs apoptosis of tumor cells

Because tumors were found to be both more numerous and larger in Min-Sash

mice, we examined tumor cell proliferation and apoptosis. Proliferation was assayed by

immunohistochemical staining for phospho-Histone H3, a marker specific for late

anaphase and mid-metaphase mitosis (Hendzel et al., 1997). Proliferation was not

statistically different between Min-Sash and Min litter mates (Figure 19A and 19B).

BrdU incorporation, a marker of DNA synthesis, produced similar results (Figure 20A).

To assess apoptosis, tumors were sectioned and immunohistochemically stained for

cleaved caspase-3, and positive cells per unit area were compared. Tumors from Sash

mice had 33% fewer apoptotic nuclei per unit area than wild type controls (Figure 19C

and 19D). TUNEL staining produced similar results (Figure 20B). The effect of mast

cell ablation on adenoma formation and growth was therefore likely due to effects on

adenoma cell apoptosis.

Tumors from mast cell deficient mice have reduced eosinophil infiltrate

Mast cells have been shown to induce apoptosis through signaling to other

immune cells. To determine if the observed difference in apoptosis was the result of

differential immune cell infiltration into the tumor, immunohistochemical staining for

eosinophils, neutrophils, macrophages, and T cells was performed. Eosinophils and

neutrophils were found throughout the tumor and adjacent tissue of both Min and Min-
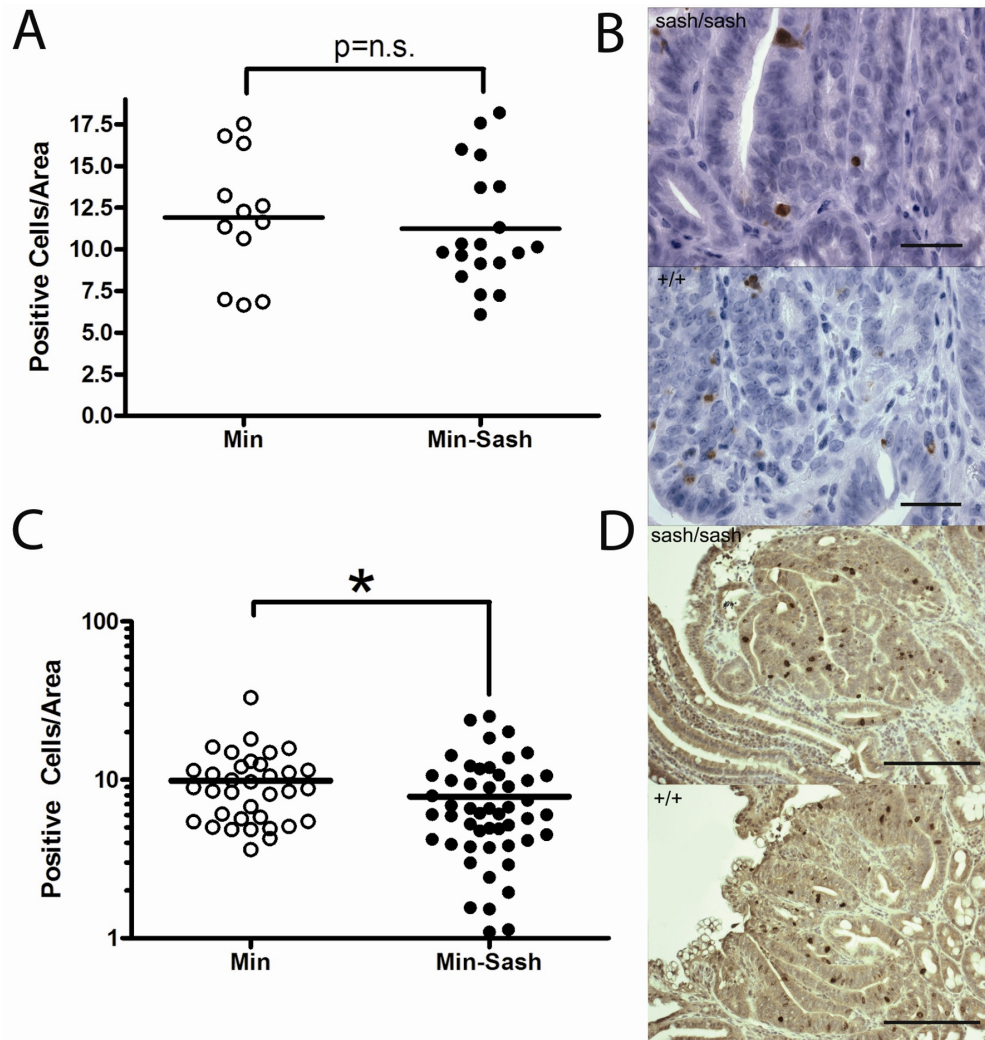
Figure 19: Apoptosis is inhibited in intestinal adenomas from Min-Sash mice compared to littermate controls; proliferation is not affected. A: Intestinal adenomas were assayed for proliferative cells by phospho-Histone H3 immunohistochemistry. Positive cells were counted per unit area using NIH ImageJ software; *hash mark*, mean, not significant. **B:** Phospho-histone H3 staining (dark brown staining) in tumors isolated from Min (*top*) and Min-Sash mice (*bottom*). Nuclei were visualized by counterstaining with hematoxylin. Scale bar indicates 100μm. **C:** Intestinal adenomas were isolated from Min (n=7) and Min-Sash (n=7) littermates and stained for cleaved caspase-3, a marker of apoptosis. Positive cells were counted per unit area as determined by NIH ImageJ software. *hash mark,* mean *, P<0.05, difference is statistically significant, Mann-Whitney two-tailed *t* test. **D:** High power photomicrograph of caspase-3 immunohistochemistry (dark brown stain) in tumors isolated from Min (*top*) and Min-Sash (*bottom*) mice (63x), scale bar represents 50μm. Nuclei were counterstained with Mayer's hematoxylin.
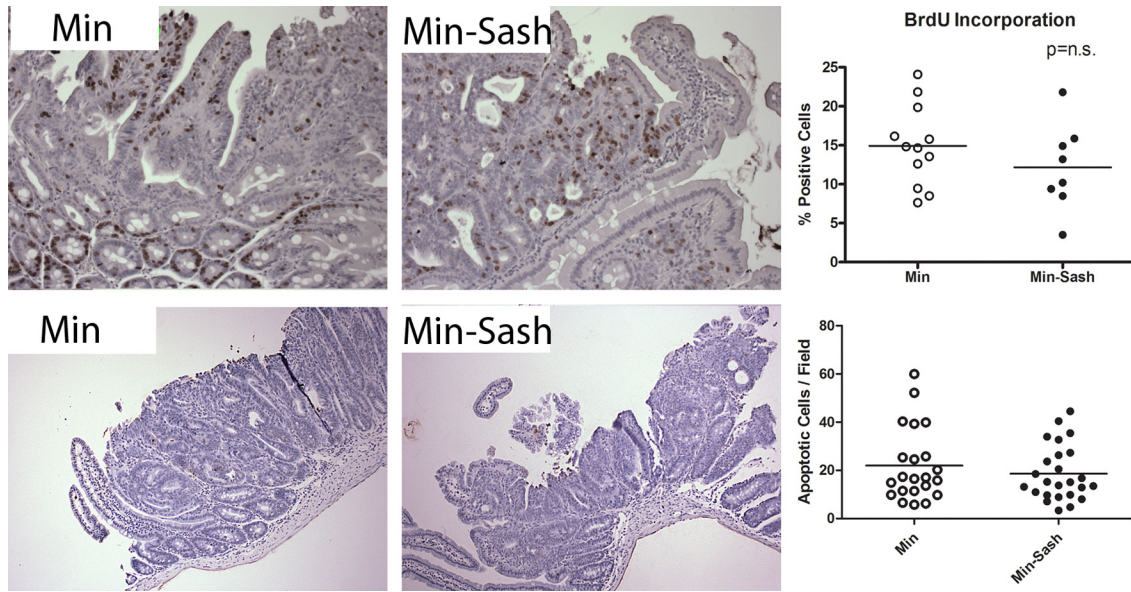
Figure 20: Alternative quantification of proliferation and apoptosis performed by histochemistry for incorporated BrdU or TUNEL staining, respectively.
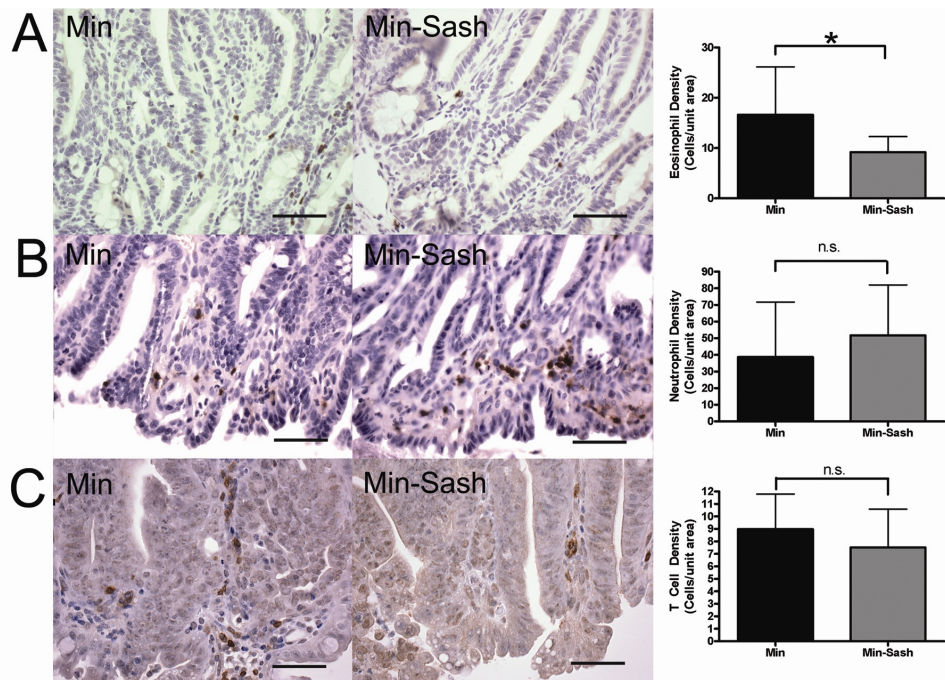
Figure 21: Eosinophils are less abundant in adenomas from Min-Sash mice compared to wild-type littermates; other leukocyte populations are not affected. Intestinal adenomas were isolated from wild-type and Min-Sash littermates and profiled for leukocyte populations by immunohistochemical staining. **A:** Immunohistochemical demonstration of MBP-positive cells indicating eosinophils in wild-type *(left)* and Min-Sash *(center)* mice. Scale bar indicates 100 μm. Quantitation of eosinophils in Min-Sash mice compared to Min controls *(right)*; *hash mark,* mean, *, P<0.05, difference is statistically significant, Student's two-tailed t-test. **B-C:** Immunohistochemical demonstration of neutrophils (anti-neutrophil+) *(B)* and T cells (CD3ε+) *(C)* in tumor tissue isolated from Min *(left)* and Min-Sash *(center)* mice. No differences were detected between groups *(right).* Scale bar indicates 100μm.

Sash mice, however, fewer eosinophils were present in tumors of Min-Sash mice compared to Min littermates (Figure 21A). No differences were detected in the number or distribution of neutrophils, (Figure 21B). T cells were primarily detected adjacent to tumor with very few intratumoral cells, and no difference was detected between Min and Min-Sash mice (Figure 21C). Macrophage staining was exceptionally sparse and virtually all macrophages detected were located in adjacent stromal tissue; no difference was detected between groups.

To confirm that mast cells were responsible for this phenotype, we attempted to repopulate the gut with bone marrow derived mast cells. To reconstitute intestinal mast cells into Min-Sash mice,1-week old Min and Min-Sash mice received a single i.p. injection of GFP tagged BMMC. At 17 weeks of age mice were euthanized and intestines were harvested and tumor multiplicity and size were measured. The presence of reconstituted mast cells was visualized by both GFP immunohistochemistry and direct GFP fluorescence. GFP-positive mast cells were rarely detected in tumors from any of the mice, though abundant GFP-positive cells were observed in intestinal lymph nodes (Figure 22). Since reconstituted mast cells did not appear to home to the tumors, at least at the 17 week time point, we asked if they altered the infiltration of endogenous mast cells or eosinophils to the tumors. The abundance of intratumoral mast cells was not significantly different between treated and previously described untreated Min or Min-Sash mice (Figure 23A). Compared to untreated mice, the eosinophil density in Min-Sash mice remained unchanged, but Min mice that received an injection of BMMC had a significantly increased density of intratumoral eosinophils (Figure 23B). Interestingly,
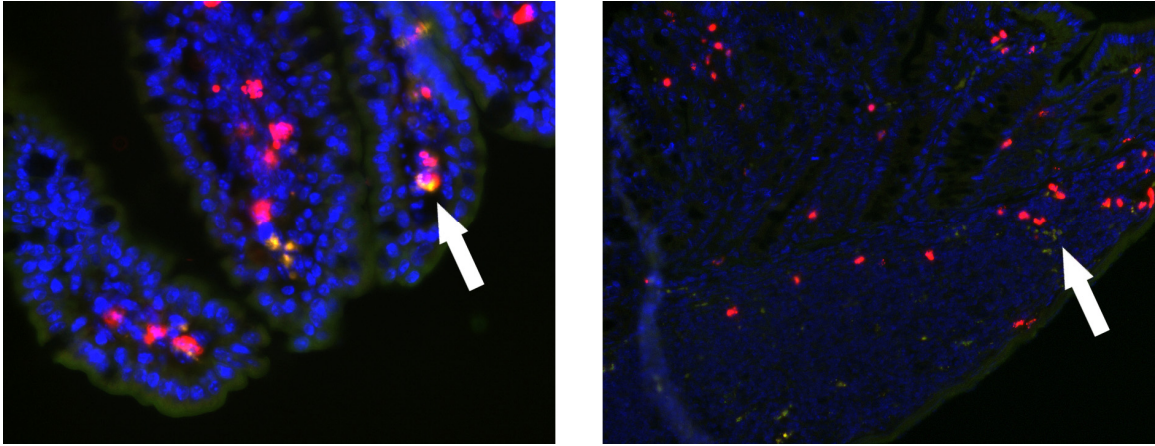
Figure 22. Cultured bone marrow mast cells preferentially migrated to intestinal lymph nodes, but not tumors. GFP tagged bone marrow mast cells were injected intraperitoneally into 1 week old Min-Sash pups in an effort to repopulate mucosal mast cells throughout the gut. Mast cells (green) were visualized by direct immunofluorescence and only rarely detected in tumors (left), but were abundant throughout intestinal lymph nodes (right) and spleen. Eosinophils (red) were detected with a monoclonal antibody to MBP and present throughout both tissues.
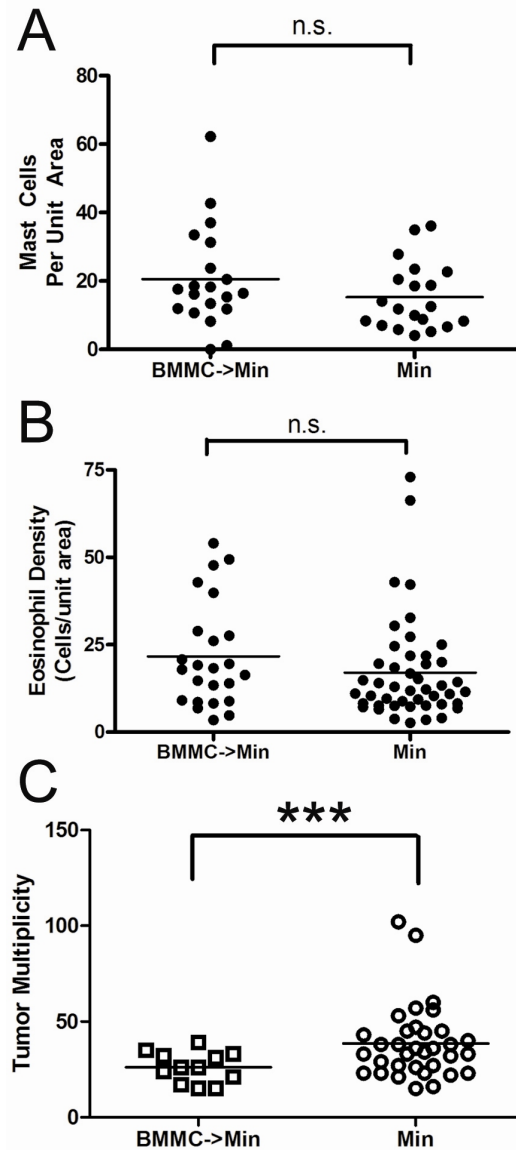
Figure 23: Bone marrow mast cell (BMMC) injection reduces adenoma formation in Min mice. A: Min mice treated with an intraperitoneal injection of bone marrow mast cells did not have an increased abundance of mast cells in tumors compared to non-treated controls; *bars,* mean; Difference is not statistically significant, Student's two-tailed *t* test. **B:** Eosinophil density is not increased in tumors isolated from Min mice that received a BMMC injection compared to non-treated controls; *bars,* mean. Difference is not statistically significant, Student's two-tailed *t* test. **C:** Tumor multiplicity in the small intestine is reduced in Min mice treated with an intraperitoneal injection of bone marrow mast cells (BMMC->Min) compared to untreated controls. ***, P<0.0005, difference is statistically significant, Poisson regression.

Min mice that received a single i.p. injection of mast cells developed 40% fewer tumors than previously described Min controls. In contrast, BMMC treated Min-Sash mice did not develop significantly fewer tumors than untreated Min-Sash controls. The difference in tumor multiplicity correlated positively with intratumoral eosinophil density (Figure 23C), suggesting a significant anti-tumor role for eosinophils.

In summary, ablation of mast cells correlated with a decrease in tumor-infiltrating eosinophils but not of any other leukocyte lineage examined. Overall, our results support a protective, *anti-tumor* role for mast cells and eosinophils in intestinal tumorigenesis and provide experimental validation of an epidemiological observation.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Discussion

The goal of the work described in this dissertation was to investigate the role of

host derived proteinases in an animal model of early intestinal tumorigenesis. In order to

generate a comprehensive profile of all proteinases expressed by intestinal tumors of the

Min mouse model system, we developed a custom Affymetrix microarray containing

probes for all known human and murine proteinases. Based upon the profile that was

generated, I tested the effect of ablating various MMPs on tumor multiplicity in the Min

system. I have demonstrated that genetic ablation of MMP-2, -12, or -19 does not affect

multiplicity or growth of intestinal adenomas in mice harboring the Min mutation.

However, deleting MMP-9 significantly reduces tumor multiplicity in this model system,

suggesting that MMP-9 plays a pro-tumorigenic role. Furthermore, immunofluorescent

co-staining has identified neutrophils as the major cellular source of MMP-9 in these

tumors. Additionally, using this microarray I detected an increase in mast cell

proteinases in Min adenomas and have shown that mucosal mast cells function in a

protective, anti-tumor role in the intestine. Although I was unable to identify the

molecular mediators responsible for this protective effect, data suggests that it may be

mediated through the recruitment of eosinophils. Taken together, these results suggest

that inflammatory mediators function in both pro- and anti-tumorigenic capacities during early intestinal tumorigenesis, a concept that will be further explored in this chapter.

Microarray Expression Analysis of MMPs in Intestinal Adenomas

Previously, using *in situ* hybridization, we generated an expression profile of the MMPs expressed by intestinal adenomas of Min mice, which suggested 1) that these tumors are heterogeneous in their expression of MMPs, and 2) that genetic ablation of MMP-7 may be functionally compensated for by increased expression of MMP-2 (Wilson et al., 1997). Since our initial screen, several additional MMP family members have been identified. Many of these genes are extremely restricted in their expression pattern, and in some cases are only produced by specific cell lineages. Further, these initial experiments used *in situ* hybridization, a time consuming technique that assays each gene of interest independently, making analysis of large families of genes expensive in terms of both labor and materials. To gain a more comprehensive characterization the MMPs expressed by these tumors, I utilized microarray analysis, which enables the analysis of thousands of genes simultaneously and allowed me to easily examine expression levels of MMPs and other types of proteinases in intestinal tumorigenesis.

Affymetrix microarray experiments produce two different, but related types of results that are commonly referred to as absolute and relative analyses. Absolute analysis generates a call of whether a gene is present or absent based upon a proprietary algorithm that compares hybridization of target sequence to "Perfect Match" and "Mismatch" oligonucleotide probes (Affymetrix, 2004). This type of analysis is most useful for comparing large sets of samples, but is prone to false negatives when there is high

background such as the case when examining genes with high sequence homology or degraded RNA. In contrast, relative analysis compares the intensity of hybridization between samples. Because transcriptional regulation of many genes functionally results in a gradient of expression rather than simple all-or-nothing binary state, this approach allows more subtle examination of gene expression (Biggar and Crabtree, 2001). Further, biological samples frequently contain numerous cell types, and while a given gene may be regulated in a binary fashion in any given cell, in the larger context of a tissue or organ the transcriptional contribution of each cell type typically resembles more of a graded response if the percent composition of cell lineages varies between samples.

Using a custom Affymetrix microarray containing probes for all currently known human and murine proteinases, inhibitors, and interacting proteins; I have generated a comprehensive profile of the expression pattern of proteinases that are present in normal murine small intestine and intestinal adenomas of the Min model system. Absolute analysis of isolated normal small intestine and Min adenomas confirmed our previously reported findings (Wilson et al., 1997), and additionally identified four additional MMP family members that had not previously been reported to be expressed by Min adenomas. Furthermore, relative comparison between these two tissues revealed three MMPs and several other proteinase genes that were significantly more abundant in intestinal adenomas than normal tissue. These findings provide additional targets for further study of the development and progression of intestinal neoplasia, and provide experimental evidence that lead to our experiments examining other MMP family members and the potential role of mast cell proteinases described in Chapters III and IV.

I have demonstrated that transcripts encoding MMPs-10, -13, and -14 are significantly more abundant in Min adenomas than normal intestinal tissue as determined by relative analysis, and that MMP-15, which is not detected in normal intestine, is present in adenomas as determined by absolute analysis. Unfortunately, I was unable to test the effect of genetic ablation of these MMPs. Mice lacking MMP-10 and -13 have been developed (Parks, 2004; Stickens et al., 2004), but were produced on different genetic backgrounds than the C57Bl/6 background that is optimal for Min studies. These MMP-deficient mice would need to be backcrossed in order to make uniform comparisons within our studies. This process has been started but was not possible to finish before the completion of my studies.

Several tumor models have detected an increase in the expression of MMP-13 that correlates with advanced progression (Wagenaar-Miller et al., 2004), and is frequently expressed by transformed epithelial cells (Blavier et al., 2006; Rath et al., 2006) and chronic, but not acute wounds (Vaalamo et al., 1997). Further, genetic ablation of MMP-13 has been shown to limit tissue damage in a model of hepatic injury (Uchinami et al., 2006), and targeted inhibition by of MMP-13 at the RNA level potently inhibits tumor growth (Ala-aho et al., 2004). Taken together, these data suggest a role for pro-tumorigenic role for MMP-13 in tumor development, which suggests that had I completed these studies that MMP-13 deficient Min mice would develop fewer tumors than littermate controls.

MMP-10 is poorly understood in the context of tumor development, though has been shown to be expressed by migrating enterocytes that border intestinal ulcerations, suggesting a role in cell motility (Salmela et al., 2004; Vaalamo et al., 1998).

Additionally, MMP-10 has been shown to confer resistance to p53 induced apoptosis (Meyer et al., 2005). MMP-10 and MMP-3 are highly homologous enzymes (Massova et al., 1998), and because of the role of MMP-3 as a mediator of initiation, I hypothesize that ablation of MMP-10 would result in decreased tumor multiplicity in the Min model. However, due to the role of MMP-10 in migration, a more significant role may be revealed by using a model capable of metastasis.

MMP-14 deficient animals exhibit a number of severe developmental abnormalities and die prematurely making them unsuitable for use in a Min study (Holmbeck et al., 1999). To date, despite several attempts, MMP-15 deficient animals have not been successfully developed (Seiki, 2004). We have however examined the contribution of four different stromal MMPs to the development of intestinal adenomas in the Min model system- MMP-2, -9, -12 and -19.

MMP-2 Does Not Contribute to Early Tumor Development in the Min Model

I had predicted that ablation of MMP-2 would reduce tumor multiplicity in the Min model. Though not detected as differentially expressed in our microarray screen, MMP-2 is expressed by stromal cells in a large percentage of Min intestinal adenomas (Wilson et al., 1997). Additionally, while MMP-7 ablation has been shown to reduce tumor multiplicity in Min mice, of the tumors that do form, all of these tumors express MMP-2 suggesting that in the absence of MMP-7 that MMP-2 may function in a compensatory role in promoting tumorigenesis (Wilson et al., 1997). MMP-2 has been shown to regulate the activity and activation of other MMPs (MMP-1, -2, -13), acts upon a wide spectrum of substrates resulting in cleavage products with pro-inflammatory (pro-

TNFα, pro-IL-1β) and mitogenic (IGFBP-3, IGFBP-5) effects (Cauwe et al., 2007), and has been shown to contribute to tumor growth in an experimental animal model of pancreatic tumorigenesis (Bergers et al., 2000), thus making MMP-2 an attractive target for further investigation.

I have demonstrated that genetic ablation of MMP-2 does not affect tumor multiplicity or size in the Min model, a finding that is in agreement with previous unpublished studies (Wagenaar-Miller, 2002). However, previous studies using mice deficient for MMP-2 in other tumor model systems, have focused on later stage disease and, in particular, the processes of angiogenesis and metastasis (Bergers et al., 2000). Angiogenesis is thought to be required once tumors achieve sufficient diameter (Naumov et al., 2006). In the Min model, while adenomas may be as large as 5mm across, the majority of tumors morphologically are sessile, therefore flat, and thus may not require extensive angiogenesis. Further, tumors in the Min model only rarely progress to invasive adenocarcinoma and never metastasize, so any contribution of MMP-2 to metastatic spread would not be relevant in this system. Nevertheless, because of the overlapping substrate specificity of proteinases, there remains the possibility that in the absence MMP-2 other enzymes are able to functionally compensate in performing roles that ultimately result in tumorigenesis.

Because of this compensatory possibility, and previous data that suggested that MMP-7 and -2 are compensatory in the Min model, I investigated tumorigenesis in MMP-2; MMP-7 double deficient Min mice. I had anticipated that ablation of both MMP-2 and MMP-7 would further reduce tumor multiplicity, compared to MMP-7 null controls. However, while MMP-2/MMP-7 double deficient mice developed slightly

fewer tumors than MMP-7 deficient littermates, this difference was not significant. To examine the possibility that ablation of specific MMPs induces compensation by other MMP family members, we used microarray and real-time PCR analysis to compare the expression profile of tumors from wild type Min mice, Min mice with MMP-7 genetically ablated, and MMP-2/MMP-7 double knockout Min mice. Surprisingly, in contrast to previous experiments, tumors are largely homogeneous in their expression of MMPs across all genotypes examined, and the only statistically significant difference detected between samples was the absence of MMP-7 and MMP-2 from animals with those MMPs genetically ablated. However, the absence of compensatory transcription of other MMP family members may be partially due to methodological limitations of microarray analysis, which emphasizes large differences in transcript abundance of genes. Further, in order to ensure that we recovered sufficient RNA to analyze; our initial microarray experiments were performed on pooled tumor tissue samples, rather than individual tumors, which homogenizes the sample pool thus making low frequency transcripts appear to be more common. However, follow up experiments performed with RNA isolated from individual tumors did not display variability, either. Taken together, these findings suggest that MMP-2 does not contribute to early tumorigenesis in the Min mouse model, and that transcriptional compensation by other MMP family members does not occur in this model system.

MMP-12 Does Not Contribute to Early Tumor Development in the Min Model System

To investigate the role of MMP-12 in the development of intestinal neoplasia, we developed Min mice lacking MMP-12. We had expected that mice lacking MMP-12

would develop more tumors than littermate controls, and that these tumors would progress to a more advanced state for a number of reasons. MMP-12 has been shown to be produced by tumor epithelial cells, though as its trivial name macrophage elastase suggests, is primarily produced by macrophages (Lavigne and Eppihimer, 2005). High levels of macrophage derived MMP-12 is commonly associated with an improved prognosis in colorectal tumors (Asano et al., 2007; Zucker and Vacirca, 2004). Additionally, a recent study by Tan, *et al.* found that low densities of tumor associated macrophages (TAMs) correlate with increased depth of invasion; and that patients with a high TAM had fewer lymph node metastasis and an higher 5-year survival rate than those whose tumors had a low density of TAMs (Tan et al., 2005). Further, TAM have been associated with increased levels of apoptotic tumor cells in colorectal cancer (Higgins et al., 1996), and have been shown to be capable of directly stimulating tumor cell apoptosis (Cui et al., 1994).

Previous work in our lab using Lewis lung carcinoma cells demonstrated that animals deficient for MMP-12 developed a similar number of tumors as did wild-type littermates, but that animals lacking MMP-12 developed large tumors more frequently (Acuff et al., 2006). These studies have correlated host MMP-12 expression with an angiostatic host response, and implicate MMP-12 as an anti-angiogenic mediator, perhaps through the degradation of plasminogen to generate angiostatin (Acuff et al., 2006; Houghton et al., 2006; Shapiro, 1999). Because of these reasons, I analyzed the tumor multiplicity and the distribution of tumor diameter in MMP-12 deficient Min mice. When compared to littermate control mice, MMP-12 developed a similar number of tumors, and tumors from both groups were similar in size. Attempts to visualize

angiogenic vasculature were unsuccessful, though given the small size of the tumors,

angiogenesis is unlikely to be required for tumor growth, a hypothesis supported by the

literature (Alferez et al., 2008). That genetic ablation of MMP-12 did not affect tumor

multiplicity or size in Min mice, indicates that host derived MMP-12 is not a critical

mediator in the development of the early stage intestinal tumors that develop in this

model organism. However, the lack of an observable effect upon MMP-12 ablation may

be due to the early nature of the tumors of the Min model, and only observable by using a

model system that develops more advanced tumors. One such model that could be used

to assess the role of MMP-12 in later stage tumors is the Smad-3 deficient mouse. Unlike

the Min model mice with Smad-3 genetically ablated develop highly aggressive tumors

of the large bowel that are much larger in size, frequently invade through the bowel wall,

and metastasize locally to regional lymph nodes (Zhu et al., 1998).   Based upon previous

literature indicating a protective role for MMP-12, and data indicating that this protective

effect is due to a decrease of anti-angiogenic factors, I predict that had I used a model

involving later stage tumors that involve angiogenesis, that ablation of MMP-12 would

result in increased tumor size.

MMP-19 Does Not Contribute to Early Tumor Development in the Min Model System

I had expected that ablation of MMP-19 in the Min model would accelerate tumor

development, and that animals lacking this enzyme would develop more tumors, that

these tumors would be larger, and potentially metastatic. Expression analysis has

demonstrated that MMP-19 is produced by activated peripheral blood mononuclear cells,

T_{H1} lymphocytes (Sedlacek et al., 1998), and throughout the intestine in both enterocytes

and occasional stromal fibroblasts and macrophages (Mueller et al., 2000). In contrast to most other MMPs, epithelial expression of MMP-19 is decreased in transformed cells (Bister et al., 2004), and lost during malignant progression (Impola et al., 2005).

MMP-19 was only marginally detected in normal murine intestine by microarray analysis, and weakly in some pooled samples of intestinal polyps. However, this detection pattern was similar to that of MMP-3, which was previously detected in approximately 50% of intestinal adenomas by *in situ* hybridization, and may reflect a large degree of heterogeneity within polyp pools. In a previous study of skin carcinogenesis that used MMP-19 deficient animals, it was shown that the depth of tumor invasion is deeper and that angiogenesis occurs earlier in MMP-19 deficient animals, suggesting a protective role for MMP-19 (Jost et al., 2006).

To examine the effects of MMP-19 on early tumorigenesis, MMP-19 deficient mice were crossed into the Min model. However, ablation of MMP-19 did not affect tumor multiplicity or size, and no evidence of metastatic spread was observed. Thus, while MMP-19 may affect later stages of tumor development, it does not influence early stage tumorigenesis, at least in the Min model.

Leukocyte Derived MMP-9 Contributes to Intestinal Tumorigenesis

We had expected that Min mice lacking MMP-9 would develop fewer tumors than littermates, but those tumors that did develop to be of a more advanced phenotype. This dichotomy has previously been observed in studies that used a mouse model of skin tumorigenesis. Animals that lacked MMP-9 developed fewer tumors than wild-type

littermates, but those tumors that did develop were more aggressive and of a higher grade (Coussens et al., 2000).

Using absolute microarray analysis, we found that transcripts encoding MMP-9 were absent from normal intestinal tissue, but were readily detected in all tumor samples analyzed. In the context of a tumor, MMP-9 (gelatinase B) is produced by a wide variety of stromal cells including neutrophils, mast cells, macrophages, fibroblasts, and epithelium (Noel et al., 2008). Further, MMP-9 expression is dramatically augmented in colorectal tumors, and tends to increase as the disease progresses (Islekel et al., 2007).

When mice with MMP-9 genetically ablated were bred into the Min model, animals that lacked MMP-9 developed significantly fewer tumors than did littermate controls, suggesting that MMP-9 functions in a pro-tumorigenic role. However, there was no difference in tumor size, nor any evidence of local or distant metastatic spread. Immunofluorescent staining revealed that neutrophils are the major source of intratumoral MMP-9. A single MMP-9 positive macrophage was observed, though this cell localized to the tumor periphery.

A well known role for MMP-9 is as a master regulator for angiogenesis. In our model, MMP-9 co-localized with neutrophils, a finding consistent with previous findings that MMP-9 is stored in the tertiary granules of neutrophils (Opdenakker et al., 2001). Neutrophils have been implicated as a key regulator of the initial angiogenic switch (Nozawa et al., 2006) by virtue of their granule contents which are unique in that they contain TIMP-1 free MMP-9 (Ardi et al., 2007). Despite several attempts to detect angiogenesis by immunohistochemistry for CD-31, we were unable to reliably demonstrate a difference in angiogenic vasculature between samples. Additionally, when

total vasculature was visualized using von Willebrand Factor as a marker, no apparent difference in vascularization was detected between tumors from MMP-9 deficient animals and wild-type controls. While an angiogenesis mechanism would explain a difference in tumor size, I didn't actually observe any such dissimilarity between wild-type and MMP-9 null Min mice. Thus, as previously discussed, the Min model is an early model of tumor development, and because adenomas that develop in the Min model are typically of flat morphology, extensive angiogenesis may not be required for tumor survival. Hence, the pro-tumorigenic effect of MMP-9 is likely to be due to something other than a pro-angiogenic factor.

The effect observed here suggests a role for MMP-9 early in the development of neoplastic lesions. One potential way that MMP-9 could impact early tumor development is through the generation of reactive oxygen species. Such a pathway has been particularly well described for MMP-3, which incidentally can also act as an activator of pro-MMP-9 (Inuzuka et al., 2000). MMP-3 expression induces an alternatively spliced form of the small GTPase *rac1* known as *rac1b*. This variant induces an increase in intracellular ROS, elevated levels of the transcription factor Snail, and ultimately genomic instability, though the initial cleavage product leading to this change has not yet been identified (Radisky et al., 2005). Importantly, this effect has also been observed for MMP-9, which has been shown to be capable of substituting for MMP-3 in this same pathway (Radisky et al., 2005). Further, rac1b has been shown to potentiate the *Wnt* pathway, thus enhance transcription of MMP genes (Esufali et al., 2007) and potentially setting up a run-away feedback loop.

Further, MMP-9 activity could be processing cytokines, a mechanism that has been shown to be a major regulator of neutrophil activity. For example, MMP-9 mediated cleavage of full length IL-8 (1-77) to a truncated form (7-77) enhances IL-8 activity by more than tenfold (Van den Steen et al., 2000). This enhanced activity stimulates neutrophils via a positive feedback loop resulting in increased IL-8 binding, migration, production of MMP-9 (Opdenakker et al., 2001) and ROS (Guichard et al., 2005), and ultimately degranulation (Van den Steen et al., 2000). Thus, it is possible that MMP-9 mediated differences in cytokine signaling are involved as upstream mediators of rac1b expression.

Another possibility is that MMP-9 is promoting tumorigenesis through the processing of growth factors, either by direct cleavage, or indirectly via the activation of other MMPs as previously discussed (Imai et al., 1997; Whitelock et al., 1996). Specifically, an MMP-9 dependent effect has been observed in the proliferation and morphogenesis of mammary endothelial cells. *In vitro* stimulation of mammary epithelial cells with TNF-α induces cell proliferation and branching morphogenesis, an effect that is blocked with MMP-9 specific inhibitor (Lee et al., 2000). However, such an effect need not necessarily be due to direct activation by MMP-9. Specifically, MMP-9 has been shown to activate other MMPs, including MMP-2 which has been shown to liberate bound IGF from binding proteins (Thrailkill et al., 1995).

Alternatively, the effect may be the result of MMP-9 influencing neutrophil behavior. MMP-9 activity has been shown to produce factors that regulate cell proliferation, migration, and apoptosis. Furthermore, it has been demonstrated that neutrophils themselves metabolize carcinogens to reactive oxygen and nitrogen species

that damage adjacent cells (Josephy and Coomber, 1998). Defects in neutrophil migration associated with MMP-9 deficiency have been reported in some (Khandoga et al., 2006), but not all (Felkel et al., 2001) model systems. In our samples, that there was no apparent difference between the number of neutrophils in Min tumors of wild-type and MMP-9 null mice suggests that MMP-9 is not essential for neutrophil recruitment or migration to tumors in the gastrointestinal tract.

In conclusion, data presented here present *pro-tumorigenic* role for inflammatory cells, in particular, neutrophils, though the molecular mechanisms responsible for this effect are as of yet unknown.

Mast Cells Function in an Anti-Tumor Capacity

I assayed intestinal tumors using a novel Affymetrix microarray to identify dysregulated proteinases that could potentially contribute to the development of tumors. Interestingly, an unexpected finding of this screen was the detection of several mast cell derived proteinases that were more abundantly expressed in tumors than normal intestinal tissue. Histochemical staining demonstrated that mast cells are more abundant in tumor than normal intestinal tissue, and because of these finding, I further explored the role of mast cells in intestinal tumorigenesis. I have demonstrated that mast cell deficient animals developed more abundant and larger benign tumors than mast cell competent littermates. Furthermore, tumors isolated from mast cell deficient mice had diminished numbers of apoptotic nuclei and fewer intratumoral eosinophils when compared to wild type littermates, though other leukocyte populations were unchanged between groups.

The prognostic significance of mast cell infiltration into tumors remains unclear with studies suggesting that high levels of mast cell infiltration is both a positive (Hedstrom et al., 2007; Nielsen et al., 1999; Rajput et al., 2007; Tan et al., 2005) and a negative (Gulubova and Vlaykova, 2007) prognosis in the setting of colorectal and other cancers. In the studies reporting a positive prognosis, subjects were grouped into cohorts of high and low mast cell density and subjects with higher mast cell densities correlated with increased survival and a reduced risk of recurrence as well as decreased incidence of local and distant metastasis. However, there remains a paucity of *in vivo* experimental tumorigenesis studies to determine the mechanisms by which mast cells exert this protective effect.

My findings are contrary to a report of a pro-tumorigenic effect of mast cells in intestinal tumorigenesis induced by the carcinogen 1-2-dimethylhydrazine (*DMH*) (Wedemeyer and Galli, 2005). However, there are significant differences between the models used in my study as compared to the study by Wedemeyer *et al*. One key difference is the stage of the tumors examined. DMH treatment results in malignant carcinomas of the colon, whereas the Min model produces benign adenomas that do not metastasize. A protective effect of the immune/inflammatory system is often observed in early-stage tumorigenesis, whereas at later stages inflammatory mediators can promote tumor progression (Coussens and Werb, 2002). In a study by Gulubova *et al.*, a high density of mast cells in association with angiogenic "hot spots" was found to correlate with decreased survival and a poor prognosis (Gulubova and Vlaykova, 2007). Coussens *et al.* reported that an increase in mast cells resulted in enhanced angiogenesis in a model of squamous epithelial carcinogenesis (Coussens et al., 1999). However, it should be

emphasized that the pro-angiogenic role for mast cells reported by this group was observed using connective tissue mast cells, which are known to express a different cohort of granular proteins than the mucosal mast cells found throughout the gut (Bradding et al., 1995; Caughey, 2007).

A second important difference between my study and data previously reported by Wedemeyer *et al.* (Wedemeyer and Galli, 2005), is the mechanism of tumor initiation. The Min mouse is the result of a single mutation at base pair 850 in the gene APC (<u>A</u>denomatous <u>p</u>olyposis <u>c</u>oli) which results in a truncated protein (Miyaki et al., 1994; Su et al., 1992). Mutations in the tumor suppressor gene APC are an early event in adenoma development (Fearon and Vogelstein, 1990), and are found in 30-70% of spontaneous adenomas (Ichii et al., 1993). In contrast, chemically-induced tumors are a result of random mutations to known oncogenes and tumor suppressor genes. For instance, tumors induced by DMH or its metabolite AOM (*azoxymethane*) lead to frequent mutations in β-catenin (Takahashi et al., 2000) and to a lesser extent K-ras (Takahashi and Wakabayashi, 2004), both of which are rarely mutated in Min adenomas (Shoemaker et al., 1997b; Suzui et al., 2002). The consequence of mast cell interactions with tumor cells may be dependent on the underlying genetic alterations in the tumor. Lastly, in my study, the mice that were used were of a pure C57Bl/6j background, whereas the previous report used a WB-C57Bl/6j F1 hybrid. Strain differences have been reported to affect tumorigenesis and progression in both the Min model and DMH/AOM induced carcinogenesis through the presence of modifier loci (Diwan and Blackman, 1980; Moser et al., 2001; Shoemaker et al., 1998). There also remains the possibility that

differences exist in the intestinal microenvironment between the two strains, and that these are responsible for the discrepancy.

My data suggest a model whereby mast cells function in a protective role by promoting apoptosis of tumor cells. Mast cells have been shown to indirectly induce apoptosis via degradation of extracellular matrix components by mast cell produced chymase and granzyme B leading to epithelial cell anoikis (Ebihara et al., 2005; Pardo et al., 2007). In addition, mast cells have been shown to directly mediate tumor cell apoptosis via secretion of soluble factors such as TNF (Latti et al., 2003) or by production of reactive peroxides (Henderson et al., 1981). While capable of direct cytotoxicity, mast cells have been shown to enhance the cytotoxicity of other granulocytes, and in particular, eosinophils (Capron et al., 1978).

There are multiple levels at which eosinophils are regulated: generation in bone marrow, emigration from bone marrow, recruitment to tissue, activation and degranulation. Evidence exists for mast cell produced factors having the ability to regulate all of these steps. For example, mast cell secreted IL-5 and stem cell factor (SCF) have been shown to control differentiation of hematopoetic precursor cells into eosinophils (Metcalf et al., 2002; Oskeritzian et al., 1996). Eosinophil migration, particularly in the intestine, is largely mediated by the chemokine Eotaxin-1, an activity that is impaired in mast cell deficient mice (Harris et al., 1997). Additionally mast cell produced chymase has been shown to induce eosinophil migration *in vivo* (He and Walls, 1998). Activation and degranulation of mature eosinophils has been shown to be dependent on mast cell produced SCF and leukotriene C_4 (Oliveira et al., 2002). Once activated, eosinophils are capable of producing several soluble factors that have been

shown to induce apoptosis of tumor cells such as eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), major basic protein (MBP), and granzyme B (Costain et al., 2001). Hence, there are multiple ways in which mucosal mast cell deficiency can impact eosinophil biology.

Based on the correlation between presence of enhanced populations of both mucosal mast cells and eosinophils in the intestine of Min mice, my data suggests a model whereby mast cells are protective in the intestine through both direct effects on tumor cell apoptosis and/or indirectly through the recruitment and activation of eosinophils. This model is supported by reports of mast cell and eosinophil function in immunosurveillance and by studies that correlate high mast cell and eosinophil infiltrates with a good prognosis in colorectal cancer (Nagtegaal et al., 2001; Pretlow et al., 1984). My studies provide experimental evidence to support the epidemiological observation of a protective effect of mast cells on colorectal tumorigenesis. Conversely, in comparison to the pro-tumorigenic effect observed for neutrophils, I have demonstrated an *anti-tumorigenic* role for mast cells.

Conclusions and Future Directions

The work in this thesis began as a study of the range of proteolytic enzymes expressed during the development of intestinal tumorigenesis. I then began to examine potential functional roles using a genetic ablation approach. During the course of these studies I have discovered that the cellular sources of many of the proteinases relevant to tumor development are inflammatory cells. Chronic inflammation has long been

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associated with the development and progression of neoplasia, and taken together, these experiments further refine the role of MMPs and inflammation in early intestinal tumorigenesis. For example, several studies have shown that patients with long-term inflammatory bowel disease have an increased incidence of colorectal neoplasia (Xie and Itzkowitz, 2008). However, the molecular mediators linking inflammation to the development of neoplasia are still obscure.

We have demonstrated that MMP-9 promotes tumor formation, and that neutrophils are the major source of MMP-9 in these tumors, although the mechanism behind this is unclear. While neutrophils are the primary intratumoral source of MMP-9, we cannot exclude the possibility that another cell type produces this enzyme much earlier in tumor development, and that initial expression is ultimately what initiates tumorigenesis.

One such pathway that has been previously implicated in MMP-9 mediated tumorigenesis is through the induction of ROS. Reactive oxygen radicals have long been implicated in the development and progression of tumors and have been demonstrated to stimulate tumor development via several mechanisms including by the promotion of genomic instability, cellular proliferation, angiogenesis, motility, and the influx of inflammatory cells. However, there exists a paucity of *in vivo* data using broad, multi-stage models to support the role of ROS in tumor development. Recently however, genetic and pharmacologic agents have been developed that inhibit ROS production and activity (Storz, 2005), and application of these models in the context of tumor development could refine the role of ROS in tumorigenesis.

While ROS activity may ultimately be responsible for tumor development, the upstream mediators that ultimately trigger this pathway remain obscure. The obvious approach to identify these candidates is through proteomic screening. However, previous attempts to identify substrates for MMP-7 using whole tumor extract at various stages produced inconclusive results. However, unlike MMP-7, MMP-9 expression is ultimately a product of bone marrow derived cells that can much more easily be manipulated, perhaps even to allow comparisons from within the same mouse.

Furthermore, these studies have clarified the spectrum of MMPs known to affect tumorigenesis in the Min model. We have now shown that MMP-7 and MMP-9 both promote tumorigenesis, while MMPs-2, -12, and -19 do not affect tumor multiplicity or size. While we did not find evidence of compensatory transcriptional upregulation between MMP family members, there exists the possibility that despite similar transcript copy number enhanced mRNA stability or translation of more protein is present in tumor compared to normal tissue. Further, there may be functional compensation across MMP or even other proteinase family members that is simply not measurable by assaying transcript abundance. In retrospect, that MMP-2;MMP-7 double deficient mice behaved similarly to MMP-7 deficient mutants is not surprising given that MMP-2 ablation did not affect tumor multiplicity. However, given that both MMP-7 and MMP-9 have been shown to affect tumorigenesis alone, it would be worth assessing them both in combination.

In this thesis, I have provided experimental evidence showing that inflammatory cell populations function in both pro- and anti-tumorigenic fashions. I propose that as illustrated in Figure 24, shifts in the balance between these two diametrically opposed
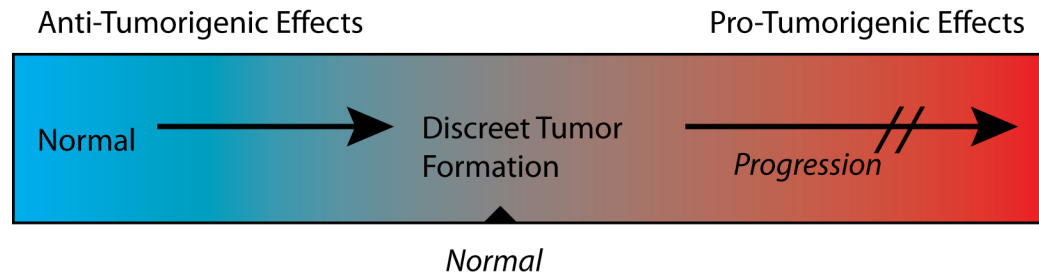
Figure 24: The balance between anti- (blue) and pro-tumorigenic (red) effects of inflammation exists as a continuum within the intestine. Neutrophil-derived MMP9 functions in a pro-tumorigenic fashion, and thus genetic ablation results in decreased tumor formation. Conversely, mast cell deficiency impairs normal anti-tumorigenic effects resulting in enhanced tumor formation.

functions ultimately determine the nascent tumor fate. Testing this hypothesis requires an appropriate mouse model system that allows the examination of various leukocyte populations throughout various stages of tumor development. Ideally, testing the role of neutrophil derived MMP-9 would be achieved using a mouse that lacks neutrophils. While there do exist mutant mice that lack mast cells, eosinophils, lymphocytes and to a degree macrophages, there are currently no known lines of neutrophil deficient mice. While it is possible to largely eliminate neutrophils by the treating mice with an anti-neutrophil depleting antibody, this effect is short lived, requires repeated treatments, and given our current understanding of tumor development in the Min mouse, would require that developing mice be treated *in utero*. Nevertheless, supposing these technical challenges could be overcome, if the major source of MMP-9 is neutrophils, then I would expect that Min mice lacking neutrophils and MMP-9 knockout Min mice would develop a similar number of tumors.

A problem with this approach is that while it does allow the direct testing of neutrophils, it supposes that neutrophils are strictly functioning as pro-tumorigenic through the release of MMP-9. It is possible that while neutrophil derived MMP-9 may promote tumorigenesis, other neutrophil derived factors function in an anti-tumor capacity, and that a relatively crude approach of simply removing all neutrophils could result in *increased* tumor burden. A cleaner system would be to develop a tissue specific, or even cleaner, a cell specific knock-out. A genetic mutation resulting in the lack of neutrophil secondary granules has been identified in mice, however, to date the genetic elements responsible for tertiary granules, the granules that contain MMP-9, have not been identified (Lekstrom-Himes et al., 1999). However, if such a gene were to be identified, it could be used to breed mice specifically lacking neutrophil derived MMP-9 while retaining other neutrophil activity for examination in the Min system.

Conversely, I have shown that ablation of mast cells results in enhanced tumor formation, presumably through an eosinophil effected mechanism, though the molecular signals behind this modulation are unknown. The most obvious next step is to investigate the effect of eosinophil ablation in our model system. Two eosinophil deficient mouse lines have been described to date, IL-5/CCL11$^{-/-}$ and ΔdblGATA that could be used to examine the role of eosinophils in this system. Assuming that mast cells activity is simply to modulate eosinophil activity, I would expect that eosinophil deficient Min mice would phenocopy Min-Sash mice. However, a more likely outcome is that eosinophil deficient Min mice would develop more tumors than eosinophil competent littermates, but less than Min-Sash mice, thus indicating that mast cells exert their anti-tumor affect through multiple cell types.

Mast cells were initially identified in our model due to an increase in the abundance of several mast cell produced proteases. It is likely that different sub-cohorts of mucosal mast cells express different combinations of mast cell proteinases (Friend et al., 1998). While I have broadly examined the effect of ablating mast cell proteinases by removing mast cells entirely from the system, the role of the individual proteinases has not been assessed. Knockout mice lacking every known mast cell proteinase individually have been developed. Unpublished data has demonstrated that mcpt-4 deficient mice develop dramatically smaller tumors in a fibrosarcoma model, a change that is likely due to a reduction of angiogenesis in the knockout animals (Stevens, 2008). However, the major mast cell proteinases that were more abundant in our tumors were mcpt-2 and mcpt-5, two chymases that have not to date been closely studied in the context of tumor development. However, chymases have been implicated in the induction of apoptosis (Heikkila et al., 2008), and since we do see a reduction in apoptosis upon mast cell elimination, it is reasonable to hypothesize that genetic ablation of these proteinases would result in a reduction in apoptosis, though because of the overlapping substrate specificity of murine mast cell proteinases (Stevens, 2008), a combination of multiple mast cell proteinases may have to be ablated to have an effect.

Alternatively, rather than focusing on specific cell lineages, it may be more beneficial to explore the role of broadly expressed inflammatory molecules in the development of intestinal tumors. For example, genetic ablation of MyD88, a toll-like receptor (TLR) adaptor molecule expressed in several leukocyte lineages, has been shown to dramatically inhibit tumor development and progression in genetic and carcinogen induced models of colorectal cancer (Rakoff-Nahoum and Medzhitov, 2007),

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suggesting that it may be more beneficial to examine specific pro-inflammatory pathways rather than specific cell populations.

In conclusion, I have presented evidence that components of the innate immune system function in both pro- and anti-tumor capacities early during tumor development. Further characterization of the molecular mechanisms responsible for these effects could lead to novel therapeutic strategies in the treatment and prevention of cancer.

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