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# The Novel Tumor Supressive Role of Epithelial-Derived Matrix Metalloproteintase 9 In Colitis Associated Cancer

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# THE NOVEL TUMOR SUPRESSIVE ROLE OF EPITHELIAL-DERIVED MATRIX METALLOPROTEINASE 9 IN COLITIS ASSOICATED CANCER

by

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Under the Direction of Professor Didier Merlin, Ph.D.

## ABSTRACT

Colitis associated cancer (CAC) is a chronic inflammation driven colon cancer among individuals with Inflammatory Bowel Disease. Its development is associated with the inflammation-dysplasia-carcinoma pathway which differs from the adenoma-carcinoma pathway of sporadic colon cancer (CRC). Matrix Metalloproteinases are zinc-dependent endopeptidases against extracellular matrix proteins expressed in the gastrointestinal tract during inflammation. We have shown that Matrix Metalloproteinase 9 (MMP9) plays a protective role in CAC contrary to its inflammatory role in acute-colitis. I hypothesize that epithelial-derived MMP9 mediates tumor suppression in CAC by regulating genomic stability via Notch1 activation. This study identifies the role of epithelial derived-MMP9 in CAC via "MMP9-Notch1-ARF-p53 axis" pathway which regulates apoptosis, cell-cycle arrest and DNA damage. This study also identifies the role of epithelial-derived MMP9 in maintaining genomic stability by reducing reactive oxygen species and activating mismatch repair mechanisms. Thus, MMP9 expression could potentially be a natural way to suppress inflammation induced colon cancer.

INDEX WORDS: Colitis Associated Cancer, Matrix Metalloproteinase 9, Tumor Suppressor, Inflammation

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College of Arts and Sciences

Georgia State University

May 2018

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**DEDICATION**

I dedicated this Dissertation to my sources of support and inspiration: my grandmother, my parents, my loving wife, and my beautiful daughter.

# TABLE OF CONTENTS

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## LIST OF ABBREVIATIONS

4',6-diamidino-2-phenylindole (DAPI)

8-hydroxy-2' -deoxyguanosine (8-OHdG)

ADP-ribosylation factor 1 (ARF1)

Adenomatous polyposis coli (APC)

Azoxymethane (AOM)

Chromosomal Instability (CIN)

Colitis Associated Cancer (CAC)

Colorectal Colon Cancer (CRC)

Crohn's Disease (CD)

Dextran Sodium Sulfate (DSS)

Extracellular Matrix (ECM)

Gastrointestinal tract (GI)

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

Hematoxylin & Eosin (H&E)

Human colon cancer cells (HCT116)

Inflammatory Bowel Disease (IBD)

Matrix Metalloproteinases (MMP)

Matrix Metalloproteinase 9 (MMP9)

Microsatellite Instability (MSI)

Mismatch Repair Pathway (MMR)

Mouse Embryonic Fibroblast (MEF)

Nonsteroidal anti-inflammatory drugs (NSAID)

Notch intracellular domain (NICD)

Proliferating cell nuclear antigen (PCNA)

Reactive Oxygen Species (ROS)

Reactive Nitrogen Species (RNI)

Superoxide dismutase **1** (SOD1)

Transgenic Matrix Metalloproteinase 9 mice (TgM9)

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL)

WT mice (WT)

Vascular endothelial growth factor (VEGF)

Ulcerative Colitis (UC)

# 1 INTRODUCTION

## 1.1 Inflammatory Bowel Disease

IBD, such as Crohn's Disease (CD) and ulcerative colitis (UC), are chronic inflammatory conditions that affects the gastrointestinal (GI) tract as well as the colonic mucosal (Molodecky, Soon et al. 2012). CD can affect any site of the GI tract from the mouth to the anus, mostly the terminal ileum, and UC occurs predominately at the distal end of the colon (Hanauer and Sandborn 2001). These diseases affect approximately 1 to 4 million individuals in the U.S (Baumgart and Sandborn 2007). IBD causes epithelial barrier disruption by affecting major components of the intestinal GI tract such as epithelial cells, goblet cells, immune cells, and intestinal commensal bacterial types (Baumgart and Sandborn 2007, Xavier and Podolsky 2007). It is characterized by relapses (acute flare) and remissions which involve immune-mediated tissue injury followed by repair (Fiocchi 1998). Acute or short-term inflammation is a beneficial response to tissue damage and pathogenic challenges. However, unregulated inflammation may lead to chronic and malignant cell transformation resulting in cancer(Lynch, Drescher et al. 2014).

## 1.2 Colitis Associated Colon Cancer

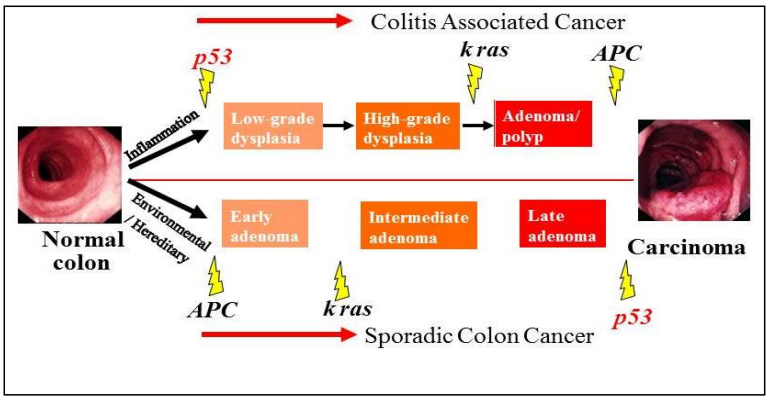


**Figure 1. Schematic of Colitis Associated Cancer Progression (above) and Sporadic Colon Cancer progression (below)**

Individuals with chronically active UC and CD have up to a 50% (depending on population cohort) risk of developing colitis-associated cancer (CAC) (Rogler 2014). The risk of developing CAC increases with the length of the disease in correlation with the severity of inflammation (Choi and Zelig 1994, Itzkowitz 2006). Although both sporadic colon cancer (CRC) and CAC are colon malignancies, several features make CAC distinct from (CRC) (Walter, Harper et al. 2013). CAC has gathered much attention due to its different molecular events associated with various stages of colon tumorigenesis including initiation, promotion and progression as compared to CRC (Terzic, Grivennikov et al. 2010). The development of CAC is associated with the *inflammation-dysplasia-carcinoma pathway* that is significantly different to the *adenoma-carcinoma* pathway of CRC(Waldner and Neurath 2015). In CAC, mutations in the tumor suppressor gene p53 are the first to occur followed by *k-ras* mutations and adenomatous polyposis coli (*APC*) mutations occur in the later stage (Walter, Harper et al. 2013). Unlike CRC that arise from adenomatous polyps, CAC develops commonly in flat dysplastic tissues in

individuals pre-exposed to IBD and have more polyp formation in the distal colon region (Terzic, Grivennikov et al. 2010). Also, CAC has a higher percentage of chromosome instability (CIN) and microsatellite instability (MSI) than CRC (Fukata and Abreu 2008). In CAC due to chronic inflammation, proinflammatory cytokines IL-1β and TNF-α secrete reactive oxidative species (ROS) and reactive nitrogen intermediates (RNI) (Kraus and Arber 2009). This leads to persistent inflammation in epithelium stimulating ROS overproduction within the epithelial cells (Terzic, Grivennikov et al. 2010). Consequently, the imbalance between generation and elimination of ROS and RNI stimulates oxidative stress inducing DNA damage (Kraus and Arber 2009).

### 1.3 Matrix Metalloproteinases

MMPs are zinc-dependent neutral endopeptidases that have proteolytic activity against extracellular matrix (ECM) proteins (Sternlicht and Werb 2001, Egeblad and Werb 2002, Okamoto and Watanabe 2005, Garg, Sarma et al. 2010). Their targets include proteinases, clotting factors, chemotactic molecules, latent growth factors, cell surface receptors, and cell-cell adhesion molecules that make them able to regulate many biological processes (Cao, Drews et al. 1998, Curci, Liao et al. 1998, Sun, Wenger et al. 1999, Sternlicht and Werb 2001). MMPs are expressed in the GI tract by immune cells (neutrophils, macrophages, and lymphocytes) as well as epithelial cells during inflammation (Baugh, Perry et al. 1999, Sternlicht and Werb 2001, Castaneda, Walia et al. 2005). They regulate innate and acquired host defenses by facilitating inflammatory cell recruitment across the epithelium and endothelial basement membrane (Ravi, Garg et al. 2007). In CRC, MMPs have been extensively studied to play a significant role in development and progression (Walter, Harper et al. 2013). However, there are no studies showing the mechanistic link between MMPs with CAC (Walter, Harper et al. 2013).

### 1.4 Matrix Metalloproteinase 9 Structure and Activation

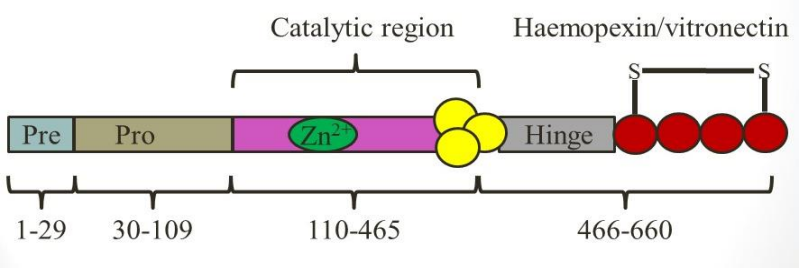


**Figure 2. 2D structure of Matrix Metalloproteinase 9**

MMP9, also called 92-kDa type IV collagenase or gelatinase B, plays a key role in ECM degradation and is involved in tissue remodeling (Yabluchanskiy, Ma et al. 2013). It is undetectable from all healthy adult tissues but is the highest expressed protease during inflammation in IBD (Baugh, Perry et al. 1999). This protease consists of an $NH_2$-terminal prodomain, a catalytic domain, a linker domain, and a COOH-terminal hemopexin domain-like domain (Chen and Greene 2004). The prodomain of contains a "bait region" in the N-terminal, that mediates activation by being cleaved by targeted serine proteinases, leading to partial activation followed by full activation via autocatalytic cleavage (Visse and Nagase 2003). Latency is maintained by an unpaired cysteine sulfhydryl group near the C-terminal end of the propeptide domain within a conserved cysteine motif, called the "cysteine switch"(Visse and Nagase 2003). This sulfhydryl acts as a fourth ligand for the active site zinc ion, that is bound to three histidine residues in the zinc binding domain of the catalytic region(Vandooren, Van den Steen et al. 2013). The catalytic domain contains consists a zinc ion, and three homologous type II fibronectin repeats (Yabluchanskiy, Ma et al. 2013). The zinc ion is coordinated with the cysteine switch motif to keep MMP-9 inactivated (Rowsell, Hawtin et al. 2002). Once activated, the zinc ion is used for proteolytic activity (Rowsell, Hawtin et al. 2002). Also, MMP-9 has a

heavily O-glycosylated fibronectin-like type II domain that is essential in binding denatured collagen and gelatin (O'Farrell and Pourmotabbed 1998, Opdenakker, Van den Steen et al. 2001). MMP9 is unique from other MMPs due to a central highly O-glycosylated domain between the catalytic and terminal hemopexin domains, which allows for their independent movements (Ravi, Garg et al. 2007). The hemopexin domain is required for substrate specificity.

Interference of the cysteine-zinc interaction in the cysteine switch leads to the activation of MMP9 (Cornelius, Nehring et al. 1998, Visse and Nagase 2003). Activation of MMP-9 by serine proteases are mediated by cleavage specificity of the bait region via an initial proteolytic attack that partially activates it (Visse and Nagase 2003). Once the part of the propeptide is removed, it will destabilize the rest of the propeptide that includes the cysteine switch zinc interaction allowing for full activation via intermolecular processing (Bini, Itoh et al. 1996). Once activated, a substrate will bind onto the catalytic site cleft, its carbonyl group of the peptide coordinates with the active-site zinc (Visse and Nagase 2003). The peptide undergoes hydrolysis, displacing water from the zinc atom, which facilitates a nucleophilic attack of the water molecule on the scissile peptide bond of the carbonyl group (Llano, Pendas et al. 1999). The specificity pocket, on the right of the catalytic zinc, accommodates the side chain of the substrate residue which becomes the new N-terminus after cleavage (Llano, Pendas et al. 1999).

### 1.5 Matrix Metalloproteinase 9 Expression

MMP9 is expressed in the GI tract by immune cells and epithelial cells during inflammation (Baugh, Perry et al. 1999, Sternlicht and Werb 2001, Castaneda, Walia et al. 2005). It is the most expressed protease in IBD as well as being expressed in several other inflammatory diseases such as asthma, rheumatoid arthritis, nonsteroidal anti-inflammatory drugs (NSAID)-induced gastric ulcers (Bailey, Hembry et al. 1994). NFκB is required for

production MMP9 (Bond, Chase et al. 2001, Okamoto, Akaike et al. 2001). AP-1, which has two binding sites on the MMP9 promoter, also stimulates the production of MMP9 from its activation (Wasylyk, Gutman et al. 1991, Woessner 1991). Cooperation between AP-1 and NFκB is required for MMP9 transcription induction because the AP-1 occupation is not sufficient. TNF-α and IL-1β are two cytokines that are shown to increase NFκB and AP-1 (He, Joiner et al. 2011). Suppression of MMP9 transcription is a result of interference with binding of NFκB and AP-1 to the promoter region such as Kiss-1, a suppressor of cancer cell proliferation and metastasis (Vandooren, Van den Steen et al. 2013).

MMP9 trafficking after translation is different in neutrophils as opposed to other cells (Vandooren, Van den Steen et al. 2013). In neutrophils, it is produced rapidly and pre-stored in zymogen granules to be secreted within minutes upon an inflammatory stimulus (Van den Steen, Proost et al. 2002). It is then used for degradation of the ECM with activation of major proangiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (Jonsson, Lundberg et al. 2011, A 2013). Other cells such as macrophages and epithelial cells rely on de novo synthesis prior to the secretion of MMP9, which takes at least several hours (Opdenakker, Van den Steen et al. 2001). Upon activation, MMP9 can be found in small Golgi-derived cytoplasmic vesicles that are associated with stable microtubules and kinesin transport proteins (Vandooren, Van den Steen et al. 2013). My lab has demonstrated epithelial not neutrophil-derived MMP9 mediates tissue damage during intestinal inflammation (Castaneda, Walia et al. 2005). The mechanism by which it mediates tissue damage is not well known (Ravi, Garg et al. 2007). Potential mechanisms by which it mediates this response could be from defective re-epithelialization, adhesion complex integrity in impaired wound healing, increased endothelial permeability, and activation of fibrin(ogen), IL-1β, and TGF-β (Alexander and Elrod

2002, Mohan, Chintala et al. 2002). We have shown exogenous MMP9 inhibits epithelial cell adhesion which is required for epithelial cells to migrate and attach to appropriate matrix after damage (Castaneda, Walia et al. 2005). During inflammation, MMP9 plays an important role in post-translation regulation of cadherin, occluding adhesive activities and proteolytic cleavage of occluding or E-cadherin ectodomain (Papon, Lechapt-Zalcman et al. 2006, Xue, Le et al. 2006). This results in the disassembly of tight and adherens junctions leading to impaired cell migrations and wound healing in cornea, skin, and endothelial cells (Stetler-Stevenson, Krutzsch et al. 1989, Matsubara, Zieske et al. 1991).

### 1.6 DUAL ROLE OF MATRIX METALLOPROTEINASE 9 IN CANCER

Studies have shown that inhibition of gelatinases (MMP9 and MMP2) results in regression of tumor growth (Lubbe, Zhou et al. 2006) (Bjorklund and Koivunen 2005). Sinnamon *et al* have shown that in Adenomatous polyposis coli (*APC*)-$^{Min}$ mice genetically deficient in MMP9 expression had fewer tumors than littermate controls due to altered proliferation by MMP9 among APC-Min adenoma cells (Sinnamon, Carter et al. 2008). On the other hand, Burg-Roderfeld *et al* (Burg-Roderfeld, Roderfeld et al. 2007) has shown that MMP9 hemopexin domain has an inhibitory effect on migration and adhesion of colorectal carcinoma cells. Another study by Pozzi *et al* (Pozzi, LeVine et al. 2002) showed that reduction of plasma levels of MMP9, in either normal or integrin alpha1-null mice, leads to decreased synthesis of angiostatin and consequent increased tumor growth and vascularization and was also supported by the study of Bjorklund and Koivunen (Bjorklund and Koivunen 2005). In melanoma, increased expression of MMP9 is found initially, but is reversed at the later stage (van den Oord, Paemen et al. 1997). It has been reported that in breast and colon cancer, MMP9 expression has been correlated with both increased and decreased survival and formation of distant metastasis (Bjorklund and Koivunen 2005) (Pacheco, Mourao et

al. 1998). Recently some studies have also shown that MMP9 has a protective role in different malignancies such as chronic inflammation in lung and liver (Liu, Turkoz et al. 2011, Gonzalez-Arriaga, Pascual et al. 2012).

Our lab has shown that MMP9 knockout animals were more susceptible to CAC (Garg, Sarma et al. 2010). Our group has also shown that MMP9 activates transcription factor Notch1 (Garg, Jeppsson et al. 2011). Notch1 plays various important roles during embryogenesis and tissue homeostasis, and also has a dual role in cancer biology (Garg, Jeppsson et al. 2011). Consequently, MMP9 could play a protective role in CAC in contrast to its inflammation mediator function in acute colitis (Talora, Sgroi et al. 2002, Talora, Cialfi et al. 2005) (Garg, Sarma et al. 2010). Also, GI cancers consist mainly of epithelial cells with p53 mutations occurring initially in CAC. **Thus, I hypothesize that epithelial derived MMP9 activates p53 via Notch1 signaling as well as regulating genomic stability to acts as a tumor suppressor in CAC.**

# 2 EXPERIMENT EPITHELIAL DERIVED MATRIX METALLOPROTEINASE 9 ACTS AS A TUMOR SUPPRESSOR IN COLITIS ASSOCIATED COLON CANCER BY ACTIVATING P53 VIA NOTCH 1 SIGNALING

(This work is mainly based on the published paper in Oncotarget. 2017;8(1):364-78. In this project, the author is obliged to Dr. Pallavi Garg and Dr. Didier Merlin for their great contributions to Colitis Associated Cancer research.)

## 2.1 Introduction

The role of inflammation in cancer development has been indicated as early as in 18$^{th}$ century (Landskron, De la Fuente et al. 2014). In the following years, an association between

chronic inflammation and cancer has been strongly highlighted which gathered much attention. Inflammation is a beneficial response to tissue damage and pathogenic challenges, though unregulated inflammation may transform into chronic and thereby inducing malignant cell transformation in the tissue environment. Inflammatory bowel disease (IBD), which primarily includes ulcerative colitis (UC) and Crohn's disease (CD), involves inflammation of all or part of digestive tract. Patients with chronically active UC and CD inevitably have significantly higher risk (as high as 50% depending on the population cohort) of colitis associated cancer (CAC) (Rogler 2014). CAC risk increases with the duration of the disease and correlates positively with the severity of inflammation. CAC is a subtype of colorectal cancer (CRC) yet uniquely different from it (Walter, Harper et al. 2013). CAC progresses through 'dysplasia-carcinoma axis' while CRC progression is through 'adenoma-carcinoma axis'(Ullman and Itzkowitz 2011). In CAC, *p53* mutations are the first ones to be detected followed, by *KRAS* and *APC* mutations respectively. On the other hand in CRC *APC* mutations are the first ones to be identified followed by *KRAS* mutations and *p53* mutations respectively (Ullman and Itzkowitz 2011, Walter, Harper et al. 2013). It is known that inflammatory responses dissect oncogenic signaling pathways such as apoptosis, proliferation, angiogenesis etc.

In this context the relation between tumor cells and its surrounding normal tissue, in which a large family of proteolytic enzymes- proteases are involved cannot be ignored. Proteases play important roles in a wide variety of biological processes as well as multiple diseases including cancer (Lopez-Otin and Bond 2008). Among the wide variety of proteases, the matrix metalloproteinases (MMPs) have significantly played the role of effectors in cancer progression due to their ability to degrade the protein components of the extracellular matrix (ECM) and the basement membranes. Thus provide an access for tumor cells to the vascular and lymphatic

systems and facilitating the generation of metastasis (Walter, Harper et al. 2013). MMPs are a family of $Zn^{2+}$ dependent extracellular matrix degrading endopeptidases that share common functional domains and activation mechanisms (Vu and Werb 2000). MMPs are the only mammalian enzymes capable of catalyzing cleavage of the interstitial collagen, types I, II, III or IV, in their native triple helical form, at the neutral pH of the extracellular space (Vu and Werb 2000). MMPs being essential regulators of ECM and basement membrane are involved at all junctures of inflammation as well as tumor progression- including proliferation, adhesion, migration, angiogenesis, senescence, apoptosis, cytokine and chemokine bioactivity and evasion of the immune system (Matsubara, Zieske et al. 1991) (Lopez-Otin and Bond 2008, Al-Dasooqi, Gibson et al. 2009, Walter, Harper et al. 2013). In recent years, studies to identify the molecular basis and pathophysiology of CAC have been attempted. However, the precise role of MMPs in mediating CAC is still unexplored except few sporadic studies (Newell, Matrisian et al. 2002, Garg, Jeppsson et al. 2011, Walter, Harper et al. 2013) (Garg, Sarma et al. 2010).

Among the 25 known mammalian MMPs (Giannandrea and Parks 2014), MMP9 is unique as its protein expression and activity is undetectable in most healthy adult tissues including the intestine and colon but is highly expressed in a variety of inflammatory states. We have shown that epithelial cell-derived MMP9 mediates tissue damage during colitis (Castaneda, Walia et al. 2005) (Garg, Vijay-Kumar et al. 2009) (Liu, Patel et al. 2013). We have also shown that despite being a mediator of acute colitis, MMP9 plays an opposite but protective role in the development of CAC (Garg, Sarma et al. 2010) (Garg, Jeppsson et al. 2011). Aim of the present study is to determine if epithelial derived- MMP9 is responsible for this contrasting but defensive role of tumor suppressor in CAC. We also sought to determine the underlying the molecular mechanism

by using MMP9 transgenic mice, Tg-villin-MMP9 (TgM9) that specifically overexpresses MMP9 in the colonic epithelium.

## 2.2 Methods

### *2.2.1 Animal Models*

All animal procedures were in compliance with the Guide for the Care of Use of Laboratory Animals from the US Public Health Service and with approval from the Animal Care Committee of Georgia State University. As described previously (Liu, Patel et al. 2013), 10 weeks old gender matched TgM9 and their wild-type (WT) littermates obtained while crossing the hetz of TgM9 of C57/B6 background were used for the study at the start of the experimental protocol. Extensive characterization of TgM9 has been published previously by us (Liu, Patel et al. 2013) and some new data has been added in supplementary Figure S1. As described previously (Castaneda, Walia et al. 2005) (Garg, Sarma et al. 2010), 10 weeks old gender matched MMP9$^{-/-}$ and WT mice of C57/B6 background were also used for the study They were maintained on a 12-hour dark-light cycle and allowed free access to nonpurified diet pellets and tap water.

### *2.2.2 Colitis Associated Cancer Induction*

Both TgM9 (n=20) and WT (n=18) were divided into two groups- one group was induced with CAC and another group was given water only. The CAC group of TgM9 (n=20) and WT (n=18) were exposed to 3% DSS (w/v) (MP Biomedicals, Salon, OH) by oral administration through their drinking water *ad libitum* for 7 days. On day 8, their water was changed to regular drinking water. On day 21, their drinking water was changed back to 3% DSS for the second cycle of a week. This was followed by two weeks of recovery period with regular drinking water. On day 85, the mice were sacrificed after 3$^{rd}$ cycle of DSS and recovery. Colonscopy images (Xenon Nova 475, STORZ, Tuttlingen, Germany) were taken as well as colons being opened longitudinally to count

the number of polyps and dysplastic lesions. We monitored body weight, stool consistency, and stool occult blood of all the mice during DSS and recovery cycles.

### *2.2.3 Hematoxylin and Eosin staining*

Swiss rolls of the colon of mice induced with CAC were collected and H&E staining was performed. H&E stained sections were used to calculate the histological score based on crypt damage, infiltration of neutrophils, and foci of ulceration in the analyzed colons (Cooper, Murthy et al. 1993, Cooper, Murthy et al. 2000).

### *2.2.4 TUNEL staining*

As described previously (Liu, Patel et al. 2013), paraffin sections of colons were deparaffinized and stained for TUNEL. Quantification of apoptosis was performed by counting number of apoptotic cells per crypt divided by total number of epithelial cells in the same crypt and was expressed as percentage. For each mouse in each group 12 crypt were counted.

### *2.2.5 Protein extraction and western blot analysis*

As described previously (Garg, Jeppsson et al. 2011), for WB analysis, mucosal stripping was obtained from the TgM9 and WT mice (n=20 per group) with and without CAC after the sacrifice. The antibodies used were anti-MMP9 (Abcam, Cambridge, MA), anti-NICD (Abcam), anti-caspase-3 (Cell Signaling, Beverly, MA) anti-p53 (Cell Signaling), anti-$p21^{WAF1/Cip1}$ (BD Bioscience, San Jose, CA), anti-cyclin D1 (Santa Cruz, Dallas, TX), anti-Cyclin A (Cell Signaling), anti-Cyclin E1 (Santa Cruz), anti-γH2AX (Abcam), anti-p19ARF (Abcam), and anti-p14ARF (Abcam). Goat anti-mouse secondary antibody (1:2000; Bio-Rad, Hercules, CA) or goat anti-rabbit secondary antibody (1:2000, Bio-Rad) were used. Densitometry graphs were generated by using image acquisition and analysis software by VisionWorksLS Analysis Software (UVP, Upland, CA).

### *2.2.6 Cell Culture and transfection*

As described previously (Garg, Jeppsson et al. 2011), MEF cells, obtained from MMP9$^{-/-}$ and WT mice. As describe previously stably transfected HCT116 cell lines (expressing wild type p53, a generous gift from Dr. VW Yang, Stony Brook University, NY) with and without MMP9 (Garg, Jeppsson et al. 2011) were used to analyze the cell proliferation assay and cell cycle arrest. They were transfected for 72 h with a pEGFP plasmid with and without the MMP9 gene in 6 well plate. The transfected clones were selected under an antibiotic (Geneticin; GIBCO, Grand Island, NY). These transfected clones were screened for MMP9 expression and the three highest MMP9 expressing clones were selected for HCT116 cell line and two highest MMP9 expressing clones were selected for MEFs, and were sorted via flow cytometry (BD Biosciences).

### *2.2.7 Cell proliferation assay*

HCT116 cells were seeded in T25 $cm^2$ flasks at the density $10^5$ per mL medium. After 24 h, 48 h and 72 h incubations, cells were counted by automated cell counter (Countess, Invitrogen, Grand Island, NY). Each experiment was done in triplicates

### *2.2.8 Statistical analysis*

As described previously (Liu, Patel et al. 2013), data are presented as means ± SE. Groups were compared by Student's t-test. P values $<0.05$ was considered statistically significant.

## 2.3 Results

### *2.3.1 Constitutive expression of MMP9 in colonic epithelium exhibited resistance to CAC*

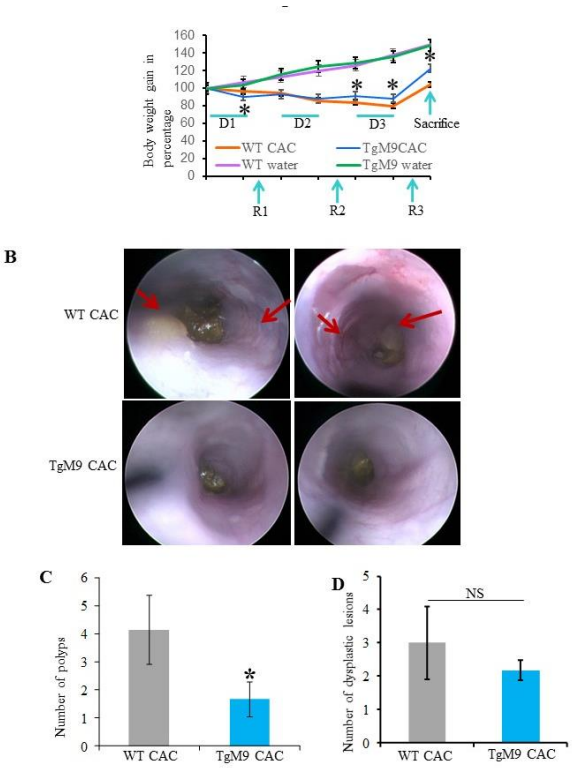


**Figure 3. Overexpression of MMP9 in colonic epithelium exhibited resistance to CAC**
(A) The line graph representation of change in body weight of TgM9 mice (blue line) and WT mice (orange line) during three cycles of DSS (D1, D2 and D3) followed by three recovery cycles (R1, R2 and R3). TgM9 mice (green line) and WT mice (red line) without CAC induction shows the body weight change. (B) The top panel shows representative colonoscopy images from two different WT mice and bottom panel shows representative colonoscopy images from two different TgM9 mice in CAC. Red arrows indicate flat polyps a characteristic feature of CAC polyps. (C) The bar graph presentation of number of polyps among TgM9 mice (blue bar) and WTs (grey bar) in CAC. (D) The bar graph presentation of dysplastic lesions counts among TgM9 mice (blue bar) and WTs (grey bar) in CAC, NS means non-significant. Each bar represents mean ± S.E., *p< 0.05

Both TgM9 and their wild type (WT) littermates were induced with CAC as described in 'Materials and Methods' section and were sacrificed after 85 days. Both groups of mice were monitored for weight loss during the entire length of the experiment. TgM9 mice showed significantly more weight loss compared to WT littermates at the end of 1$^{st}$ cycle of dextran sodium sulfate (DSS) (Figure 1A). However, at the end of 2$^{nd}$ cycle of DSS there were no significant changes in the body weight of TgM9 mice compared to WTs. On the other hand, TgM9 mice started displaying a significant weight gain at the end of 2$^{nd}$ recovery cycle (Figure 1A). They also exhibited significantly lower body weight loss compared to WTs at the end of 3$^{rd}$ cycle of DSS. This trend continued until the end point of the protocol i.e. till the day of sacrifice. TgM9 and WT mice exhibited a parallel and comparable increase in body weight without CAC as a control group (Figure 1A). Figure 1B shows the colonoscopy view indicating that thickening of mucosal layer due to inflammation and number of polyps (as shown by red arrows), were significantly lesser among TgM9 mice compared to WTs in CAC. Figure 1C is the bar graph presentation of tumor incidence among TgM9 supporting the colonoscopy data. There was significantly less number of polyps among TgM9 mice (1.7±0.64) compared to WT littermates (4.1±1.2) in CAC. In Figure 1D, TgM9 showed a trend of fewer dysplastic lesions (2.2±0.31) compared to their WT littermates (3±1.09) in CAC, however the difference was not significant. These data together indicates that TgM9 mice overexpressing MMP9 in colonic epithelium were more protected compared to WTs in CAC.

### *2.3.2 Constitutive expression of MMP9 in colonic epithelium was associated with lower histological score and apoptosis*

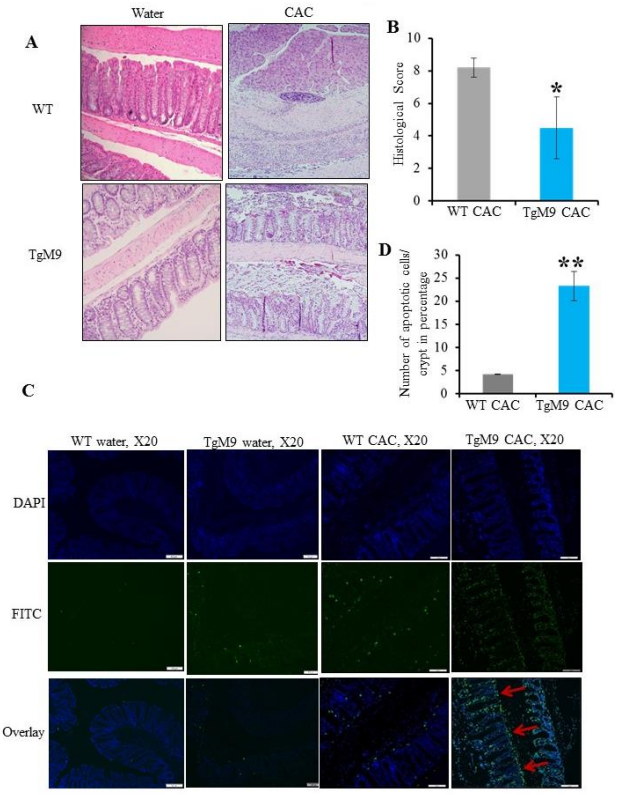


**Figure 4. Overexpression of MMP9 in colonic epithelium was associated with lower histological score and apoptosis in CAC.**
(A) H&E staining of Swiss rolls of the colons from TgM9 and WT mice in CAC indicating less damage to crypt architecture. Left panel shows X20 magnification and right panel shows X10 magnification. (B) The bar graph presentation of histological score calculated on three parameters: infiltration of neutrophils, loss of crypt architecture and foci of ulceration of TgM9 mice (blue bar) and WTs (grey bar) in CAC. (C) The top panel shows DAPI blue colored staining for nucleus, middle panel shows TUNEL staining as green fluorescent nuclei and lower panel shows the overlay images of greenish yellow nuclei of apoptotic cells as indicated by red arrow, magnification used was X10. (D) The bar graph representation of the quantification of apoptosis as the percentage of apoptotic nuclei/total number of cells per crypt among TgM9 mice

(blue bar) and WTs (grey bar) in CAC, number of crypts counted were 12 for each mice. Each bar represents mean ± S.E., *p< 0.05 and **p< 0.005.

Figure 2A represents the Haematoxylin and Eosin (H&E) staining indicating decreased crypt architecture damage, lesser infiltration of neutrophils, fewer foci of ulceration and dysplastic lesions among TgM9 mice compared to WTs. Figure 2B is the bar graph presentation of the histological score based on the parameters described in 'Materials and Methods' section, indicating that TgM9 mice had significantly lower histological score (4.5±1.9) compared to WTs (8.2±0.6) in CAC. Abnormal apoptosis and/ or irregular proliferation are the characteristic features of tumor cells. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to assess apoptosis in the colonic epithelium of TgM9 mice. In Figure 2C top panel shows blue colored Diamidino-2-Phenylindole, Dihydrochloride (DAPI) staining for nuclei, middle panel shows the green fluorescent cells as apoptotic cells and lower panel is the merged/ overlay image of greenish yellow fluorescence indicating that there was a significant increase in apoptosis (as represented by red arrows at the apical surface of crypts) among the TgM9 mice compared to WTs in CAC. Figure 2D is the bar graph presentation of the quantification of apoptotic cells among TgM9 mice and WTs in CAC, indicating that TgM9 mice had significantly higher (23.29±3.2) compared to WTs (4.24±0.07) in CAC. Together, these results indicate that in CAC epithelial derived- MMP9 maintained crypt architecture, mucosal layer integrity and associated with fewer dysplasia and increased apoptosis compared to WTs.

### *2.3.3 TgM9 mice exhibited altered protein expression of active Notch1, p53, p21 $^{WAF1/Cip1}$ and Cyclin E*

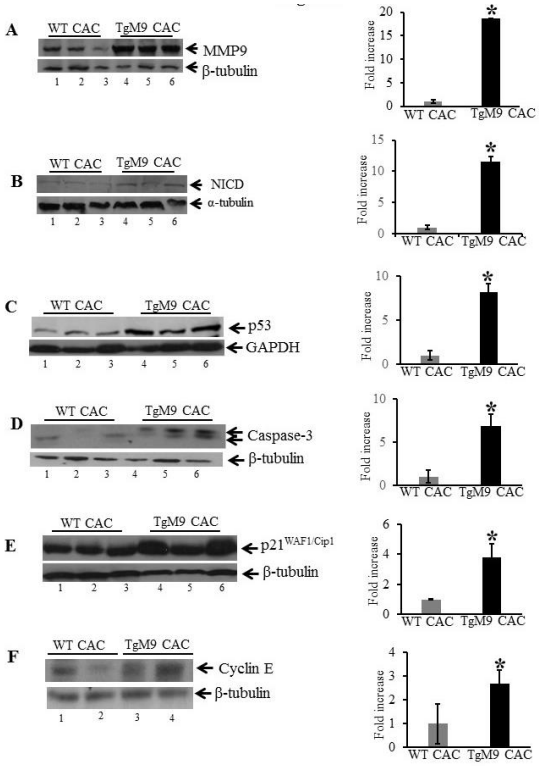


**Figure 5. Overexpression of MMP9 in colonic epithelium exhibited altered protein expression of NICD, p53, caspase-3, p21 $^{WAF1/Cip1}$ and Cyclin E.**
WBs of protein (25µg/lane) from mucosal stripping of the TgM9 and WTs with CAC were performed and probed with (A) anti-MMP9; (B) anti-NICD; (C) anti-p53; (D) anti- Caspase-3; (E) anti-p21WAF1/Cip1 and (F) anti-Cyclin E. The loading control for each blot was β-tubulin or GAPDH. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *$p< 0.05$.

Our next step was to dissect the mechanistic pathway by which epithelial derived-MMP9 mediates protection by exhibiting increased apoptosis in CAC. It is a well-recognized fact that

p53 is the most common tumor suppressor gene which is mutated in almost all kinds of cancers (Leroy, Anderson et al. 2014). Further, p53 mutations are the first ones to occur in CAC (Cooks, Harris et al. 2014). We performed western blot (WB) analysis to investigate the levels of protein expression of wild-type p53 (non-mutated) in association with MMP9 overexpression in colonic epithelium. We observed increased apoptosis through TUNEL staining hence we wanted to explore apoptosis associated proteins. There was a significant increase in the protein expression of MMP9 among TgM9 mice (Figures 3A, lanes 4-6) compared to their WT littermates (Figure 3A, 1-3) in CAC. This blot also confirms the authenticity of MMP9 overexpression in our TgM9 mice model. There was also a significant increase in the protein expression of NICD (Notch intracellular domain/ active Notch1) among TgM9 mice (Figures 3B, lanes 4-6) compared to their WT littermates (Figure 3B, 1-3) in CAC. WB analysis of apoptosis associated proteins (Figures 3C and 3D) showed significantly increased protein levels of p53 and caspase-3 (Figures 3C and 3D) among TgM9 mice (lanes 4-6) compared to WTs (Figures 3B and 3C, lanes 1-3 respectively). $p21^{WAF1/Cip1}$ is a well-documented protein involved in cell cycle regulation and is also a downstream target of p53 (Harper, Adami et al. 1993). Therefore, WB analysis was performed to assess the protein expression of $p21^{WAF1/Cip1}$ among TgM9 mice in CAC. Figure 3E shows that there was a significant increase in the protein expression of $p21^{WAF1/Cip1}$ among TgM9 mice (lanes 4-6) compared to their WT littermates (lanes 1-3) in CAC. Cyclin E, which is a cell cycle regulatory protein and forms a complex with $p21^{WAF1/Cip1}$ (Harper, Adami et al. 1993) was also observed to be significantly higher among TgM9 mice (Figure 3F, lanes 4-6) compared to WTs (Figure 3F, lanes 1-3). Each blot was normalized for loading by immunoblotting with housekeeping genes- β tubulin or GAPDH. Supplementary Figure3 represents the protein expressions of MMP9, NICD, p53, Bax1, $p21^{WAF1/Cip1}$ and Cyclin E among TgM9 compared to

WT both exposed to water only (without CAC). These results together indicate that in CAC, epithelial derived- MMP9 is associated with activation of p53. p53 activation may lead to caspase-3 dependent apoptotic pathway and cell cycle cell cycle arrest via activation of its downstream target $p21^{WAF1/Cip1}$.

### *2.3.4 Re-expression of MMP9 in $MMP9^{-/-}$ mouse embryonic fibroblast (MEFs) resulted in increased expression of NICD, p53, $p21^{WAF1/Cip1}$, Bax1, and Cyclin A*

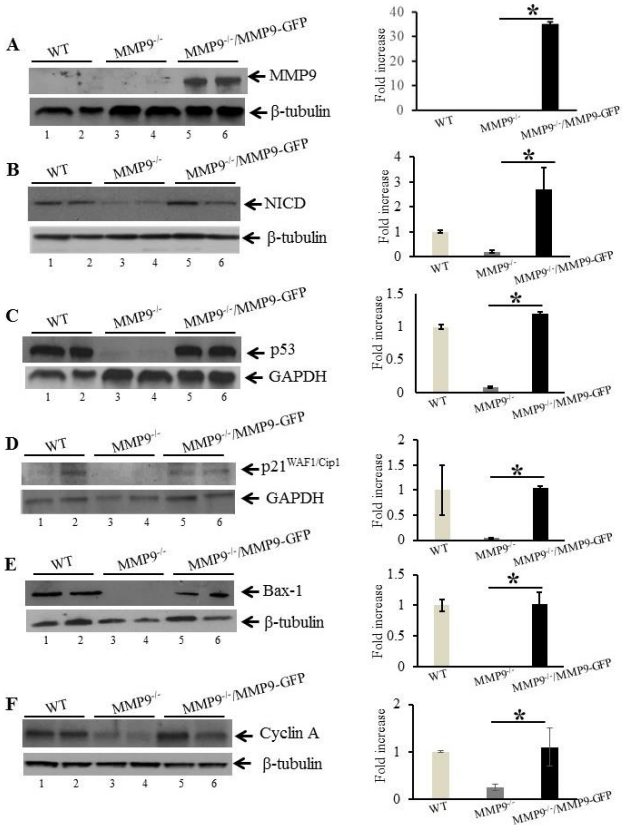


**Figure 6. Overexpression of MMP9 in $MMP9^{-/-}$ MEFs resulted in increased expression of NICD, p53 and $p21^{WAF1/Cip1}$.**

*WBs of protein (25µg/lane) from whole cell lysate of stably transfected MMP9-/- MEFs with and without pEGFP-MMP9 plasmid were performed and probed with (A) anti-MMP9; (B) anti-NICD; (C) anti-p53; (D); anti-p21WAF1/Cip1 (E) anti-Bax-1; and (F) anti-Cyclin A. The loading control for each blot was β-tubulin or GAPDH. Each blot was a representation of three individual experiments. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *p< 0.05.*

As a proof of principle model, to support our hypothesis that MMP9 plays a protective role in CAC and activates p53 resulting in increased apoptosis and cell cycle arrest, $MMP9^{-/-}$ MEFs stably transfected with and without MMP9-GFP plasmids were used and results were compared with WT MEFs. Figure 4A shows the transfection efficiency of the MMP9-GFP plasmid into the $MMP9^{-/-}$ MEFs cells by WB, indicating a significant increase in MMP9 expression (lanes 5-6, slightly higher band due to pEGFP plasmid) compared to vector control (lanes 3-4) and similar to WT MEFs (lanes 1-2). Figures 4B, 4C and 4D displays that re-rexpression of MMP9 in $MMP9^{-/-}$/MMP9-GFP MEFs ($MMP9^{+/+}$) was associated with significantly increased expression of NICD , p53 and $p21^{WAF1/Cip1}$ (lanes 4-6) compared to vector control (lanes 3-4) and similar to WT MEFs (lanes 1-2) respectively. Figures 4E and 4F shows that MMP9 overexpression in $MMP9^{-/-}$/MMP9-GFP MEFs ($MMP9^{+/+}$) was also associated with significantly increased expressions of, pro-apoptotic factor Bax-1 and cell cycle protein Cyclin A (lanes 4-6) compared to vector control (lanes 3-4) and similar to WT MEFs (lanes 1-2) respectively. Each blot was normalized for loading by immunoblotting with housekeeping genes- β tubulin or GAPDH. Taken together, our *in vitro* data using stably transfected $MMP9^{-/-}$ MEF cell line with and without MMP9 indicate that MMP9 activates p53 and $p21^{WAF1/Cip1}$ via Notch1 resulting in increased apoptosis and cell cycle arrest respectively.

### *2.3.5 Overexpression of MMP9 in human colon carcinoma cell line HCT116 displayed decreased cell proliferation and cell cycle arrest in S phase of cell cycle*

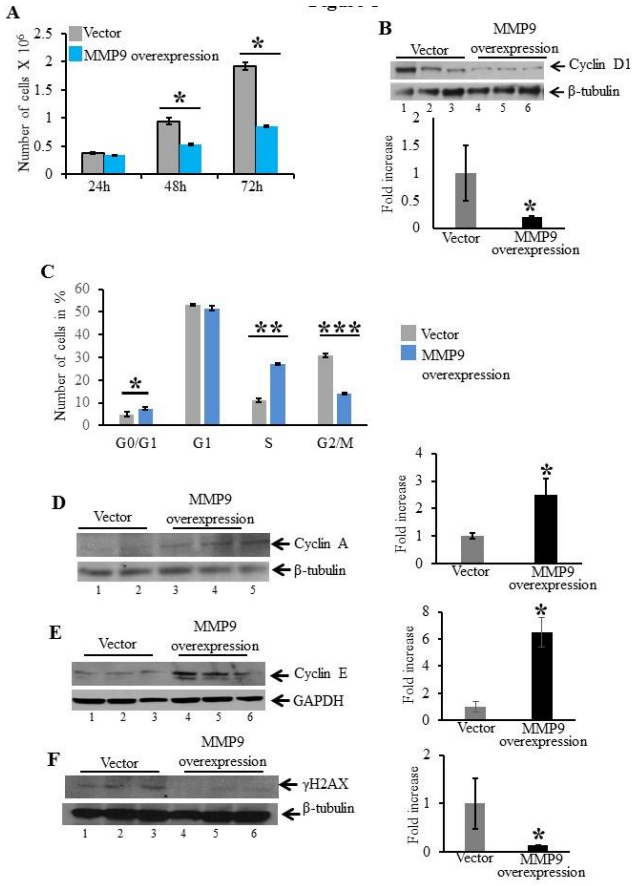


**Figure 7. Overexpression MMP9 in human colon carcinoma cell line HCT116 displayed decreased cell proliferation, initiates cell cycle arrest in S phase and decreased DNA damage**.

(A) The bar graph presentation of the number of stably transfected HCT116 cells with MMP9 (blue) and without MMP9/vector (grey) at 24, 48 and 72 hours respectively. Each bar represents mean ± S.E., *p< 0.005. (B) WB of protein (25µg/lane) from whole cell lysates of stably transfected HCT116 cells with and without MMP9 were performed and probed with anti-Cyclin D1. (C) The bar graph presentation of FACS data showing number of cells in different phases of cell cycle- G0/G1, G1, S and G2/M of stably transfected HCT116 cells overexpressing MMP9 compared to vector control. Each bar represents mean ± S.E., **p< 0.0003 and ***p< 0.0005.

WBs of protein (25µg/lane) from whole cell lysate of stably transfected HCT116 cells with and without MMP9 were performed and probed with; (D) anti-Cyclin A; (E) anti-Cyclin E; and (F) anti-γH2AX. The loading control for each blot was β-tubulin or GAPDH. Each blot was a representation of three individual experiments. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *p< 0.05.

As another mechanistic approach to assess cell proliferation, we determined the role of MMP9 in cell cycle regulation. We used stably transfected colon carcinoma human cell line HCT116 with and without MMP9 (Garg, Jeppsson et al. 2011) without γ-radiation. As described in 'Material and Methods section' equal number of HCT116 cells stably transfected with a pEGFP plasmid with or without the MMP9 gene were seeded in $T25^2$ flask and were counted after 24h, 48h and 72 h. Figure 5A is the bar graph presentation of the number of stably transfected HCT116 cells with and without MMP9, indicating that HCT116 cells overexpressing MMP9 (as indicated by blue bars) had significantly lower number of proliferating cells compared to the vector control at 48 and 72 hours (as indicated by grey bars). Figure 5B indicates that overexpression of MMP9 is associated with significantly lower levels of Cyclin D1 (another marker of cell proliferation (Mamay, Schauer et al. 2001) (Ohta and Ichimura 2000)) (lanes 4-6) compared to vector control (lanes 1-3) respectively. Cyclins are the proteins that are involved in regulating cell cycle progression (John, Mews et al. 2001) (Grana and Reddy 1995). To recognize the link between MMP9 overexpression and cell cycle progression, HCT116 cells with and without MMP9 were stained with propidium iodide and were analyzed for different phases (G0/G1, G1, S and G2/M) by fluorescence activated cell sorting (FACS) (as described in Material and Methods section) technique. Figure 5C displays the bar graph presentation of the percentile of cells in each phase of the cell cycle indicating that HCT116 cells overexpressing MMP9 had a significant cell cycle arrest (28%±0.71) in S phase compared to vector (28%±0.83) resulting fewer (16%±0.71) MMP9

overexpressing cells reaching to G2/M phase compared to vector (29%±0.88). FACS analysis of G0/G1 phase indicated a significant increase in apoptotic cells among MMP9 overexpressing cells (8%±0.62) compared to vector control (5%±0.92). The lower number of apoptotic cells was due to the fact that HCT116 cell line being a carcinoma cell line is apoptosis resistant and requires γ-radiation (12 Gy) to induce apoptosis (Garg, Jeppsson et al. 2011). Figure 5D and 5E shows that MMP9 overexpression is associated with significant increase in protein levels of Cyclin A (lanes 4-6) and Cyclin E (lanes 4-6) compared to vector control (lanes 1-3) respectively. Each blot was normalized for loading by immunoblotting with housekeeping genes- β tubulin or GAPDH. Histone H2AX, a variant form of histone H2A, undergoes phosphorylationon serine 139 of its C terminal tail in response to DNA damage (Scully and Xie 2013) as an early cellular response. Figure 5F displays significant decrease in DNA damage at endogenous levels as indicated by decreased expression of γH2AX among MMP9 overexpressing cells (lanes 4-6) compared to vector control (lanes 1-3). These data together, demonstrate that MMP9 regulates cell proliferation by initiating cell cycle arrest in S phase of the cell cycle, associated with increased expressions of Cyclin A and Cyclin E and also protects from DNA damage at endogenous levels.

### *2.3.6 Attenuation of MMP9 in MMP9$^{-/-}$ mice is associated with decreased expression of p19$^{ARF}$ an upstream regulatory molecule of wild type/non-mutated p53*

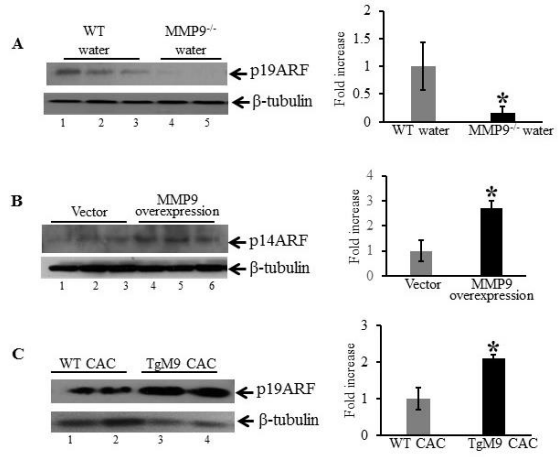


**Figure 8 MMP9$^{-/-}$ mice showed decreased expression of p19ARF an upstream regulatory molecule of p53.**
WB of (30µg/lane) proteins **(A)** from the mucosal stripping of the colons of WT and MMP9$^{-/-}$ mice (n=10 each group) probed with anti-p19ARF; **(B)** from HCT116 overexpressing MMP9 probed with anti-p14ARF (human homologue of p19ARF). The loading control for each blot was β-tubulin. Each blot was a representation of three individual experiments. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *p< 0.05.

It has been well documented that ARFs (alternative reading frame, termed p19ARF in mouse cells and p14ARF in human cells) and MDM2 (mouse double minute 2 homolog) are main regulatory proteins upstream of p53 and regulates its expression (Sharpless and DePinho 1999) (Martelli, Hamilton et al. 2001) (Canepa, Scassa et al. 2007). ARF binds to MDM2 and inhibits its binding with p53 by sequestering it to nucleus. Once sequestered in nucleus it cannot bind to p53 and cannot degrade p53 by ubiquitylation (Khan, Guevara et al. 2004) (Zindy, Williams et al. 2003). To identify the mechanistic link by which MMP9 activates wild type/non-mutated p53, we

used WB analyses of upstream regulatory molecules of p53 and observed that at basal level MMP9$^{-/-}$ mice (Figure 6A, lanes 4-5) compared to WT mice (Figure 6A, lanes-1-3) had significantly decreased protein expression of p19ARF. Figure 6B shows significantly increased expression of p14ARF (human homologue of p19ARF) among stably transfected HCT116 overexpressing MMP9 (lanes 4-6) compared to vector (lanes 1-3). However, at the basal level we didn't observe any changes in the MDM2 expression among MMP9$^{-/-}$ mice (Supplementary Figure S2). We also observed that TgM9 mice (Figure 6C, lanes 3-4) had significantly increased expression of p19ARF compared to WT littermates (Figure 6C, lanes 1-2) in CAC. These results suggest that the tumor suppressive role of epithelial derived-MMP9 is through a novel mechanistic pathway "MMP9-Notch1-ARF-p53 axis" in CAC (Figure 7).

## 2.4 Conclusion

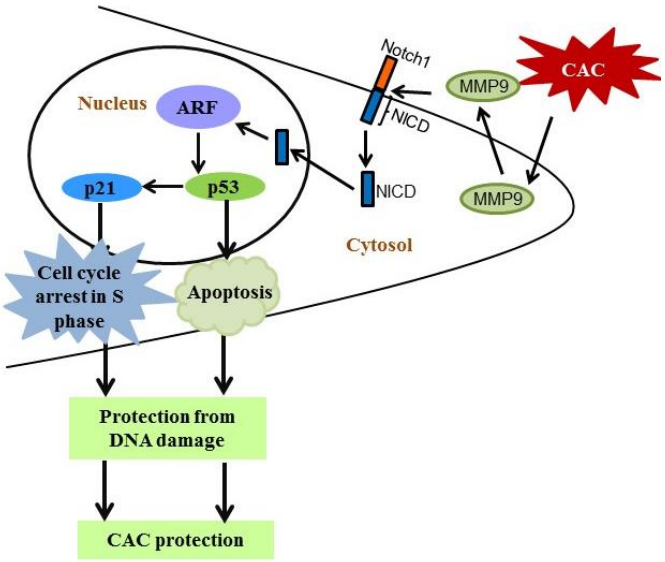


**Figure 9 Schematic representation of "MMP9-Notch1-ARF-p53" mechanistic pathway of epithelial derived MMP9 mediated protection in CAC.**
Abbreviations used are: NICD, Notch1 intracellular domain; ARF, alternative reading frame; CAC, colitis associated cancer.

CAC is chronic inflammation driven carcinogenesis process, though the exact molecular mechanism by which chronic inflammation triggers CAC is still unexplored. Further, in CAC the physiological and pathological progression of inflammation to dysplasia and finally to carcinoma makes it tough to determine the rate limiting step for therapeutic strategies. It has been reported that more than 20% of IBD patients develop CAC within 30 years of disease onset, and less than 50% of these die from CAC (Lakatos and Lakatos 2008, Walter, Harper et al. 2013). Therefore, identification and prognosis of disease risk genes in CAC has been a big hurdle for the efficacy of the therapeutic interventions in CAC.

Individual roles of MMPs with the initiation and perpetuation of intestinal inflammation as well as with the progression of CRC have long been cited in the literature. However, there are few sporadic studies discussing the precise role of MMPs in CAC (Newell, Matrisian et al. 2002), (Garg, Sarma et al. 2010), (Garg, Jeppsson et al. 2011), (Walter, Harper et al. 2013). At the onset of inflammation, similar to the release of chemokines and cytokines, MMP9 is also active and highly up-regulated. Although its role has been well understood and documented in contexts of acute inflammation and CRC individually. However, the precise role of MMP9 has never been studied in the setting of chronic colonic inflammation/CAC. We were the first ones to report the novel protective role of MMP9 in CAC (Garg, Sarma et al. 2010) against its convention role of a mediator of acute inflammation. We have reported that in CAC attenuation of MMP9 gene was associated with increased susceptibility to tumor incidence and tumor burden compared to WTs. We have also shown that MMP9$^{-/-}$ mice had significantly higher proliferation and lower apoptosis. It has been known that epithelial cells and neutrophil cells are the most important cells involved in secreting MMP9 in any tissue/organ. Therefore, to understand the precise mechanism of tumor suppressive role of MMP9 in CAC, it is very important to know which MMP9 'epithelial-derived' or 'neutrophil-derived' is involved.

In the present study, we used TgM9 mice which can overexpress MMP9 in colonic epithelium (Liu, Patel et al. 2013) to identify the role of epithelial derived MMP9 in CAC. We observed that overexpression of MMP9 in colonic epithelium is associated with lower tumor incidence, and retained crypt architecture and mucosal layer integrity compared to WT littermates indicating that TgM9 mice were more protected from ulceration in CAC. In this study we have also shown that protection from CAC in TgM9 mice was associated with increased apoptosis compared to WTs. To identify the underlying molecular mechanism by which MMP9 mediates

protection in CAC, as *in vivo* model we used TgM9 and WTs and observed that epithelial derived-MMP9 mediates activation of p53 dependent caspase-3 apoptotic pathway as well as modulated the expressions of cell cycle regulatory proteins $p21^{WAF1/Cip1}$ and Cyclin E via Notch1 signaling. Through our 'Proof of Principle' model we verified the direct participation of MMP9 as a tumor suppressive molecule, by using stably transfected $MMP9^{-/-}$ MEFs with MMP9-pEGFP. We observed that by 'knocking in *MMP9* gene' among $MMP9^{-/-}$ MEFs we could restore the protein expressions of NICD, p53, Bax-1, $p21^{WAF1/Cip1}$ and Cyclin A (which otherwise were either absent or low in $MMP9^{-/-}$ MEFs). Our *in vitro* model of human colonic carcinoma cell line HCT116 stably transfected with MMP9 displayed decreased proliferation compared to vector supporting the *in vivo* data of increased apoptosis. FACS analysis indicated cell cycle arrest in S phase among MMP9 overexpressing cells compared to vector control. We also observed increased expressions of cell cycle regulatory proteins (specifically associated with S phase of the cell cycle) Cyclin A and Cyclin E. The most striking and significant data in our eyes were decrease in endogenous levels of γH2AX with MMP9 expression as indicated by our HCT116 *in vitro* model. These results together delineate the underlying molecular mechanism of the protective role of epithelial derived-MMP9 in CAC. Our *in vivo* and *in vitro* data showed direct correlation between MMP9 expression and ARF expression suggesting the mechanistic pathway by which MMP9 regulates p53 expression via Notch1 activation (Figure 7). Taken together the data suggest that epithelial derived-MMP9 acts as a tumor suppressor by activating MMP9-Notch1-ARF-p53 axis which results in increased apoptosis, initiates cell cycle arrest via activating $p21^{WAF1/Cip1}$ as well as keep a check on DNA damage.

p53 is a well-established tumor suppressor that plays an important role in the development of CAC as well as sporadic colon cancers. $p53^{-/-}$ mice are highly susceptible to CAC (Donehower

1996) (Chang, Coudry et al. 2007). Cellular stress signals like DNA damage, UV light and oncogene activation trigger p53 activation and nuclear translocation. The activation of p53 modulates tumor suppression by initiating a transcriptional program that results in regulating apoptosis through caspase-3 activation or promoting cell cycle arrest or senescence. Among cell cycle regulatory proteins that are activated following cellular stress signals, the Cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1/Cip1}$ (also a downstream target of p53) plays essential roles in maintaining cellular integrity by inducing cell cycle arrest. Bimolecular complexes of CDKs and their Cyclin partners send the signals to responder molecules (e.g., $p21^{WAF1/Cip1}$) to move the cell trough growth and division cycle (Harper, Adami et al. 1993). Any damage to a cell's genome induces the activation of $p21^{WAF1/Cip1}$, which thereby blocks the activity of Cyclin-CDK complex halting the cell cycle progression, until the damage has been repaired. Therefore, the cell cycle does not progress and inadvertently stall the copying of damaged DNA sequences.

It has been well documented that ARF is the positive and MDM2 is the negative upstream regulatory proteins of p53. ARF functions as a tumor suppressor by directly binding and interfering with the p53-negative regulator MDM2, resulting in stabilization and activation of p53 (Tago, Funakoshi-Tago et al. 2014). It is worth to mention here that *p19ARF* sequences are intertwined with *p16INK4A* (inhibitor of the CDK4 and CDK6 kinases that plays a role in cell cycle arrest). The INK4a/ARF is the second most commonly altered gene locus in human cancer after p53 (Kanellou, Zaravinos et al. 2008). Since in our study we have observed cell cycle arrest in S phase and p16INK4A is responsible for G1/S phase cell cycle arrest, in future we would also explore the correlation between MMP9 and p16INK4A expressions (Kanellou, Zaravinos et al. 2008). This will help in understanding that the inclination of protection equilibrium is more towards apoptosis or is more for cell cycle arrest.

Genomic instability is critical for tumor progression. Uncontrolled endogenous DNA damage due to physiological cellular processes, chronic inflammation or exposure to carcinogens encourages faster growth of cancer cells over normal healthy cells by compromising regular cellular functions. Genomic instability is therefore directly associated with oncogene activation and inactivation of tumor suppressors (Tian, Gao et al. 2015). Studies have suggested that γH2AX detection is an efficient biomarker in monitoring cancer progression (Mah, El-Osta et al. 2010). Cancer cells typically have increased endogenous γH2AX levels compared to normal cells (Mah, El-Osta et al. 2010) (Sedelnikova and Bonner 2006). The downregulation of H2AX can be governed by the ARF/p53 pathway. Studies have shown that mutations in the ARF/p53 module, have elevated H2AX levels and exhibit accelerated growth (Yoshioka, Atsumi et al. 2015) (Atsumi, Inase et al. 2013). Our study shows that in CAC, MMP9 protects from genotoxicity as indicated by decreased endogenous levels of γH2AX and acts a tumor suppressor.

MMP9 mRNA, protein level and activity are increased in human and animal models of CRC and potentiate colon cancer metastasis (Zucker and Vacirca 2004). In general, metalloproteinase inhibitor as well as inhibitors of gelatinases, decrease colon cancer progression in animal models (Mook, Frederiks et al. 2004). In mice, it has been reported that genetic ablation of MMP9 in $APC^{Min+/-}$ mice resulted in 40% fewer tumors than littermate controls, although tumor size distribution remained unaffected (Sinnamon, Carter et al. 2008). On the other hand, some studies have shown that in breast and colon cancer, MMP9 expression has been correlated with both increased and decreased survival and distant metastasis (Bjorklund and Koivunen 2005). However, in recent years, new protective role of MMP9 has also been reported in CRC. It has been observed that MMP9 hemopexin domain has an inhibitory effect on migration and adhesion of colorectal carcinoma cells (Burg-Roderfeld, Roderfeld et al. 2007). Another study has shown that

reduction of plasma levels of MMP9 in either normal or integrin alpha1-null mice leads to decreased synthesis of angiostatin and consequent increased tumor growth and vascularization (Pozzi, LeVine et al. 2002). MMP9 exhibited protective role against lethal inflammatory mass lesions in mouse colon in absence of plasminogen (Hald, Rono et al. 2011) has also been documented.

In very recent years' protective functions of MMP9 have also been described in other cancers/malignancies. It has been reported that MMP9 promotes liver recovery from ischemia and reperfusion injury (IRI) by activating TGF-β and suggested that it plays dual roles (bad and good) in liver IRI, depending on the stage of the disease (Feng, Wang et al. 2013). Protective roles of MMP9 have also been identified in oral cancers depending on the stage of the disease (Vilen, Salo et al. 2013). MMP9 displayed protective function in chronic kidney disease (Okada, Kawai et al. 2012), lung cancer (Gonzalez-Arriaga, Pascual et al. 2012) and systemic autoimmune disease (lymphoproliferation and lupus) (Cauwe, Martens et al. 2011).

We have shown that in colon MMP9 activates Notch1 (Garg, Ravi et al. 2007) whose signaling is important for cell fate determination, stem cell potential and lineage commitment and importantly in carcinogenesis. Notch1 function is highly context-dependent, and can either be oncogenic (van Es, van Gijn et al. 2005) (Korinek, Barker et al. 1997) (El Khatib, Bozko et al. 2013) or have a tumor suppressor function (Liu, Turkoz et al. 2011) (Devgan, Mammucari et al. 2005) (Talora, Cialfi et al. 2005) (Banerjee, Hernandez et al. 2015). Different studies have shown a link between Notch1 regulation p53 activation, although clear cut association is yet to be established (Licciulli, Avila et al. 2013) (Guan, Gong et al. 2013) (Artavanis-Tsakonas, Matsuno et al. 1995). Once Notch1 signaling is active by releasing intracellular domain of the Notch receptor (NICD), it is then translocated to the nucleus, and forms a complex with transcription

factor proteins forming a multiprotein complex, which directly activates transcription of downstream genes (Artavanis-Tsakonas, Matsuno et al. 1995, Guan, Gong et al. 2013). We hypothesize that Notch1 may mediate tumor suppression by regulating p53 stability.

Our study therefore establishes the unique role of epithelial derived-MMP9 in CAC as a defensive molecule against its conventional role of a mediator of acute inflammation. We have also identified that activation of MMP9-Notch1-ARF-p53 axis is responsible for the two plausible mechanistic pathways- p53 dependent apoptosis or p21 mediated cell cycle arrest in mediating the protective role of epithelial derived-MMP9 and thereby controls or eliminates cells with damaged DNA. Outcome of this study highlights the paradox of using MMP9 inhibitors in current therapies to treat CAC patients and explains the failure of such treatments in the setting of chronic inflammation. Thus, MMP9 can potentially be used in novel therapies in the setting of CAC.

# 3 RESULTS MATRIX METALLOPROTEINASE 9 PLAYS A ROLE IN REGULATING GENOMIC INSTABILY BY REDUCING REACTIVE OXYGEN SPECIES AND SUPPRESSING DNA DAMAGE VIA MISMATCH REPAIR MECHANISM ACTIVATION

## 3.1 Introduction

Inflammatory Bowel Disease (IBD), which includes both Crohn's Disease (CD) and ulcerative colitis (UC), is a chronic inflammatory conditions that affects the gastrointestinal (GI) tract as well as the colonic mucosal (Molodecky, Soon et al. 2012). IBD causes epithelial barrier disruption by affecting major components of the intestinal GI tract such as epithelial cells, goblet cells, immune cells, and intestinal commensal bacterial types (Baumgart and Sandborn 2007, Xavier and Podolsky 2007). CD can affect any site where in GI tract from mouth to anus, mostly the terminal ileum, and UC occurs predominately at the distal end of the colon (Hanauer and Sandborn 2001). These diseases affect approximately 1 to 4 million individuals in the U.S (Baumgart and Sandborn 2007). Acute or short-term inflammation is a beneficial response to tissue damage and pathogenic challenges. However, unregulated inflammation may lead to chronic and malignant cell transformation resulting in cancer(Lynch, Drescher et al. 2014). Thus, individuals with chronically active UC have up to a 50% (depending on population cohort) risk of developing colitis-associated cancer (CAC) (Rogler 2014). Therefore, The risk of developing CAC increases with the length of the disease in correlation with the severity of inflammation.

Although both sporadic colon cancer (CRC) and CAC are colon cancers, several features make CAC distinct from CRC (Walter, Harper et al. 2013). CAC and CRC have different molecular events including initiation, promotion and progression during the various stages of tumorigenesis (Terzic, Grivennikov et al. 2010). The development of CAC is associated with the

progressive stages of dysplasia that is significantly different to the pathway of CRC(Waldner and Neurath 2015). Unlike CRC that arise from adenomatous polyps(Walter, Harper et al. 2013), CAC develops commonly in flat dysplastic tissues in individuals pre-exposed to IBD and polyps are mainly localized in the distal colon region (Terzic, Grivennikov et al. 2010, Walter, Harper et al. 2013).

In CAC, proinflammatory cytokines consistently secrete ROS which stimulates its overproduction within the epithelial cells (Terzic, Grivennikov et al. 2010). The accumulation of ROS from chronic inflammation produces DNA damage which accelerates cancer cell growth over healthy cells (Lakatos and Lakatos 2008, Erie and Weninger 2014). Increased ROS levels lead to DNA damage response (DDR) dysregulation which compromises DNA repair mechanisms (Waldner and Neurath 2015). DDR regulates cell proliferation via cell cycle arrest which protects against the proliferation of defective DNA (Nardella, Clohessy et al. 2011). Therefore, genomic instability from an excess of ROS is directly associated with tumorigenesis (Tian, Gao et al. 2015).

Matrix Metalloproteinases (MMPs) are zinc-dependent neutral endopeptidases that have proteolytic activity against extracellular matrix (ECM) proteins (Sternlicht and Werb 2001, Garg, Sarma et al. 2010). Their targets include proteinases, clotting factors, chemotactic molecules, latent growth factors, cell surface receptors, and cell-cell adhesion molecules that make them able to regulate many biological processes (Cao, Drews et al. 1998, Sternlicht and Werb 2001). MMPs are expressed in the GI tract by epithelial cells as well as immune cells (neutrophils, macrophages, and lymphocytes) during inflammation (Baugh, Perry et al. 1999, Castaneda, Walia et al. 2005). They regulate innate and acquired host defenses by facilitating inflammatory cell recruitment across the epithelium and endothelial basement membrane (Ravi, Garg et al. 2007). MMP9, also known as 92-kDa type IV collagenase or gelatinase B, plays a key role in ECM degradation and

is involved in tissue remodeling (Yabluchanskiy, Ma et al. 2013). It is undetectable in all healthy adult tissues but the most predominantly expressed MMP in the GI tract by epithelial cells and immune cells in several inflammatory diseases such as IBD, asthma, rheumatoid arthritis, nonsteroidal anti-inflammatory drugs (NSAID)-induced gastric ulcers (Bailey, Hembry et al. 1994, Castaneda, Walia et al. 2005, Walter, Harper et al. 2013).

We have previously shown that MMP9 knockout animals were more susceptible to CAC (Garg, Jeppsson et al. 2011). We have also shown that MMP9 activates p53 via transcription factor Notch1 (Garg, Jeppsson et al. 2011, Walter, Harper et al. 2013). Consequently, epithelial not neutrophil derived MMP9 could play a protective role in CAC in contrast to its inflammation mediator function in acute colitis (Liu, Patel et al. 2013, Walter, Harper et al. 2013, Walter, Pujada et al. 2017). Recently, our lab has shown that constitutive expression of MMP9 in the colonic epithelium maintains tight junction and mucosal lining integrity in mice during CAC. However, the role of MMP9 in the regulation of genomic stability remains unknown. The aim of this study is to examine that epithelial derived MMP9 plays a role in genomic stability by DNA damage. In this study, we used MMP9 transgenic (TgM9) mice that constitutively expresses MMP9 in the colonic epithelium under the villin promoter. As "a proof of principle" model, we evaluated stably transfected HCT116 cell lines (expressing wild type p53, a generous gift from Dr. VW Yang, Stony Brook University, NY) with and without MMP9 to analyze genomic stability as an *in vitro* model.

## 3.2 Methods

### *3.2.1 Animal Models*

All animal procedures were performed in accordance with the Guide for the Care of Use of Laboratory Animals from the US Public Health Service and with the approval from the Animal

Care Committee of Georgia State University. As described previously (Liu, Patel et al. 2013), gender matched 8-10 weeks old Transgenic mice with MMP9 overexpression in the colonic epithelium (TgM9) and their wild-type (WT) littermates were obtained while crossing the hetz of TgM9 of C57/B6 background were used in this study by the start of the experimental protocol. Our lab has previously published the extensive characterization of TgM9 (Liu, Patel et al. 2013) . The mice were maintained in a 12-hour dark-light cycle and allowed free access to nonpurified diet pellets and tap water.

### *3.2.2 Colitis Associated Cancer Induction*

TgM9 (n=19) and WT (n=21) were separated into two groups in which one group was induced with CAC and another group was given water only. The CAC group of TgM9 and WT were injected i.p. with 7.6 mg/kg Azoxymethane (AOM, Sigma, St. Louis, MO) on day 0. On day 7, WT and TgM9 mice were exposed to 3% (w/v) Dextran Sodium Sulfate (DSS, MP Biomedicals, Solon, OH), given through drinking water *ad libitum* for 7 consecutive days. On day 14, their water was switched back to regular drinking water. On day 28, their drinking water was again changed to 3% DSS for an additional 7-day cycle of DSS exposure. Afterwards, the mice were returned back to regular drinking water and sacrificed on day 56. Their colons were opened longitudinally. Body weight, stool consistency, and stool occult blood data of all mice were collected during both DSS treatment and recovery phase.

### *3.2.3 Hematoxylin and Eosin Staining*

The swiss rolls of the colon from both mice groups were fixed in 10%-buffered formalin at room temperature and embedded in paraffin. Tissues were sectioned at 6-μm thickness and H&E staining was performed.

### *3.2.4 Generation of in vitro models (Cell Culture and Transfection)*

As describe previously stably transfected HCT116 cell lines (expressing wild type p53, a generous gift from Dr. VW Yang, Stony Brook University, NY) with and without MMP9 overexpression were used to analyze genomic instability (O'Sullivan, Gilmer et al. 2015). They were transfected for 72 h with a pEGFP plasmid with and without the MMP9 gene in 6 well plate. The transfected clones were selected under an antibiotic (Geneticin; GIBCO, Grand Island, NY). These transfected clones were screened for MMP9 expression and the three highest MMP9 expressing clones were selected for HCT116 cell line and were sorted via flow cytometry (BD Biosciences).

### *3.2.5 Reactive Oxygen Species Assay*

Fresh whole colonic tissues were collected from mice in each group and immediately homogenized with cold Phosphate-buffered saline (PBS). Afterwards, the lysates analyzed independently using flurescent commercial kit (Cell Biolabs Inc., San Diego, CA) according to the manufacturer's instructions as described previously (Feng, Yan et al. 2016). This kit uses Dichlorodihydrofluorescin (DCFH) as a florigenic probe. This probe reacts with ROS species and oxidized to fluorescent 2', 7'-dichlorodihydrofluorescein (DCF). Thus, Fluorescence intensity is proportional to the total ROS levels within the sample. The fluorescence was analyzed with a fluorometric plate reader at 480/530 nm

### *3.2.6 Protein Extraction and Western blot analysis*

As described previously (Garg, Jeppsson et al. 2011), for WB analysis, mucosal stripping was obtained from the TgM9 and WT mice with and without CAC induction after the sacrifice. The antibodies used were anti-MMP9 (Cell Signaling, Beverly, MA), anti-γH2AX (Abcam), anti-MLH1 (Abcam), anti-MSH2 (Abcam), anti-PCNA (Abcam), anti-cyclin D1 (Santa Cruz, Dallas,

TX), anti-Cyclin A (Cell Signaling), anti-Cyclin E1 (Santa Cruz), anti-γH2AX (Abcam). Goat anti-mouse secondary antibody (1:3000; Bio-Rad, Hercules, CA) or goat anti-rabbit secondary antibody (1:6000, fAbcam) were used. Densitometry graphs were generated by using image acquisition and analysis software by VisionWorksLS Analysis Software (UVP, Upland, CA)

### *3.2.7 Immunofluorescence staining*

The colons in each group were fixed in formalin and paraffin-embedded. They were washed with 1X PBS (with Tween 20) and then blocked with 3%BSA in 1X PBS for 1 hour at 37˚C. Next, tissues were stained by phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma-Aldrich Corp) for 1 hour at room temperature. After washing with 1X PBS (with Tween 20), tissues were incubated overnight at 4˚C with anti-γH2AX (Abcam, Cambridge, MA), anti-8OHdG (Santa Cruz Biotechnology, Dallas, TX) and anti-SOD1 (Santa Cruz Biotechnology). After washing, tissues were incubated with their appropriate FITC secondary antibody for 1 hour at room temperature. After washing, the tissues were mounted with ProLong Antifade mounting medium (Thermo Fischer Scientific, Waltham, MA) and were analyzed by the fluorescent microscope.

### *3.2.8 Statistical analysis*

As described previously (Viennois, Ingersoll et al. 2016), data are presented as means ± SE. Groups were compared by Student's t-test. P values $<0.05$ was considered statistically significant.

## 3.3 Results

### *3.3.1 TgM9 mice exhibited lower levels of ROS production in CAC*

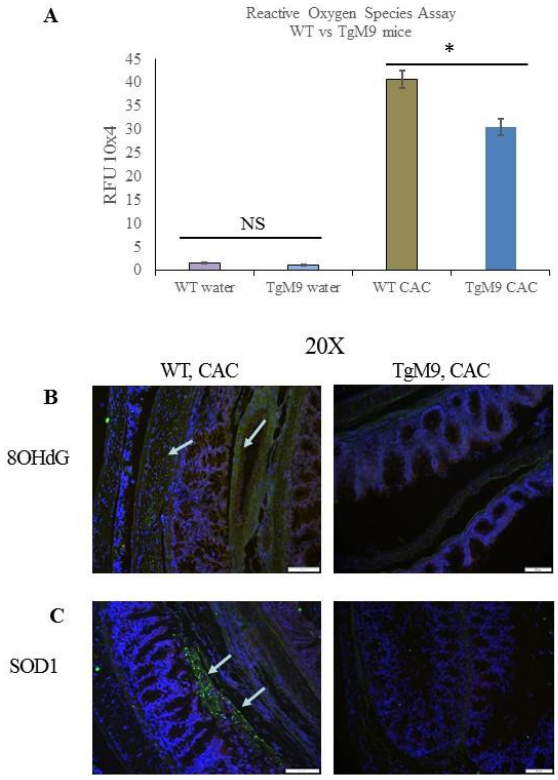


**Figure 10. Constitutive expression of MMP9 in colonic epithelium was associated with decreased ROS production in CAC**
(A)Bar graph of ROS levels that were generated and analyzed through DCF-DA from WT and TgM9 mice with water and CAC. (B) Immunofluorescence staining of the Swiss rolls from WT and TgM9 mice in CAC probed with (B) 8OHdG and (C) SOD1.

TgM9 and their WT littermates were induced with CAC as described in 'Materials and Methods' section and were sacrificed after 56 days. It is well-documented that elevated levels of

ROS due to chronic inflammation produces endogenous DNA damage which accelerates cancer cell growth over healthy cells (Mook, Frederiks et al. 2004). Our previous study has established epithelial derived-MMP9 as a defensive proteinase in CAC via p53 induced apoptosis causing DNA in cells (Walter, Pujada et al. 2017). Thus, we examined if MMP9, being a secretory proteinase, plays an important role in the reduction of ROS levels to maintain genomic stability. Figure 1A shows a significantly lower ROS levels among TgM9 mice compared to WT mice in CAC by using dichlorodihydrofluorescin (DCFH) as a florigenic probe. Next, immunofluorescence was performed using two markers of oxidative DNA damage 8OHdG (Figure 1B), a modified base that occurs in DNA due to attack by hydroxyl radicals that are formed as byproducts and intermediates of aerobic metabolism and during oxidative damage, and SOD1 (Figure 1C), a superoxide dismutases responsible for destroying free superoxide radicals in the body by converting superoxide radicals to molecular oxygen and hydrogen peroxide, were used to validate MMP9 overexpression reduces the amount of ROS in CAC (Sea, Sohn et al. 2015). The results show TgM9 mice exhibited less amount of both 8OHdG and SOD1 ROS markers within the colonic epithelium during CAC. Together, these results indicate that in CAC epithelial derived-MMP9 expression is associated with a significantly lower levels of ROS/oxidative stress compared to WTs littermates.

### *3.3.2 MMP9 overexpression suppresses the amount of DNA damage in CAC*

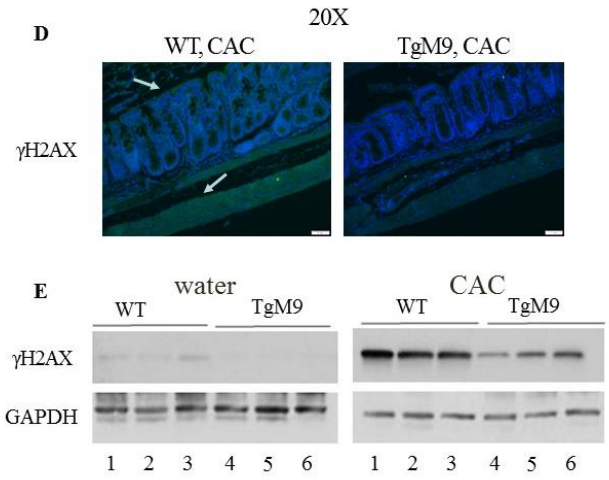


**Figure 11. Constitutive expression of MMP9 in colonic epithelium was associated with decreased DNA damage in CAC.**
(A)Immunofluorescence staining of the Swiss rolls from WT and TgM9 mice in CAC probed with γH2AX as indicated by white arrows. (B) WB of protein (25 µg/lane) from mucosal stripping of TgM9 and WTs with water and CAC was performed and probed with anti-γH2AX. The loading control for the blot was GAPDH. Densitometry evaluations of the WB is representing by the adjacent bar graph. Each bar represents mean ± S.E., *p< 0.05. Microscopic images were taken at 20X magnification.

DNA consistently undergoes oxidative damage by oxygen free radicals which are produced inside the cell as a result of ROS production (Sea, Sohn et al. 2015). This causes the formation of double-strand breaks (DSBs), which triggers tumorigenesis, which leads to the phosphorylation of the histone variant H2AX (γH2AX) (Podhorecka, Skladanowski et al. 2010). Next, we investigated the amount of γH2AX in TgM9 mice as compared to its WT littermates. First, we performed an immunofluorescent staining using γH2AX to examine the amount of DNA damage present within the colon during CAC. The immunofluorescence staining showed a significantly higher presence of γH2AX (Figure 12A) as indicated by white arrow within WT compared

TgM9 mice during CAC. Next, western blot analysis was done to validate the results of our immunofluorescent staining. This also showed a significantly lower amount of γH2AX in TgM9 mice (12B lanes 4-6) as compared to WT mice (Figure 12B lanes 1-3) within both water and CAC conditions. Taken together, our results suggest epithelial-derived MMP9 plays a protective role in CAC by reducing the amount of DSBs in the colonic epithelium.

### *3.3.3 TgM9 mice exhibited an increase expression of mismatch repair proteins in CAC*

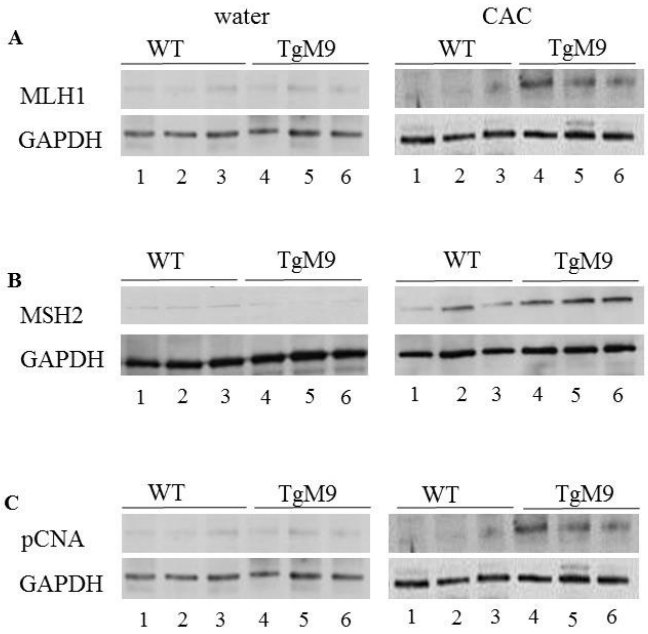


**Figure 12. TgM9 mice exhibited increased protein expression of MMR proteins MLH1, MSH2, and pCNA**
WBs of protein (25/μg/lane) from mucosal stripping of the TgM9 and WTs with CAC were performed and probed with (A) anti-MLH1; (B) anti-MSH2 and (C) anti-pCNA. The loading control for each blot was GAPDH. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *p< 0.05.

ROS downregulates MMR proteins by NO-induce upregulation of DNA methyltransferase which results in cytosine base methylation of MLH1 as well as silencing the promoter and loss of gene expression at the protein level and displacing the MSH2/MSH6 dimerization (Bridge, Rashid et al. 2014). In CAC, MMP9 may play a significant role in reducing genomic instability by potentially regulating MMR genes (Garg, Sarma et al. 2010). Thus, our next step was to examine the protein expression of MMR proteins with and without MMP9 overexpression in the colonic epithelium in both basal and CAC conditions. The western blot analysis of mismatch repair associated proteins (MMR) showed a significant increase of MLH1 (Figure 13A lanes 4-6) and pCNA (Figure 13C lanes 4-6) in TgM9 mice during CAC compared to WT mice (Figure 13A lanes 1-3 and Figure 13C). Also, Figure 13B showed that TgM9 mice had an increase of MSH2 expression (lanes 4-6) as compared to WT mice (lanes 1-3). Each blot was normalized for loading by immunoblotting with housekeeping genes GAPDH. Taken together, our results indicate epithelial derived MMP9 expression promotes the activation of MMR genes which could play a role in the reducing of DNA damage during CAC.

### *3.3.4 Overexpression of MMP9 in human colon carcinoma cell line HCT116 displayed decreased expression of γH2AX and MDC1 and increased expression of MLH1*

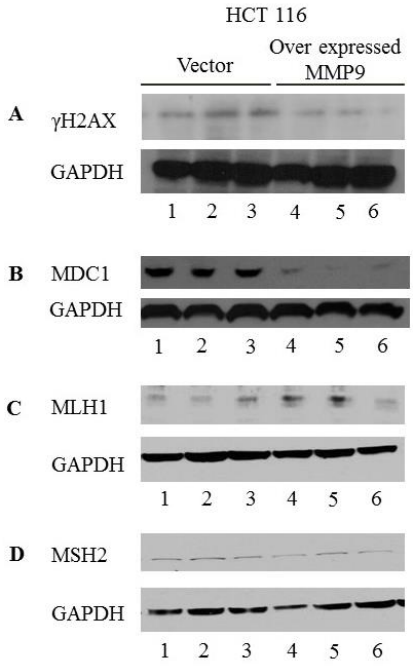


**Figure 13. Overexpression MMP9 in human colon carcinoma cell line HCT116 showed decreased DNA damage and increased MMR protein expression.**
WB of protein (25/µg/lane) from whole cell lysate of stably transfected HCT116 cells with or without MMP9 were performed and probed with; (A) anti-γH2AX; (B) anti-MDC1; (C) anti-MLH1; and (D) anti-MSH2. The loading control for each blot was GAPDH. Each blot was a representation of three individual experiments. Bar graphs are the representation of densitometry evaluations of the western blots. Each bar represents mean ± S.E., *p< 0.05.

The DNA damage response (DDR) is initiated to restore genomic stability from ROS lead mutations. Furthermore, MMR gene are inactivated at the protein level from ROS oxidation during inflammation based carcinogenesis (Colotta, Allavena et al. 2009, Terzic, Grivennikov et al. 2010). Alternatively, to support our hypothesis that MMP9 reduces DNA damage, we used

stably transfected colon carcinoma human cell line HCT116 with (vector) and without MMP9 expression. Figure 3A and 3B show a decrease of DNA damage marker γH2AX and its adaptor protein MDC1 in MMP9 overexpressing cells (lanes 4-6) as compared to vector (lanes 1-3) respectively. We also observed a significant increase of mismatch repair gene MLH1 in MMP9 overexpressing cells (Figure 3C lanes 4-6) than the vector (Figure 3C lanes 1-3). However, Figure 3D shows no significant difference of protein expression of another mismatch repair protein MSH2 between MMP9 overexpressing cells. Taken together, both *in vivo* and *in vitro* data supports epithelial derived MMP9 expression plays an indispensable role in reducing of DNA damage as well as in activating MMR machinery in CAC.

### 3.4 Conclusion

The risk of individuals developing CAC with IBD is generally attributed to UC patients as opposed to individuals suffering from CD (Morson 1966, Nieminen, Jussila et al. 2014). It is reported that individuals with UC have a 2.4-fold increased risk of developing CAC 15 years after acquiring the disease (Jess, Rungoe et al. 2012). The development of CAC is referred to as the *inflammation-dysplasia-carcinoma pathway* in which low to high grade dysplasia in intestinal inflammation leads to subsequent invasive carcinoma (Zisman and Rubin 2008). Thus, the physiological and pathological CAC progression leads to difficulty in the development of therapeutic strategies.

Chronic inflammation in response to an infection or tissue damage eliminates dead cells and promotes tissue restoration by activation of stem and myofibroblast cells, cell proliferation, angiogenesis. However, an excess oxidative stress is a key component of chronic inflammation from innate immune cells that release various ROS into the tissue microenvironment upon activation(Waldner and Neurath 2015). Oxidative damage such as DNA sequence rearrangement, DNA lesions, and activation of oncogenes that play a key role in the initiation

and progression of tumorigenesis (Waris and Ahsan 2006). These mutations in DNA from ROS include oxidized nucleotides, alkali labile sites, single and double strand breaks (Jaruga, Theruvathu et al. 2004). Guanine to Thymine transversion are the most frequent mutations in the p53 tumor suppressor which is the first mutation in CAC (Hollstein, Sidransky et al. 1991). Also, 8OHdG, an oxidative stress-depend base modification, is often found in dysplastic tissue as well as increased concentrations of nitric oxide in IBD tissue (Waldner and Neurath 2015).

Matrix metalloproteinases (MMPs) have known as key mediators of the extracellular matrix (ECM) which promotes and restricts ECM assembly, structure, and quantity (Weber and Saftig 2012, Apte and Parks 2015, Kessenbrock, Wang et al. 2015). Among the 25 known MMPs, MMP9 is unique as it is undetectable in healthy adult tissues but highly expressed in various inflammatory diseases such as asthma, rheumatoid arthritis and nonsteroidal anti-inflammatory drugs (NSAID)-induced gastric ulcers (Bailey, Hembry et al. 1994, Baugh, Evans et al. 1998, Ravi, Garg et al. 2007). Distinctly, MMP9 has three type II fibronectin repeats, containing an elongated linker between catalytic and hemopexin like domain. This domain is essential in binding to denatured collagen or gelatin (Opdenakker, Van den Steen et al. 2001). Our lab has shown that epithelial not neutrophil-derived MMP9 mediates tissue damage during intestinal inflammation and inhibits epithelial cell adhesion, which is required for epithelial cells to migrate and attaches to appropriate matrix after damage (Castaneda, Walia et al. 2005). However, our lab has also reported the tumor suppressor role of epithelial based MMP9 by activating p53, which mediates cell-cycle arrest and apoptosis, via Notch 1 signaling in CAC (Walter, Harper et al. 2013, Walter, Pujada et al. 2017). We have also shown that $MMP9^{-/-}$ mice exhibited lower amount of apoptosis with significantly more cell proliferation (Garg, Sarma et al. 2010). Our previous studies of inhibiting Notch1 signaling using difluorophenacetly-L-alanly-S-phenylglycine t-butyl ester

(DAPT) as a pharmacological blocker displayed decrease protein expressions of p53, p21, and activated caspase-3 in WT mice further validated our research findings (Garg, Jeppsson et al. 2011). MMP9 is secreted by both epithelial and immune cells in various tissues during inflammation. Recently, our lab has shown epithelial derived MMP9 also preserves the epithelial barrier and sustains the mucosal lining of the colonic epithelium in CAC (ADANI paper). Thus, it is important to understand the effects of epithelial derived MMP9 being a tumor suppressor in CAC.

In this study, we used TgM9 mice that constitutively express MMP9 in the colonic epithelium (Liu, Patel et al. 2013), to examine the role of epithelial derived MMP9 in maintaining genomic stability during CAC. We observed a significantly decreased amount of ROS in mice that exhibited constitutive expression of MMP9 in the colonic epithelium compared to their wildtype littermates. We have also shown less genomic instability from CAC in TgM9 by a significant decrease in DNA damage as well as activation of Mismatch Repair mechanisms compared to WTs. Our *in vivo* model demonstrated lower levels of ROS in TgM9 mice using the florigenic probe dichlorodihydrofluorescin (DCFH) as well as lesser amounts of ROS damage markers 8OHdG and SOD1 present in the colonic epithelium compared to their WT littermates. The TgM9 also displayed a significant decrease in protein levels of γH2AX and an increased protein expression of mismatch repair mechanisms as opposed to their WT littermates. Our *in vitro* model of human colonic carcinoma cell line HCT116 stably transfected with MMP9 shows a decrease of γH2AX and its adaptor protein MDC1 as well as an increase of MLH1 as compared to vector which supports the *in vivo* data of decreased DNA damage and MMR activation. Taken together, these results show epithelial derived MMP9 maintains genomic stability during CAC. Our *in vivo* and *in vitro* data showed direct correlation MMP9 expression and γH2AX reduction and MLH1

activation indicating mechanisms MMP9 promotes genomic stability by DNA damage reduction and MMR activation. Overall, the data suggest that epithelial derived-MMP9 maintains genomic stability minimizes the amount of ROS in the colonic epithelium which reduces the amount of DNA damage and allows for the activation of MMR mechanisms in CAC.

The DNA damage response (DDR) is activated to repair RONS-induced mutations which comprises genomic integrity. It regulates cellular proliferation from cell senescence activation, an irreversible cell cycle arrest which provides protection against defective DNA amplification and mutation (Nardella, Clohessy et al. 2011). Also, inflammation induced carcinogenesis leads to MMR gene repression allowing for ROS to oxidize the inactivated mismatch repair genes at the protein level (Colotta, Allavena et al. 2009, Terzic, Grivennikov et al. 2010). For instance, MLH1 expression is inhibited in enterocytes via histone deacetylase and epigenetic repression of the Mlh1 promoter via DEC-1 mediation within $Gi\alpha2^{-/-}$ mice which spontaneously develop inflammation and cancer in the colon (Edwards, Witherspoon et al. 2009). Genetic mutations of MLH1 are also found within patients suffering from Lynch syndrome, which is a hereditary non-polyposis colon cancer (HNPPC) (Heinen 2014). These mutations have been identified in roughly 50% of individuals with Lynch syndrome (Heinen 2014). Our data suggest MMP9 expression plays a role in genomic stability within the colonic epithelium which represses the oxidation of MLH1 from ROS during CAC.

Our study focuses on the novel role of epithelial derived-MMP9 in CAC as playing a protective role instead of its conventional role as a mediator or acute inflammation. We have established that epithelial derived MMP9 expression plays a role in maintaining genomic stability in CAC by reducing the amount of ROS, DNA damage, and the potential regulation of MMR genes. Furthermore, our study highlights the shortcomings of MMP9 inhibitors in recently used

therapies to treat CAC patients. Thus, our proposal of the protective role of MMP9 in CAC will be able to extend research to the clinical practice by establishing the inhibition of MMP9 should be avoided in chronic inflammatory conditions. Also, this study underlines the importance of avoiding humanized versions of MMP9 inhibitors or neutralizing antibodies for CAC patients. Understanding this defensive role of MMP9 in CAC is an important way to improve human healthcare costs as being a helpful proteinase.

## 4 CONCLUSIONS

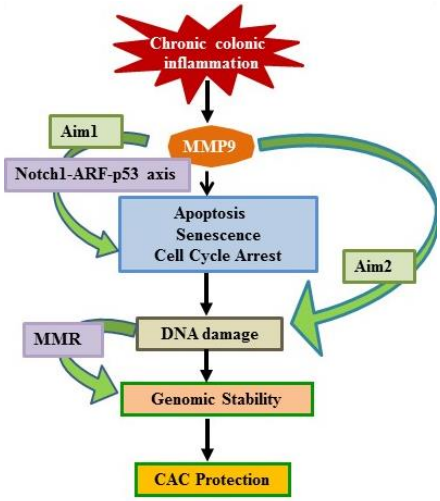


**Figure 14. Schematic Overview of Epithelial derived MMP9 plays a protective role during CAC**

Inflammatory bowel diseases (IBDs) are incurable, chronic inflammatory conditions of the gut and disproportionately affect highly industrialized regions such as North America and Europe, making them a growing, worldwide concern (Molodecky, Soon et al. 2012). It is well-known that chronic inflammation predisposes tissues to oncogenic mutations in its constituent cells, and in the gut this manifests as CAC. My lab has shown MMP9 is highly expressed in

chronic inflammation and MMP-9$^{-/-}$ models to have increased susceptibility to CAC (Garg, Sarma et al. 2010). Understanding this role of MMP9 will assist in the drive for treatments to prevent carcinogenesis in chronically inflamed tissues. My overall hypothesis is that epithelial derived MMP9 mediates tumor suppression in CAC via activation of Notch1 and regulating genomic stability. Thus, our hypothesis was tested with two aims, which could 1) significantly impact future efforts to target key cells or gene pathways important to treat CAC development/severity, and 2) bring an unprecedented specificity of treatment to cancer prevention in IBD patients.

In CAC, p53 is the first oncogenes to be mutated (Ullman and Itzkowitz 2011). Wildtype p53 plays an important role of tumor suppressor in regulating cancer progression. In normal cells ARF (alternative reading frame, termed p19$^{ARF}$ in mouse cells and p14$^{ARF}$ in human cells and an upstream regulator of p53), binds with active Mdm2 molecules, resulting in increased levels of non-mutated p53. In the absence of ARF protein, Mdm2 binds with non-mutated p53 and degrades it by ubiquitination (Zindy, Williams et al. 2003). Hence suppression of ARF function by oncogenic signaling pathways favors tumor formation. The activation of non-mutated p53 modulates tumor suppression by initiating a transcriptional program that results in regulating apoptosis or promoting cell cycle arrest or senescence. Thus, we investigated the tumor suppressive role of epithelial derived-MMP9 by identifying a novel mechanistic pathway "MMP9-ARF-p53 axis" via Notch1 activation in CAC. Our results showed thickening of the mucosal layer due to inflammation and number of polyps were significantly lesser among TgM9 mice compared to WTs in CAC. Also, TgM9 displayed slightly fewer dysplastic lesions as compared to their WT littermates in CAC. Thus, the results indicate that villin-MMP9 mice express functional MMP9 in the colonic mucosa which was more protected against neutrophil

infiltration and tumorigenesis in CAC compared to WT littermates which is the same as CAC patients. Furthermore, western blot analysis to show an increased expression of activated Notch1 and p53 within TgM9 vs. WT mice. In response to oncogene activation, $p14^{ARF}$ can activate p53 expression and is a key negative regulator of the E3 ubiquitin ligase, MDM2, that targets p53 acetylation(Reisman and Loging 1998).

We have also shown an increase in apoptosis in TgM9 mice with CAC as the two regulatory cellular functions to remove damaged or abnormal cell as well as a significant amount of cell cycle arrest with HCT116 cells overexpressing MMP9 as it is predicted to so during CAC. The bimolecular complexes of CDK and their Cyclin partners send the signals to responder molecules, such as $p21^{WAF1/Cip1}$, to move through cell growth and division (Harper, Adami et al. 1993). Among the cell cycle regulatory proteins activated following cellular stress signals, we have shown $p21^{WAF1/Cip1}$, which is a downstream target of p53, was increased during the overexpression of MMP9 in CAC which is a Cyclin-dependent kinase (CDK) inhibitor that plays a significant role in maintaining cellular integrity. There was also increased expression of Cyclins A and E during MMP9 overexpression as well as cell cycle arrest occurring in the S phase in HCT116 cells overexpressing MMP9. These results together should outline an underlying molecular mechanism of the protective role of epithelial-derived MMP9 by mediating cell cycle arrest and apoptosis in CAC. Thus, epithelial derived MMP9 has a defensive role in inflammation-associated cancer colorectal cancer by activating the MMP9-ARF-p53 axis.

It is well-documented that an excess of reactive oxygen species and nitrogen compounds (RNOS) from chronic inflammation produces endogenous DNA damage which accelerates cancer cell growth over healthy cells (Mook, Frederiks et al. 2004). Therefore, genomic instability is directly associated with tumorigenesis (Tian, Gao et al. 2015). MMP9 being a

secretory proteinase may play an important role in the down-regulation of genomic instability. Thus, I examined if MMP9 plays a defensive role from genotoxicity by detecting endogenous levels of initial DNA repair sensors (γH2AX) and regulation of initial mismatch repair genes in CAC. The immunofluorescent staining results will reveal TgM9 CAC mice to have a decrease in γH2AX expression as opposed to the WT CAC mice. Western blot analysis confirmed an increased expression of the initial DNA damage repair (DDR) sensor γH2AX and MDC1 (its adaptor protein) within WT CAC mice as opposed to TgM9 CAC mice. These results were support by using γ-radiated HCT116 and MEF models to support the *in vivo* outcomes. These results indicated that MMP9 protects from genotoxicity by decreased endogenous levels of γH2AX in CAC.

The DNA mismatch repair (MMR) pathway and human cancer are been linked together since MSH2 mutations were discovering in patients with hereditary non-polyposis colon cancer (Fishel, Lescoe et al. 1993, Heinen 2014). MMP9 may also play important role in down regulation of genomic instability by potentially regulating MMR genes in CAC (Garg, Sarma et al. 2010). Thus, I investigated the role of MMP9 in suppressing DNA damage due to activation of MMR genes. Western blot analysis exhibited an increase of MLH1, MSH2, and pCNA outcomes in TgM9 mice as compared to WT mice during CAC. MMP9 decreases DNA damage by increasing expression of MMR genes during CAC. *In vitro* models mentioned above were used to support by the *in vivo* data. Taken together, these results indicate that overexpression of MMP9 will play a defensive role in CAC by reducing ROS and activating the MMR pathway. Thus, epithelial derived MMP9 mediates tumor suppression via activation of Notch1 and regulating genomic stability in Colitis Associated Cancer (CAC).

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