THE CONTRIBUTION OF INFLAMMATORY PATHWAY SIGNALING AND MICRORNA CHANGES TO COLON CANCER PROGRESSION

by

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SIGNED: Benjamin Chidi Onyeaguch
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DEDICATION

In loving memory of my grandmother Chimezie Onyeagucha and all who died from cancer, it is my goal to work and hope that on-going research in cancer will lead to improved diagnosis and development of effective therapeutic strategies.

This dissertation is dedicated to my father Obinna Amanze Onyeagucha and mother, Agnes Chiatogu Onyeagucha. Their enormous encouragement and love made this dissertation possible. Special dedications to my brothers, Iyke, Okey, James, Uche, Joe Onyeagucha, and sisters, Joy and Chinma for their support and love. Also, I dedicate this work to my loving wife Ijeoma Chika Onyeagucha and my children for all their love and supports.
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ABSTRACT

Inflammation and aberrant microRNAs expressions promote colon cancer growth and progression. However, the molecular mechanisms that link these pathways remain to be determined. In this dissertation, the causal relationship between inflammation and aberrant microRNAs expressions were explored.

Elevated expression of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) receptor EP4 has been seen in human colon cancer. However, the mechanism by which EP4 receptor protein is deregulated is not known. Experiments in this dissertation demonstrate, for the first time, that the EP4 receptor is negatively regulated by miR-101.

In previous work, we show that S100P is induced by stimulation of the PGE\textsubscript{2}/EP4 receptor signaling pathway. S100P is a ligand for Receptor for Advance Glycation End-products (RAGE). However, little is known about the downstream targets of S100P/RAGE signaling. Here, we demonstrated that S100P/RAGE receptor signaling induces expression of miR-155 via the transcription factor AP-1. In addition, we investigated the genes that are downstream of S100P/RAGE/miR-155 pathway. Our microarrays and bioinformatics analyses identified two novel miR-155 targets, WNK1 and ZNF493 that are down-regulated upon activation of the S100P/RAGE/miR-155 pathway.
Lastly, we investigated whether inhibition of S100P/RAGE signaling pathway would be beneficial as a cancer therapy using methyl-2-acetamidoacrylate (M2AA). M2AA treatments decreased colon cancer cells viability and also suppressed colon tumor growth and metastasis in vitro and also in the CAM assay in vivo. Taken together, our results suggest that modulation of S100P/RAGE signaling by M2AA offers therapeutic potential as anti-metastatic agents.

In summary, this dissertation provides new insights on the molecular events that link inflammation pathways and microRNAs to colon cancer as well as show that therapeutic strategies targeting these pathways could be effective in treatment of neoplasia.
CHAPTER 1:
INTRODUCTION AND BACKGROUND

The Epidemiology of Colon Cancer

Like other types of cancer, colon cancer is defined as uncontrolled proliferation of malignant cells due to genetic and epigenetic alterations. In the past two decades, colon cancer mortality has decreased (Jemal, Simard et al. 2013). Deaths due to colon cancer decreased by 3.9% per year from 2002 to 2006 in men and also decreased by 3.4% per year from 2001 to 2006 in women in United States (Edwards, Ward et al. 2010). This can be attributed to improved understanding about the pathogenesis of colon cancer, as well as, advancements made in the colon cancer diagnosis and treatment. However, colon cancer incidence remains the 3rd highest among other types of cancer and mortality due to colon cancer is the 2nd leading cause of cancer related death in the United States. Approximately 142,820 new cases of colon cancer and nearly 50,830 deaths are reported annually (Howe, Wu et al. 2006; Jemal, Siegel et al. 2010).

There are disparities in colon cancer incidence and mortality rates among African Americans and Caucasian Americans. The African American male and female have the highest incidence and mortality rate of colon cancer cases compared to their Caucasian American counterparts (Alexander, Waterbor et al. 2007; Jemal, Siegel et al. 2008). The incidence from 2000 to 2004 among African American male was 73.0 and female 55.0 whereas Caucasian male and female were at 60.4 and 44.0, respectively (Jemal, Siegel et
Also, 5 year relative survival rates among African Americans patients were lower than Caucasian Americans (Jemal, Siegel et al. 2008). This may be in part, due to the fact that African Americans experience more severe disease as well as poor response to treatment compared to other racial groups. Nonetheless, little is known about the subtle difference between African American and Caucasian American tumor biology.

During the past decades, numerous epidemiological reports have shown substantial variation in the incidences of right-sided and left-sided colon cancer. The variation in location and etiology of tumors often presents different clinical challenges and outcomes. Men have higher incidence of right-sided colon cancer than women (Elsaleh, Joseph et al. 2000). Among 53,801 colon cancer patient, 67% had right-sided colon tumor and 33% had left-sided colon tumor (Weiss, Pfau et al. 2011). Also, patients with right-sided colon cancer were mostly older with a median age of 73 whereas left-sided colon cancer patients were younger with a median age of 69 (Meguid, Slidell et al. 2008; Weiss, Pfau et al. 2011). Right-sided colon cancer displays 20% level of microsatellite instability (MSI) compare to 1% for left-sided (Elsaleh, Joseph et al. 2000). MSI occurs due to loss or impairment of DNA mismatch repair (MMR) genes thus causing frequent mutation on small repeated DNA sequence. Mutation and hypermethylation are the mechanisms of loss of MMR gene. MSI is a key feature in hereditary nonpolyposis colorectal cancer (HNPCC) (reviewed in (Giardiello, Brensinger et al. 2001)). Currently, the proportion of right-versus left-sided colon cancers cases has changed, with a growing incidence of right-sided colon cancer (Meguid, Slidell et al.
Thus understanding the differences in the etiology can lead in the progression of new treatment modalities.

Patients with right-sided colon tumor are more likely to be diagnosed with more advanced stage and the tumors are poorly differentiated. Furthermore, right-sided colon cancer patients are more likely to experience abdominal pain, weakness due to anemia, lethargy and distinct abdominal mass. Left-sided colon cancer patients are predominantly found in patients with adenomatous polyposis coli colon cancer (APC). Patients with left-sided colon cancer often present with constipation and rectal bleeding, in part due to bowel obstructions. Lastly, patients with right-sided cancer are expected to have poor prognosis (Meguid, Slidell et al. 2008) and reduced survival rates for all stages except for stage II (Weiss, Pfau et al. 2011). Yet, left-sided tumor patient displays higher frequency of hepatic and pulmonary metastases while right-sided shows high degree of peritoneal carcinomatosis (Benedix, Kube et al. 2010).

Several factors may contribute to the divergence in the frequency between the right-sided and left-sided colon cancer. The colon developed from two different embryonic regions of the primitive gut (Jacobs, Thompson et al. 2007). The midgut gave rise to the right-side colon whereas the left-side colon originated from hindgut. The residual water from the incoming contents is absorbed in the right-sided colon through haustral shuttling. Lastly, genetic profiling of normal right and left colon indicates major difference in gene expression (Glebov, Rodriguez et al. 2003). More than 165 genes
were up-regulated beyond 2 fold and another 49 more were also up-regulated by more than 3 fold in the normal right sided compared to normal left-sided colon.

**Pathogenesis of Colon Cancer**

Colon cancer arises due to the transformation of normal colonic epithelium by multistep events, overtime. These events comprise both genetic and epigenetic alterations of several crucial genes (Hanahan and Weinberg 2000). Sequential accumulations of genetic and epigenetic alterations overtime are understood to initiate the early events that lead to the transformation of normal colonic epithelium (Hanahan and Weinberg 2000). Although a fixed sequence of events might be preferred during the transformation and progression, the total accumulation of changes overtime are more important than the order of events. The events resulting from the alterations are responsible for changes in tissue architectures. These chronological events can be shown by histological staining (Grady and Carethers 2008). Transformed cells acquire numerous advantages over the surrounding normal cells that include growth, morphological features, migratory and survival potential (Hanahan and Weinberg 2000; Grady and Carethers 2008).

Fearon and Vogelstein proposed a model for series of events leading to colon carcinogenesis. According to Fearon and Vogelstein, colon cancer results from the activation oncogenes or inactivation of tumor suppressors (Fearon and Vogelstein 1990) (Figure 1.1). The oncogene activation confers growth advantage and drives the normal cells transformation. In normal cells, the oncogenes expressions are tightly regulated by
the tumor suppressors that maintain the genomic integrity of the cells. The mechanisms leading to oncogene activation are point mutations, deletions and translocations. On the other hand, inactivations of tumor suppressors occur through mutations, deletions, truncations, epigenetic modifications and viral inactivation. Activation of a single oncogene would not lead to cancer, if there is a normal functioning tumor suppressor present. Both alleles of a tumor suppressor must be inactivated in order for transformation of the cells to occur, according the Knudson two hit hypothesis. Loss of a single tumor suppressor allele can lead to a heterozygous state. In some heterozygous state, the presence of a single normal allele can fail to function adequately and thus leads to the phenomenon termed as halopinsufficiency, a typical example is the retinoblastoma. With the same token, inactivation of tumor suppressor alone would not lead to cancer unless there is an oncogene present to drive the transformation.
Figure 1.1: Molecular events in colon cancer progression
The early events in colon cancer progression include the loss of impairment of APC genes, a known tumor suppressor and up-regulation of β-catenin and/or COX-2 leading to formation of adenoma. The second event includes the activation of k-Ras oncogene which promotes cells proliferation. However, the impairment of p53 results to full transformation of the cells and formation of carcinoma, resulting in them becoming highly metastatic and allowing them to undergo EMT.
Sequential accumulation of genetic alterations that includes the activation of oncogenes and inactivation of tumor suppressors leads to higher propagation of a subset of cells and eventually metastasis. Approximately half of the patients diagnosed with colon cancer will develop liver metastasis (Sheth and Clary 2005), which is the major cause of death in cancer patients and is largely considered incurable due to a lack of effective therapy other than hepatic resection (Elias, Liberale et al. 2005; Jemal, Siegel et al. 2010). Metastasis is a complex multi-factorial and multi-step process which promotes the detachment, migration, and proliferation of malignant lesions from the primary tumor site to distant site (Tse and Kalluri 2007; Gout and Huot 2008).

For successful metastasis to occur the tumor cells must undergo a series of molecular and morphological changes that enable them to break-through the basement membrane, and invade neighboring and distant tissues (Figure 1.2). The process leading to the dissemination of tumor cells from their primary location is known as epithelia-mesenchymal transition (EMT). Tumor cells are polarized, however during EMT, the cells lose their polarization by acquiring mesenchymal phenotype that enables them to become migratory and invasive (reviewed in (Geiger and Peeper 2009)). Preceding this event is the down-regulation of prominent epithelia proteins such as E-cadherin, occludin, claudins, and cytokeratins that help facilitate the EMT process. Beside this, mesenchymal proteins including N-cadherin, vimentin, and tenascin C expressions are up-regulated. Several other important molecular changes also occur during EMT such as the rearrangement of the cytoskeleton to help the transformed cells be able to manipulate
their morphology, so as to facilitate movement through the basement membranes. After the cells have successfully metastasized, they undergo mesenchymal-epithelial transition (MET) where the cells re-acquire their original features. This enables the tumor cells to thrive in their new niches. Most colon cancer cells metastasize to the liver and lungs.
Figure 1.2: Complex multi-nature process of colon cancer metastasis

The primary tumor bed contains a heterogeneous population of cells with different mutation sites. Only a subpopulation of the tumor cells under additional alterations that enable them to undergo EMT. The cells evade the basement membrane and intravasate into the blood vessel, allowing them to travel to the liver and extravasate. In the liver, the cells undergo MET and thrive. The liver is the common site for metastatic colon.
**Role of Inflammation in Colon Cancer Progression**

Inflammation is involved in colon cancer progression. Inflammation is an innate immunological response that occurs in the presence of an antigen or stimuli. Studies have shown that up-regulation of inflammation promotes gene alterations by increasing DNA damage and genomic instability (reviewed in (Schetter, Heegaard et al. 2010)). Risks of acquiring cancer are elevated in patients with inflammatory diseases. A study has linked inflammatory disease to tumorigenesis of colon epithelium (Vaiopoulos, Papachroni et al. 2010). Patients with inflammatory bowel disease (IBD) are at higher risk of acquiring colon cancer (reviewed in (Klampfer 2011). Approximately 15% of all cancers are caused by chronic inflammation (Kuper, Adami et al. 2000). Some of the mechanisms of inflammation driven cancer are well known; however, little is known about the role of inflammation and microRNA (miRNA) in colon cancer progression. During chronic inflammation, intrinsic mediators of inflammatory responses such as pro-inflammatory cytokines that are secreted by the immune cells and reactive oxygen species (ROS) resulting from the activities of immune cells could induce genetic and epigenetic alterations, including point mutations, deletions and methylation events (Coussens and Werb 2002; Chiba, Marusawa et al. 2012). High levels of ROS cause DNA damage, which could result to inactivation of tumor suppressors and/or activation of oncogenes. Elevated level of inflammation plays a role in dysregulation of miRNAs expressions (Rossato, Curtale et al. 2012; Yunta, Nieto-Diaz et al. 2012).
The relationship between inflammation and cancer are intertwined. In order to sustain cancer progression, cancer cells secrete soluble factors like cytokines and chemokines that promote inflammation in the tumor microenvironment (reviewed in (Klampfer 2011)). In tumor microenvironment, inflammatory cells such as tumor-associated macrophages (TAMs) are constantly releasing immune factors to promote cells proliferation (reviewed in (Sica, Allavena et al. 2008; Klampfer 2011)). In addition to this, inflammatory pathways like NF-kappa B (NF-kB) (reviewed in (Klampfer 2011)) are activated by inflammatory molecules and oncogenes such as k-RAS in tumor cells for the production of several pro-inflammatory mediators cytokines. Inflammatory molecules like TNFα have been shown to activate the transcription factors, AP-1 and NF-kappa B in the tumor cells (reviewed in (Terzic, Grivennikov et al. 2010)). Both AP-1 and NF-kappa B regulate genes that control numerous processes including cell proliferation, tumor growth, apoptosis, angiogenesis, tumor progression and invasion. Also, AP-1 and NF-kappa B are inducers of positive signaling loops that increase the production of chemokines and cytokines, thus leading to the constant recruitment and activation of inflammatory cells in the tumor microenvironment (reviewed in (Terzic, Grivennikov et al. 2010)).

Cyclooxygenase-2 (COX-2) Linked in Colon Carcinogenesis

The cyclooxygenase-2 (COX-2) provides a classic example on the role of inflammation in cancer progression. The COX-2 is a rate-limiting enzyme in the synthesis of prostaglandins from arachidonic acid (Rao, Yang et al. 2004). COX-2
catalyzes the conversion of arachidonic acid to prostaglandin (PG) H$_2$. PGH$_2$ is further converted by PG synthase into PGD$_2$, PGE$_2$, PGF$_2$, PGI$_2$ and thromboxane A$_2$ by prostaglandin isomerase (reviewed in (Cha, Solnica-Krezel et al. 2006)). The PG isoforms are pro-inflammatory lipid that plays critical role in cancer progression (Wang and Dubois 2010). COX-2 is readily induced by cytokines, LPS, growth factors and tumor promoters. Induction of COX-2 during chronic inflammation or carcinogenesis leads to aberrant metabolism of arachidonic acid and the subsequently pro-inflammatory prostanoid production (reviewed in (Wang and Dubois 2010)).

Evidences from genetic and pharmacological studies have demonstrated that over-expression of COX-2 promotes colon cancer progression. Up-regulation of COX-2 leads to increased levels of its metabolite products and subsequently causes pleotropic effects that are associated with cancer. Knockout of COX-2 decreases the number of polyps in APC delta 716 “knockout” mutant mice, (Oshima, Dinchuk et al. 1996). Inhibition of COX-2 expression with non-selective COX-2 inhibitor, nonsteroidal anti-inflammatory drugs (NSAIDs) reduces breast (Takkouche, Regueira-Mendez et al. 2008), colon (Chan, Ogino et al. 2007), esophageal (Abnet, Freedman et al. 2009), gastric (Abnet, Freedman et al. 2009), lung (Van Dyke, Cote et al. 2008), and prostate cancers (Jacobs, Thun et al. 2007). Celecoxib, a selective COX-2 inhibitor significantly reduces the size and number of intestinal adenomas in familial adenomatous polyposis (FAP) (Steinbach, Lynch et al. 2000; Arber, Eagle et al. 2006). Taken together, these observations suggest that COX-2 plays a pivotal role in colon carcinogenesis.
Importance of PGE2 in COX-2-Induced Colon Cancer

PGE$_2$ is the most abundant and potent member of the COX-2 derived prostanoids. Several lines of evidence demonstrate the functional importance of overproduction of COX-2 and PGE$_2$ in many human cancers including colon cancers (Brown and DuBois 2005; Chell, Kaidi et al. 2006; Cha and DuBois 2007). Preclinical studies implicated both COX-2 and PGE$_2$ in many of the hallmark characteristics of malignant disease including metastasis (reviewed in (Chell, Kaidi et al. 2006; Cha and DuBois 2007). For example, in vitro studies show that PGE$_2$ stimulates colon cancer cell proliferation, invasion, and resistance to apoptosis (Chell, Witherden et al. 2006; Cherukuri, Chen et al. 2007; Hawcroft, Ko et al. 2007). In addition, in vivo studies demonstrate that PGE$_2$ can drive intestinal tumorigenesis in animal models of colon cancer (reviewed in (Wang and Dubois 2010)).

PGE$_2$ Signaling via EP4 Receptor Promotes Cancer Progression

PGE$_2$ signals are transduced via four G-protein coupled cell surface receptors, termed as EP1, EP2, EP3, and EP4 receptors (Harris, Padilla et al. 2002; Cha and DuBois 2007). The EP1 is coupled $G_q$ and upon activation increases the level of accumulation of intracellular calcium levels. The EP2 and EP4 receptors are coupled to stimulatory $G_{as}$ protein. Ligand binding of PGE$_2$ stimulates intracellular second messenger cyclic AMP (cAMP) levels, which regulates genes expression. Lastly, EP3 is coupled to inhibitory $G_{ai}$, which suppresses cAMP activities. Indications from studies have shown that EP3 receptor has anti-proliferative effect whilst EP1, EP2, and EP4 are considered to
contribute to cell proliferation (Sonoshita, Takaku et al. 2001; Mutoh, Watanabe et al. 2002; Shoji, Takahashi et al. 2004; Kashiwagi, Shiota et al. 2013).

There is a growing appreciation that the EP4 receptor is an important transducer of PGE$_2$ signaling leading to cell invasion and motility during tumorigenesis. Interestingly, constitutive expression of EP4 promotes proliferation and anchorage-independent growth (Ma, Kundu et al. 2006), demonstrating that the EP4 receptor may also be a key regulator of tumor progression. Also EP4 receptor signaling appears to be important for cell movement and motility during progression. Studies in zebrafish show that the EP4 receptor transduces PGE$_2$ signaling to regulate appropriate speed of cell migration during gastrulation, demonstrating that regulation of cell motility by EP4 receptor signaling is evolutionarily conserved (Cha, Kim et al. 2006). PGE$_2$ stimulates the proliferation and motility of LS174T adenocarcinoma cells through the EP4 dependent activation of phosphatidylinositol 3-kinase/AKT signaling (Sheng, Shao et al. 2001). Consistent with this, PGE$_2$ inhibits apoptosis in human Caco-2 adenocarcinoma cells through an EP4 dependent pathway (Leone, di Palma et al. 2007). Furthermore, premalignant aberrant crypt foci formation in EP4 deficient mice following azoxymethane treatment is suppressed compared to the EP4 wild type mice (Mutoh, Watanabe et al. 2002). This study also showed a reduction in colon adenomatous polyp formation in mice wild-type for the EP4 receptor treated with the EP4 receptor antagonist ONO-AE2-227 (Mutoh, Watanabe et al. 2002). Treatment with another EP4 antagonist, ONO-AE3-208, decreased liver metastases after intrasplenic injection of MC26 colon
cancer cells (Yang, Huang et al. 2006). In addition, *in vitro* studies by our group and others indicate that PGE$_2$/EP4 receptor signaling via ERK activation promotes tumorigenic behavior of colon cancer cells (Pozzi, Yan et al. 2004; Cherukuri, Chen et al. 2007). Finally, forced expression of EP4 receptors promotes the tumorigenic behaviors of HT-29 colon cancer cells (Hawcroft, Ko et al. 2007). Also there is a limited amount of evidence suggesting that increased EP4 expression occurs in human colon cancers compared with normal colorectal mucosa (Chell, Kaidi et al. 2006). However, exactly how EP4 expression becomes deregulated in colon cancer remains to be clarified.

**Pro-inflammatory Protein, S100Passociated with Colon Cancer Progression**

S100P, a 95 amino acid protein which was first purified from human placenta with a restricted cellular distribution, is a member of the S100 family of calcium-binding proteins of the EF-hand type (Becker, Gerke et al. 1992). Marked expression levels of S100P have been reported in both primary and metastatic colon cancer lesions (Mousses, Bubendorf et al. 2002; Arumugam, Ramachandran et al. 2006; Wang, Platt-Higgins et al. 2006; Bulk, Hascher et al. 2008; Parkkila, Pan et al. 2008; Chandramouli, Mercado-Pimentel et al. 2010). A number of studies have strongly linked S100P to cell proliferation, invasion, and migration in several cancer types, including colon cancer (Arumugam, Simeone et al. 2004; Arumugam, Simeone et al. 2005; Wang, Platt-Higgins et al. 2006; Fuentes, Nigavekar et al. 2007; Barry, Chelala et al. 2012). S100P is among three signature genes that were shown to promote liver metastasis in an orthotopic mouse model of colorectal cancer (Ding, Chang et al. 2011). The over expression of S100P
is associated with poor prognosis and survival in patients with breast and lung cancer (Beer, Kardia et al. 2002; Wang, Platt-Higgins et al. 2006).

On the contrary, blockage of S100P inhibits colon cancer growth and metastasis, while also improving mice survival (Arumugam, Ramachandran et al. 2006; Jiang, Lai et al. 2011; Arumugam, Ramachandran et al. 2012). Our group has previously shown that S100P expression is regulated by the PGE$_2$/EP4 signaling through cAMP response element-binding protein (CREB) activation in colon cancer cells (Chandramouli, Mercado-Pimentel et al. 2010). S100P is known to bind to the receptor for Advanced Glycation End-products (RAGE), a member of the immunoglobulin superfamily of cells surface molecules. RAGE can also be activated by other ligands, including other S100 family members, to activate the MAP-kinase and NF-kappa B pathways (Arumugam, Simeone et al. 2004; Donato 2007; Fuentes, Nigavekar et al. 2007). S100P activation of RAGE stimulates growth, invasion and migration in colon cancer cells. However, the downstream signaling events in the S100P/RAGE signaling axis remain to be identified.

**The Role of miRNAs in Tumor Progression**

The miRNAs are a class of small noncoding RNA of about 22 base pairs that have emerged as a key player in various cellular and pathogenic processes that includes cellular progression, immunological response, tumorigenesis, invasion and metastasis. For two decades since they were first discovered, miRNAs have emerge as important regulators of gene expressions and implicated in many human cancers (Calin, Liu et al. 2004; Calin
Accumulating evidences are indicating that the dysregulation of miRNAs contributes to the progression of left-sided and right-sided colon cancers (Yantiss, Goodarzi et al. 2009; Earle, Luthra et al. 2010; Schickel, Park et al. 2010; Pavicic, Perkio et al. 2011; Kamatani, Nakagawa et al. 2013). The miRNAs are transcribed by RNA polymerase from their host genes and subsequently processed by the drosha in the nucleus and dicer in the cytoplasm to produce a matured (effector) miRNAs (Figure 1.3). The pairing of mature miRNAs with the mRNAs of their target genes carrying a partially complementary sequence in the 3’-UTR results in translation repression and/or degradation of mRNA and thus leads silencing of correlated genes (Doench and Sharp 2004; Garzon, Marcucci et al. 2010).
Figure 1.3: Schematic representation of microRNA biogenesis

The miRNAs are transcribed by RNA polymerase II from their host gene in an about 75 bp primary miRNAs (pri-miRNAs) that are recognized by drosha and cleaved to produce a pre-miRNAs. Exportin 5 recognized and export the pre-miRNAs from the nucleus to the cytoplasm where it is further process by the drosha into matured miRNAs. The matured miRNAs are incorporated to the RISC complex. Binding of the mature miRNA to the 3’ UTR of mRNA leads to the degradation or translation inhibition of both the miRNAs and their targets.
Some miRNAs play important roles during inflammation and cancer in many different ways. The miRNAs such as miR-155 are critical for proper innate and adaptive immune cells response (reviewed in (Schetter, Heegaard et al. 2010)). Evidence has demonstrated that the expression of several miRNAs are stimulated by LPS and cytokines (reviewed in (Lu and Liston 2009; Schetter, Heegaard et al. 2010)). Other studies have shown that miRNAs such as miR-146 and miR-155 can induce the production of pro-inflammatory cytokines like IL1 and TNF-α (reviewed in (Ochs, Steinhilber et al. 2011)). In adaptive immune response, miR-155 is important for B cell maturation. Many miRNAs are found downstream of inflammatory pathways such as NF-kB, P13K/AKT and MAP-kinase (reviewed in (Ochs, Steinhilber et al. 2011)). In addition, many of these miRNAs contain NF-κB and AP-1 binding elements and thus are transcriptionally regulated by them.

Dysregulation of the expression of miRNAs that regulated genes involved in cellular proliferation, differentiation or apoptosis, has been observed in different human cancers, including colon cancers (Valeri, Gasparini et al.). MiRNAs 199 and 101a can regulate the expression of COX-2 during embryo transplantation (Chakrabarty, Tranguch et al. 2007). However, miR-101 expression is down regulated in endometrial, hepatocellular carcinomas, and prostate cancers (Hiroki, Akahira et al. ; Varambally, Cao et al. 2008; Su, Yang et al. 2009). In addition, decreased miR-101 has been implicated in the over expression COX-2 in human colon cancers (Strillacci, Griffoni et al. 2009). In the present study, we assess whether EP4 expression could be regulated by one or more
miRNAs and whether elevated EP4 expression in human colon cancer could be the result of an aberrant expression of miRNAs.

MiR-155 has been implicated in the pathogenesis of colon cancer as well as other malignancies (Volinia, Calin et al. 2006). Having both oncogenic and inflammatory properties, miR-155 is a prime example of a miRNA that links inflammation and cancer (Tili, Michaille et al. 2011). With respect to colon cancer, elevated levels of miR-155 have been observed in primary colon cancers and metastatic lesions (Shibuya, Iinuma et al. 2010). Elevated level of miR-155 promotes microsatellite instability, which is predominantly observed in HNPCC (Valeri, Gasparini et al. 2010; Svrcek, El-Murr et al. 2013). Although miR-155 has been shown to target transcripts involved in DNA repair (Tili, Michaille et al. 2011), the targets important for the metastatic phenotype associated with colon cancer are not known. Furthermore, the upstream signaling events that regulate the expression of miR-155 in colon cancers cells remain to be elucidated.
**Statement of the Problem**

For over 4 decades, significant knowledge has been gained on events leading to colon cancer pathogenesis. At the same time, great advancement has been made on the treatment of primary and malignant tumors. Despite all these, the incidence and mortality rates of colon cancer remain very high. Colon cancer still ranks the 3rd most frequent occurring type of cancer and the 2nd leading cause of death due to cancer. The role of inflammation on the pathogenesis of cancer is well documented. Inflammatory pathways such as the PGE$_2$/EP4 and S100P/RAGE signaling up-regulation are linked to several hallmarks of cancer. Up-regulation of both or either of these two pathways promotes cell proliferation, invasion and metastasis. However, the downstream targets that mediated the effects of the inflammation are not fully known (Figure 1.4). Identification of these targets is necessary to fully understand the mechanisms associated with colon cancer progression. Furthermore, determining these targets could lead to therapeutic intervention for human colon cancer.

**Overall Hypothesis:**

Aberrant expression of miRNA influences inflammatory pathway signaling events (prostaglandin EP4 and RAGE signaling pathways) during colon cancer progression.

**Specific Aims:**

1. To identify a miRNA that negatively regulates prostaglandin EP4 expression in human colon cancer.
2. To determine whether activation of RAGE by S100P leads to the induction of miR-155 expression.

3. To identify miR-155 target genes in the S100P/RAGE signaling pathway.

4. To determine whether M2AA treatment is efficacious against human colon cancer growth.
Figure 1.4: Proposed model linking inflammatory pathways to colon cancer progression.
CHAPTER 2: MATERIALS AND METHODS

Cell culture and Maintenance of Cell Lines

DLD-1, HEK-293T, LS174T and SW480 cell lines were obtained from the American Type Culture collection (ATCC Manassas, VA). All the cell lines were maintained as previously described (Chandramouli, Mercado-Pimentel et al. 2010; Chandramouli, Onyeagucha et al. 2012). HEK-293T, LS174T, and SW480 cells were cultured in 1X high glucose Dulbecco’s modified Eagle’s medium (DMEM) with sodium pyruvate (Invitrogen) supplemented with 10% fetal Bovine serum (FBS), and 1% penicillin-streptomycin (P/S). DLD-1 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% FBS and 1% P/S. SW480 cells stably transfected with S100P (SW480/S100P) or empty vector (SW480/pcDNA) were maintained in 1X DMEM supplemented with 10% FBS, 1% P/S and 250 µg/mL G418 selections. Also, LS174T cells with stable S100P knockdown (LS174T/ShS100P) or empty vector (LS174T/PLKO1) were maintained in 1X DMEM supplemented with 10% FBS, 1% P/S and 2 µg/mL puromycin. The empty vector or miR-155 sponge transduced SW480/S100P cells were maintained in 250 µg/mL G418 and 2 µg/mL puromycin supplemented medium. DLD-1 and LS174T cells transduced with miR-155 sponge or empty vector were maintained in RPMI 1640 and 1X DMEM (respectively) that were supplemented 10% FBS, 1% P/S and 2 µg/mL puromycin.
Immunohistochemistry

Immunohistochemistry was performed using a monoclonal EP4 antibody or isotype control antibody. Staining for EP4 receptor protein expression on formalin fixed paraffin embedded colon cancer specimens was performed by methods previously described (Doldan, Chandramouli et al. 2008). The intensity of the immunohistochemical signals in each core is graded as (0) negative, (1) weak, (2) moderate, or (3) strong. The proportion of cells staining positive is also evaluated as a percentage of the total cell population. The score is then calculated as the numbers representing intensity multiplied by the percentage of cells stained (Doldan, Chandramouli et al. 2008). Specimens were scored in a blinded fashion by two investigators (M.N. and A.B.)

In situ Hybridization

Digoxigenin-5’ and 3’-labeled LNA (locked nucleic acid) DNA probe was used to detect miR-101 expression (Exiqon, MA). Hybridization of the LNA probe (40 nM) was performed at 45°C in hybridization chamber. After stringent washes, the sections for the chromogenic in situ were blocked for 1 hr and incubated overnight at 4°C with anti-Digoxigenin-AP (ROCHE, CA) and for the fluorescence in situ, sections were incubated with anti-Digoxigenin-HRP (ROCHE, CA). Chromogenic in situ were reacted with BCIP/NBT to visualized miR-101 expression and counterstained with eosin, dehydrated and mounted with Cytoseal XYL mounting media. To visualize miR-101 and EP4 expression in the fluorescence in situ, tyramide-conjugated fluorochromes (NHS-Rhodamine and NHS-Fluorescein) was applied to the slides by performing two tyramide
signal amplification reactions. Slides were stained with DAPI and mounted with ProLong Gold anti-fade reagent (Invitrogen, CA). Analysis and photography was done by Deconvolution microscopy.

**Chorioallantoic Membrane Assay**

The chorioallantoic membrane (CAM) assay was performed as described previously [31, 32]. Five million HCA-7 cells were implanted on 10-day old chicken embryo chorioallantoic membrane (CAM). After 24 h, tumors were treated daily with 10 µM of EP4 receptor antagonists for 4 days or M2AA for 5 days. At the termination of the experiment, tumors were resected, trimmed and weighed. For the M2AA experiment, 5 million SW480 cells were implanted on 10-day old chicken embryo CAM. The cells were allowed to form tumor for 3 days then treated with M2AA 140 µM daily for 5 days. Afterward, the tumor was resected, trimmed and weighed.

**Isolation of Total RNA from Mammalian Cell Lines**

The control and experimental cells were disrupted with RLT buffer. Disrupted cells were passed through Qiashredder columns (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and treated with DNase enzyme to avoid genomic DNA contamination before elution. The isolated RNA concentration was estimation using the Nanodrop (Thermo Scientific, West Palm Beach, FL, USA).
Isolation of RNA from Fresh Frozen Tissue Specimens

Approximately 0.5 cm block was cut out of tissue specimens obtained from the Pathology Department Archive. Each tissue sample was placed in 1 mL of Trizol and minced using a sterile tissue trearor. All instrument for RNA extraction were washed thoroughly first in RNase-Away solution (Invitrogen, Carlsbad, CA) and then in RNase-free water. The tissue sonicator was also washed with RNase-Away and then in trizol reagent before used. After tissue cells were properly disrupted, 100 µL of chloroform was added and the samples were vortexed very well to produce a homogenous mixture. Samples were spun at fun speed at 4°C for 10 minutes to pellet cell debris. The aqueous solution was carefully recovered and mixed thoroughly with equal amount of 70% ethanol (prepared in DEPC-treated de-ionized autoclaved water) and immediately placed in RNease Mini spin columns. Further steps in RNA extraction were performed according to the manufacturer’s directions. Total RNA yield was estimated using Nanodrop (Thermo Scientific, West Palm Beach, FL, USA).

Small RNA Isolation and qRT-PCR Analysis

Small RNA from both experimental and control treated cells were isolated using the mirVana™ miRNA Isolation Kit (Ambion, Austin, Texas, USA) according to the manufacturer direction. Briefly, experimental and control treated cells disrupted with Lysis/Binding Buffer, directly on the plate. The cells were then scraped into the 1.5 mL microcentrifuge tube and miRNA Homogenate Additive was subsequently added. Next, the cells were vigorously vortexed and incubated on iced for 10 minutes. Afterward,
Acid-Phenol:Chloroform was added to the cells and the mixture vortex for 1 minute. Next, the cell mixture was subjected to centrifugation for 5 minutes at 10,000 RPM. The upper phase containing the aqueous layer was removed and placed into a new 1.5 microcentrifuge tube. To immobilize total RNA, one third volume of 100% ethanol was added to the aqueous solution, vigorously vortexed and placed into filter cartridges. The content was centrifuge briefly for 10 seconds with the collection tube in placed, to separate the immobilized total RNA from the small RNA. To immobilize the small RNA, two thirds volume of 100% ethanol was added to the volume of the small RNA then vortexed briefly. The solution was once again placed into a new filter cartridge with the collection tube in place. Immobilized small RNA was separated by centrifuging at 10,000 RPM for 10 seconds. Contaminants were removed through washing with miRNA Wash Solution 1 and Wash Solution 2/3. Finally, small RNA was eluted with 95 ºC RNA –free water. Once again, small RNA concentration was obtained using Nanodrop (Thermo Scientific, West Palm Beach, FL, USA).

Small RNA qRT-PCR Analysis

The isolated small RNAs were subjected to reverse transcriptase reaction using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Pittsburgh PA) reverse transcriptase kit and protocol. Gene specific stem loop primers for miR-155, GTCGTATCCAGTGAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT and U6, GTCGTATCCAGTGAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATATG (IDT, Chicago, IL) were added to the reverse transcriptase reaction mix. The
reaction mixtures were incubated for 10 min at 25°C followed by 50°C for 30 min, and 85°C for 5 min. The cDNA (50 ng) was added to 20 µL reaction mix containing 0.5 µM of forward miR-155 primer, GCGGTTAATGCTAATCGTGAT or U6 primer, GCGCGTCGTGAAGCGTTC (IDT) in 1X Roche Master mix for realtime PCR reaction. The reaction mix were incubated at 95 ºC for 3 minutes, 95 ºC for 10 seconds, 60 ºC for 20 seconds and 72 ºC for 30 seconds. Matured miR-155 transcript levels were determined by comparative Ct method ($2^{-\Delta\Delta Ct}$). The $\Delta\Delta Ct = \Delta Ct$ miR-155$\text{target} - \Delta Ct$ U6$\text{reference}$. The results were normalized using the empty vector or vehicle control.

**Isolation of Total and Nuclear Proteins from Human Cell Lines**

Cells were washed twice with 1X PBS and lysed by adding cell lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Sodium deoxycholate and 1% Protease Inhibitor Cocktail, 1 mM PMSF, 1 mM Sodium orthovanadate, and Sodium Fluoride) to the cells on the plate. Following lysis, the samples were transferred into 1.5 microcentrifuge tubes and centrifuged at 10,000 X g for 10 minutes at 4 ºC. The supernatant were collected and place into a new microcentrifuge tube. Nuclear proteins were isolated with NE-PER extraction reagent (Pierce) according to manufacturer’s Protein concentrations were measured using the Bradford assay (Bio-Rad) instruction.

**Expression and Isolation of Human Recombinant S100P Protein**

The expression and purification of human recombinant S100P protein were performed as previously described (Arumugam, Simeone et al. 2004). Briefly, full-
length human S100P cDNA was cloned into pTrcHis2 vector and chemically transformed into one shot TOP10 competent Escherichia coli (E. coli) cells (Invitrogen). Transformed E. coli cells were cultured on a 270 RPM shaker and at 37°C to $A_{600} = 0.6$, at that point 1 mM isopropyl-1-thio-β-D-galactopyranoside was added and cells were cultured for another 4 hours at 37 °C and 270 RPM. The cells were harvested by centrifuging at 3000 g at 4°C and stored at -80°C for later use. Next, the bacterial cells were sonicated for 13 minutes with cooling to release the proteins. His-S100P and other proteins were then separated from cellular debris using centrifugation. The His-S100P protein isolation was performed under Native condition using Probond resin kit, according to the manufacturer protocol (Invitrogen). In order to avoid purification of nonspecific proteins, the probond resins were washed 4 times with 50 mM Imidazole and another 4 times with 100 mM Imidazole. The His-S100P protein was then eluted with 250 mM Imidazole in native buffer and concentrated using Amicon Ultra-15 (Millipore, Billerica, MA, USA), estimated and then analyzed using SDS-PAGE and Western blot. Purified S100P proteins were stored at -20 °C in 5% glycerol. The proteins from the empty vector-transformed TOP10 cells were used as a control to monitor for protein purity.

**Purification and Isolation of Plasmid DNA**

Plasmids were enriched in TOP-10 chemically competent bacterial cells. Prior to this, the TOP-10 cells were chemical transformed with experimental or vector control plasmid using the heat shock approach. Briefly, competent cells were incubated plasmids on ice for 30 minutes followed by 45 seconds incubation (heat-shock) in a 42 °C water
bath. The cells were placed again on ice for another 2 minutes and SOC medium (Invitrogen, Carlsbad, CA, USA) were added to revive the cells. The chemically transformed cells were cultured for 1 hour on 270 RPM shaker at 37 °C to allow for new cell replication. Cells were then streaked on LB-agar plate that containing the appropriate antibiotic and incubated at 37 °C overnight to allow for growth of the transformed cells. Next, individual colonies collected with inoculated loop and were used to inoculate 5 mL sterile LB broth containing the appropriate antibiotics. The bacterial cells were cultured at 37 °C on shaker overnight. Afterward, 1 mL of the bacterial culture was added to 500 mL of LB medium and cultured until bacterial growth reached 0.6 OD. Finally, plasmid DNAs were extracted using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendation. Purified plasmid DNA was quantified using nanodrop (Thermo Scientific, West Palm Beach, FL, USA).

**Western Blotting**

The same amount of proteins isolated from human colon cancer cell lines were resolved by means of electrophoresis in a 10% SDS-polyacrylamide gel for all proteins blots. Resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membrane by wet electric transfer at 20 volts overnight. After the transfer, membrane was block with 5% non-fat dry milk prepared in 1X TBST buffer (Tris, NaCl, 0.05% Tween-20). Next the membrane was probed with appropriate primary antibody with gentle shaking for 2 hours, followed by 5 minutes gentle washing with 1X TBST
solution. The wash was repeated a total of three times. The membrane was then probed with appropriate horseradish-peroxidase linked secondary antibodies for 1 hour. Again the membrane was washed for 15 minutes three times with 1X TBST. Protein signal was detected by electro chemiluminescence (ECL) reagent as directed by the manufacturer’s (Perkin Elmer, Waltham, MA, USA).

**Antibodies for Western Analysis**

The anti EP2 and EP4 receptors primary antibodies were raised in rabbit and purchased from Cayman Chemical (Ann Arbor, MI, USA). The antibodies were used at a dilution of 1:1,000 in TBST solution containing 5% BSA, 0.1% NaZ. Also, the primary antibodies against c-Fos, phosphor-c-Fos (ser32), c-Jun, phosphor-c-Jun (ser73), NF-kB p65 and phospho-NF-kB-p65 (ser536) were raised in rabbit and purchased from Cell Signaling (Danvers, MA, USA) and prepared at 1:1,000 dilution in TBST solution containing 5% BSA, 0.1% NaZ. Primary antibodies against S100P and GAPDH were raised in Goat and mouse, respectively. Anti-S100P antibody was purchased from R & D Systems (Minneapolis, MN, USA) and used at 1:1000 dilutions whereas the anti-GAPDH antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diluted at 1:1,000 in TBST solution. The antibody against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204) and phospho-IκBα (ser32) were purchased from Cell Signaling and prepared as described above. The secondary antibodies used were anti-rabbit (raised in goat), anti-goat (raised in donkey), and anti-mouse (raised in Donkey). All the secondary
antibodies were purchased from Santa Cruz Biotechnology and diluted at 1:7,000 in 5% milk/TBST solution.

**Pharmacological Study**

Cell lines were seeded in different sized plates in medium containing 10% FBS and incubated overnight. The cells were serum starved in OptiMEM for 24 hours before treatment with indicated concentrations of 10 µM U0126 (Sigma Aldrich, St. Louis MO) for 30 minutes or 0.5 µM CAY10512 (Sigma Aldrich) for 1 hour or different concentration of M2AA (Sigma Aldrich). Experimental controls were carried by treating the cells with the vehicle (water or DMSO).

**Luciferase Assays**

LS174T and SW480 cells were transfected using Lipofectamine 2000 Reagent or TransIT (Mirus Bio LLC, Madison WI) according to the manufacturer’s specification. A total of 1x 10^6 cells were seeded in each well in six-well plate and incubated overnight. For the miR-101 experiment, the cells were co-transfected overnight with pGL3-EPWt, pRL-null and pcDNA3.1-miR-101 or pGL3-EPmut, pRL-null and pcDNA3.1-miR-101. Subsequently, the activity of luciferase was assayed using the Dual Luciferase Reporter™ Assay (Promega, Madison, WI, USA) according to the directions provided. Briefly, cells were first lysed in 1X Passive Lysis Buffer. In a sterile tube, 100 µL of LARII regent (firefly luciferase substrate) was added to 2 – 5 µL of the cell lysate. Using the Sirus, the measurement of the luminescence was obtained. Immediately after this,
100 µL of the Stop&Glo® reagent was added in order to quench firefly fluorescence, rapidly and introduce the Renilla luciferase substrate. The second measurement of the luminescence represents the background luminescence of samples. Promoter activities measure by this method represented as a ratio between the Firefly luciferase over Renilla Luciferase activities. For miR-155 experiment, the cells were miR155HGpromoter/pGL3 and renilla or pGL3basic and renilla. The transfected medium was replaced with fresh optiMEM medium and incubated for 24 hours. Next, the cells were pre-treated with pharmacological inhibitor for MEK (10 µM U0126) or NF-kappa B (0.5 µM CAY10512) for 4 hours. The cells were then subjected to 200 nM human recombinant S100P treatments for 1 hour and later harvested with luciferase 1X passive lysis buffer. The luciferase promoter activities were performed as described above.

**Chromatin Immunoprecipitation**

For chromatin immunoprecipitation, 2 X 10^6 of LS174T or SW480 colon cancer cells were seeded in each well in a six-well plate and cultured for 24 hours. Next the cells were starved with 2 mL 1X OptiMEM medium for 24 hours and then treated with 200 nM of purified human recombinant S100P or vehicle for 1 hour. Cells were then fixed with 1% formaldehyde for 10 min and 0.125 M glycine was added to stop fixing and avoid over fixing of the cells. Next, the cells were washed twice with cold 1X PBS, harvested by spinning down at 1000X g for 1 min, then suspended in cold-RIPA buffer and stored at -80 °C. To fragment the DNA, the cells were sonicated to obtain fragmented DNA of about 600 bp, repeatedly for 10 seconds with 50 seconds cooling for
8 times at 90 amplitude. The lysates were subjected to immunoprecipitation with anti-human antibodies for c-Fos (Cell signaling, catalog number-5348) using the EpiSeeker ChIP kit and protocol (ABCAM). Realtime PCR was performed to assess enrichment of AP-1 at miR155HG promoter using AP-1forward primer – TGTAGGTTCCAAGAACA GGCAGGA and reverse primer - ACTTGTGACTCATAACCGACCAGG; GAPDH forward primer - GCTACTAGCGGTTTTACGGG and reverse primer - TGACTGTGCA ACAGGAGGAG.

**Generation of Stable MiR-155 Knockdown (sponge) Cell Lines**

Transient transfection of retroviral particles and transduction were performed as previously described (Yin, McBride et al. 2008). Briefly, retrovirus particles were generated through transient cotransfection of HEK-293T cells with retroviral expression vectors plus packaging vectors using modified calcium phosphate precipitation procedure. HEK-293T cells were seeded at 2.5 million cells onto 100 mm tissue culture dish that was pre-coated with poly-D glycine (Sigma-Aldrich, St Louis, MO, USA) for 30 minutes and cultured overnight at 37 °C with 10 mL of freshly supplemented high glucose DMEM medium. Later, plasmid DNA was precipitated by mixing 10 μg of retroviral vector, 10 μg of vesicular stomatitis virus G protein expression vector, and 10 μg of pVPack dGI packaging vector with ethanol and 3 M of sodium acetate and then stored at 4 °C overnight. The following day, 30 μL of 2.5 M CaCl₂ was added to re-suspend the DNA. Another round of DNA precipitation was carried out by the addition of 0.5 mL of 2X HEPES-buffered saline (0.5% HEPES, 0.6% NaCl, 0.1% dextrose,
0.01% anhydrous Na$_2$HPO$_4$, 0.37% KCl [pH 7.10]) and mixing. The precipitates were incubated at room temperature for 20 minutes and then added in dropwise fashion to the cells. The cells were cultured at 37°C with 5% CO$_2$ for 20 h before the medium was replaced with 12 mL of freshly supplemented DMEM medium. At 48 hour the viral supernatant was collected, spun for 1.5 minutes at 1000X g and then filtered with 0.45-μm-pore-size surfactant-free cellulose acetate filter. The filtered supernatants were used to infect DLD-1, LS174T and SW480/S100P cells. A day prior to infection, 500,000 cells were seeded on six-well plates and incubated overnight. The medium was changed with fresh supplemented DMEM (10% FBS and 1% P/S). Polybrene® (Sigma-Aldrich, St. Louis, MO, USA) was added to the viral supernatant, as well as, the transfection medium to the final concentration of 4 μg/mL. At this point, the viral supernatant was then added to the cells. After 8 hours, 3 mL of fresh completed medium were added to the cells and cultured for additional 48 hours. The infected cells were selected with 2 μg/mL puromycin.

**Colony Formation Assay**

Transduced miR-155 sponge or empty vector cells were seeded in 100 mm dish plates in the amount of 1000 cells per dish. The cells were then incubated in 20 mL 1X DMEM supplemented with 10% FBS, 1% P/S, 2 ug/mL and incubated at 37 °C and 5% CO$_2$ for 3 weeks. At the end of the incubation, the culture medium was aspirated and colonies were stained in methylene blue dye (0.5% methylene blue and 50% methanol) at
room temperature for 10 minute. The plates were gently rinsed with water and visible colonies counted.

**Motility and Invasion Assay**

Cell motility and invasion assays were performed as previous described (Chandramouli, Mercado-Pimentel et al. 2010; Chandramouli, Onyeagucha et al. 2012). In a 24 well plate, 600 µL cultured medium was added to each well prior to the placement of 8 µm pore Falcon transwell. The cells bearing stable miR-155 knockdown or empty vector were resuspended with culture medium to make a final volume of 100 µL and then seeded into the transwell and cultured for 24 hours or 48 hours at 37 ºC, 5% CO₂. Next, the incubation medium at the upper and bottom chambers was aspirated and the insert placed upside down. The surfaces of the inserts were quickly stained with crystal violet stain (0.5% crystal violet in 20% methanol) for 1 minute at room temperature. The crystal violet solutions on the inserts were removed by gently dipping and rinsing them in distilled water three times. Non-motile cells inside the inserts were removed with wet cotton swap, repeatedly. The inserts were then allowed to air dry overnight. The experiment was repeated one more time. The number of motile cells (cells penetrating through the membrane) and invaded cells (cells that had passed through the membrane) were counted under a light microscope.

**Genomic DNA Isolation from Animal Tissue and Alu qPCR**
Genomic DNA was isolated from harvested chick embryo lung tissues using BD tract™ Genomic DNA Isolation Kit (Maxim Biotech Inc, San Francisco CA) according to manufacturer instruction. The isolated DNA was measured using the nanodrop and subjected to Alu qPCR. Briefly, human Alu sequences were amplified by qPCR using 100 ng of the genomic DNA template in a 20 µL reaction containing Roche SYBER Green master mix and 0.5 µM of forward Alu primer (5’- ACGCCTGTAATCCCAGCA CTT-3’) and reverse Alu primer (5’-TCGCCAGGGCTGGAGTGCA-3’). The qPCR was carried out at 95 °C for 3 minutes, followed by 30 cycles at 95 °C for 10 seconds cycles and 60 °C for 30 seconds and 72 °C for 30 seconds). Each assay included with negative and positive controls.

**Statistical Analysis**

The experiments are repeated at least three times. Results are presented as means of ± S.E.M. Statistical comparisons between two groups of data were made using two-tailed unpaired Student’s t test. A \( p \)-Value of < 0.05 was considered statistically significant. Any \( p \)-Value of < 0.05 is denoted as *, \( p \)-Value of < 0.01 as **, and \( p \)-Value of < 0.001 denoted ***.
CHAPTER 3:
MICRORNA-101 POST-TRANSCRIPTIONALLY REGULATES THE EXPRESSION OF EP4 RECEPTOR IN COLON CANCER

Introduction

Several lines of evidence demonstrate the functional importance of overproduction of COX-2 and one of its major metabolites, PGE$_2$ in many human cancers including colorectal cancers (Brown and DuBois 2005; Chell, Kaidi et al. 2006; Cha and DuBois 2007). PGE$_2$ transduced its signal via the prostanoid receptors, EP1-4. The EP2 and EP4 are mainly responsible for mediating PGE$_2$-driven pro-inflammatory and pro-malignant signals (reviewed in (Menter, Schilsky et al. 2010)). Among these two receptors, activation of EP4 receptor has the most potent effect in promoting cancer. EP4 receptor protein is elevated in cancer however the EP4 receptor mRNA remains unchanged in colon cancer and thus, suggests that EP4 receptor protein expression is regulated at the post-transcriptional level. Nonetheless, little is known about how EP4 receptor expression is regulated in colon cancer. Since miRNAs have been demonstrated to regulate gene expressions at the post-transcriptional levels, we investigated whether a miRNA can regulate EP4 receptor at the post-transcriptional level.
Results

Prostaglandin EP4 receptor expression increases during tumor progression

We first examined EP4 receptor expression in human colon cancer specimens. There were low levels of EP4 protein in normal mucosa located mainly on membrane surfaces (Figure 3.1A). Similar findings were seen in adenomas (Figure 3.1C). In contrast malignant lesions (i.e. adenocarcinoma and metastatic lesions) stained strongly for EP4 receptor expression (Figure 3.1B). EP4 receptor expression was elevated in malignant lesions compared to adenoma and normal colonic epithelium (Figure 3.1C). The lack of staining in the stromal tissue in both normal as well as cancerous lesions indicates specificity of staining to colonic cells. In addition, there was an increased staining in high grade carcinomas (Figure 3.1C). Interestingly, in one case, malignant colon cancer cells that had penetrated into the intravascular space displayed strong EP4 staining (data not shown). However, consistent with previous reports, we did not observe a significant increase in EP4 mRNA in malignant lesions compared to normal tissues (data not shown) (Gustafsson, Hansson et al. 2007). Collectively, these data suggest that elevated EP4 receptor protein expression might occur by post-transcriptional mechanism.
Figure 3.1: Elevated EP4 receptor expression in human colon cancer specimens. Immunohistochemistry (IHC) staining of EP4 receptor protein was performed in human colon tissue specimens. (A) The staining of normal colonic mucosa shows low EP4 receptor protein expression on the apical membrane. (B) Elevated EP4 staining was observed in adenocarcinomas lesions. (C) Differences between IHC scores for normal, adenomatous, and cancerous lesions are shown as a box and whisker plot. The distribution of pair-wise comparisons of differences in EP4 staining scores shows a significant difference in EP4 receptor staining in malignant colonic lesions compared with normal and adenoma lesions (*p < 0.0001). Each dot represents IHC score (intensity of stain X percentage of cells stained) generated after staining formalin-fixed paraffin embedded tissues for EP4 antibody by IHC.
An evolutionarily conserved target sequence for miR-101 binding is present in the 3’-UTR of EP4 gene sequence

The PTGER4 mRNA encodes for the human EP4 protein. To identify miRNA target sites located within the 1.3 kb 3’-UTR of the human PTGER4/EP4 mRNA, predictions from target predictor algorithms were compiled and compared. We searched TargetScan, PicTar, Pita, and miRanda-mirSVR for microRNAs capable of binding to the 3’-UTR of the EP4 receptor. A 100% match at position +18 to +27 in the 3’-UTR was observed in multiple database searches (Figure 3.2A). Comparing the human sequence for interspecies homology, we found that the miR-101 target seed sequence at nt 18 to 27 of the PGTER4/EP4 -3’-UTR is highly conserved among several species including chimpanzee, rhesus, cattle and dog (Figure 3.2B). Because miR-101 has been found to regulate COX-2 in human colon cancer, we focused our studies on miR-101 (Strillacci, Griffoni et al. 2009).
Figure 3.2: Highly conserved miR-101 seed sequence within the PTGER4 mRNA (A) Computational prediction of miR-101 seed region on PGTER4 mRNA. The miR-101 target sequences are located on nucleotides 18–24 in 3' -UTR of PTGER4. The target region is denoted with bold letters. (B) The miR-101 seed sequence is highly conserved across five species.
**EP4 protein expression correlates inversely with miR-101 levels in colon cancer cell lines and resected patient samples**

First, we determined expression levels of miR-101 and EP4 protein in several colon cancer cell lines. In cell lines with low miR-101, as measured by qRT-PCR, there were high amounts of EP4 protein (Figure 2). Conversely, there were low levels of EP4 protein in cell lines with high miR-101 expression. Next, we sought to corroborate our initial findings in colon cancer cell lines by analyzing resected tumors and corresponding normal tissues of 12 patients with colon cancer for EP4 protein (Western) and miR-101 (qRT-PCR) levels. Representative examples are shown in Figure 3. As reported for other tumor entities (Strillacci, Griffoni et al. 2009; Su, Yang et al. 2009), we observed that miR-101 showed a higher expression in normal colonic tissues compared to corresponding colorectal tumor lesions. However, EP4 protein was still observed to be high in colon tumor samples as previously observed by us (Figure 1B) and others (Chell, Witherden et al. 2006). Moreover, on normal and colon cancer tissue sections, *in situ* hybridization revealed expression of miR-101 in normal colonic epithelium and a loss of miR-101 expression in malignant epithelial cells (Figure 4I). The absence of staining with a scrambled probe demonstrates the specificity of the technique (Figure 4). To clarify the clinical significance of the altered miR-101 expression in colon cancer tissue, we performed the sensitive fluorescence-based *in situ* hybridization method together with the routine clinical immunohistochemical assay to visualize the co-expression of miR-101 and EP4 protein in the same tissue section. This data identified malignant epithelial cells expressing significant EP4 levels with low miR-101 levels and cells expressing
higher EP4 levels with no detectable miR-101 expression (Figure 4II. B, C and D), confirming that in the later cells the absence of miR-101 expression has allowed the full expression of EP4 levels and therefore that miR-101 represses EP4 expression. Taken together our data from both colon cancer cell lines and colon cancer tissue support the notion that the EP4 receptor expression is negatively regulated by miR-101 at the post-transcriptional level.
Figure 3.3: Inverse relationship between EP4 receptor protein and miR-101 expression levels in colon cancer tissues

(A) Western analysis characterizing EP4 receptor protein levels in different colon cancer cell lines. (B) Characterization of miR-101 expression levels in various colon cancer cell lines using qRT-PCR. (C) Western analysis of EP4 receptor protein isolated from paired normal (N) and colon tumor (T) tissue specimens. Tumor colon specimens display higher EP4 receptor protein levels. (D) qRT-PCR analysis showing relative levels of miR-101 in paired normal colon and cancer specimens. The miR-101 expression levels of tumors (T) were normalized against their corresponding levels in normal (N) colon tissues. Note, loss of miR-101 in the tumor specimens is associated with increased EP4 protein expression. **p < 0.01.
Figure 3.4: Analysis of miR-101 expression in human colon tissue by in situ hybridization

(A) (I) Paraffin embedded, formalin-fixed non-malignant colon epithelial tissue stained with H&E. (II) Non-malignant colon tissue hybridized with LNA scrambled probe (III) Non-malignant human colon tissue hybridized with LNA miR-101 probe. Note miR-101 expression was observed in normal colon epithelial cells and surrounding tissue. (IV) Colon adenocarcinoma tissue stained with H&E. (V) Colon cancer tissue hybridized with LNA scrambled probe. (VI) Colon cancer tissue hybridized with LNA miR-101 probe. Malignant adenocarcinoma cells show considerably low miR-101 expression levels or none. Images captured at 20x magnification. (B) (I) DAPI-labeled nuclei. (II) miR-101 expression detected with HRP-DIG-labeled miR-101 probe reacted with tyramide conjugated fluorescein. (III) EP4 expression revealed with HRP-EP4 antibody reacted with tyramide-rhodamine. (IV) Co-detection of EP4 and miR-101. Two cell populations are visible in the malignant epithelium, cells co-expressing low miR-101 levels permitting significant EP4 expression (white arrow) and cells that had shut down miR-101 expression allowing high levels of EP4 expression (yellow arrowheads). A third group of cells expressing high miR-101 levels and no EP4 expression is identifiable surrounding the malignant epithelium (white arrowheads). De-convoluted images captured at 40x (water).
The EP4-3’-UTR is a target for miR-101

Given the hypothesis that the EP4 receptor might be a target of miR-101, we asked whether the 3’-UTR of EP4 is a functional target of miR-101. We cloned the miR-101 seed sequence of wildtype-EP4-3’-UTR into a luciferase reporter construct at a position immediately downstream to the luciferase gene (Figure 3.5). In parallel, we generated a second reporter construct in which the conserved targeting region of miR-101 within the EP4-3’-UTR was specifically mutated in a way predicted to abolish binding (Figure 3.5). The relative luciferase activity of the reporter that contained wild-type 3’-UTR was significantly suppressed when pcDNA3.1-miR-101 was co-transfected (Figure 3.5B). In contrast, the luciferase activity of mutant reporter was unaffected by simultaneous transfection of pcDNA3.1-miR-101 (Figure 3.5B). Similar findings were observed with a EP4-3’-UTR in which seed sequence was specifically deleted (data not shown). Furthermore, we examined the effect of miR-101 on the endogenous expression of EP4 by transfecting pcDNA3.1-miR-101 plasmid for long periods of time and demonstrated a time-dependent decrease in the endogenous EP4 receptor protein levels in colon cancer cell lines (Figure 3.5C). These data indicate that miR-101 may regulate the expression of the EP4 receptor at the posttranscriptional level by directly targeting the 3’-UTR sequence of the PTGER4/EP4 mRNA.
Figure 3.5: The EP4 receptor is a direct target of miR-101

(A) Predicted miR-101 binding sequence in the human PTGER4 39-UTR, encoding EP4 receptor. The PTGER4 wildtype and mutant constructs were cloned into the pGL3 plasmid and downstream of the Luciferase gene at the Xba1 restriction site. The location of the wild-type constructs in the plasmid is denoted with yellow and mutant with red. Ninety percent of the seed sequence is mutated in the mutant construct. (B) The LS174T colon cells were co-transfected with pGL3 carrying the constructs (wild-type or mutant EP4-39-UTR and either a miR-101 expressing plasmid or empty vector, and Renilla. Co-transfection of the wildtype construct and miR-101 suppressed EP4 Luciferase expression. The relative luciferase activities were measured and normalized against their control. (C) Western analysis of endogenous EP4 receptor protein expression levels after ectopic miR-101 expression in LS174T cells. The level of endogenous EP4 receptor protein decreases in a time-dependent manner.
Suppression of colony formation and motility in vitro by miR-101

The significant reduction of miR-101 expression in colon cancer samples and the observations from our luciferase assays prompted us to explore the functional significance of miR-101. As an initial step, the capacity of colony formation was evaluated in LS174T colon cancer cells, followed by analysis of motility in transwell motility assays. First, we observed that ectopic expression of EP4 receptor caused an increase in the number of colony forming units. Next, we also saw an enhancement in the percentage of motile cells. In contrast to these observations, we demonstrated that enhanced miR-101 expression suppressed colony formation ability as well as tumor cell motility of colon cancer cell lines. Interestingly and more importantly, co-expression of EP4 receptor effectively and significantly restored tumorigenic and motile behavior of high miR-101 expressing colon cancer cell lines, that otherwise remained anti-tumorigenic (Figure 3.6A and 3.6B). Moreover, genetic silencing of the EP4 receptor (by RNA interference) or pharmacological inhibition of the EP4 receptor signaling (by using EP4 specific antagonist, L-161,982), recapitulated the anti-tumorigenic effects of miR-101 (Figure 3.6C and 3.6D). Thus, these functional studies support the notion that EP4 receptor is negatively regulated by miR-101 at the post-transcriptional level.
Figure 3.6: The biological effects of miR-101 and EP4 inhibition in vitro and in vivo
(A) Ectopic expression of miR-101 in LS174T cells suppressed EP4 expression colon tumor cell motility, whereas co-expression of EP4 could overcome miR-10 suppression of motility. (B) Enforced expression of miR-101 attenuated LS174T cell motility, whereas co-expression of EP4 rescued cells from the inhibitory effects of miR-101. (C) RNAi-based silencing of EP4 receptor expression using shRNA (shEP4) reduced colony formation relative to cell containing vector scrambled sequence (shCtrl). (D) Administration of the EP4 receptor antagonist LS161982 inhibited the growth of colon cancer cells (LS174T) grown in the chorioallantoic membrane (CAM) assay. **p < 0.01.
Discussion

This is the first study to show that the pro-tumorigenic activity of the prostanoid EP4 receptor is negatively regulated by miR-101 at the post-transcriptional level, via a specific target site within the 3'-UTR. It is also the first study to demonstrate that miR-101 inhibits cell proliferation and migration of colon cancer cells \textit{in vitro}. Finally, we also show for the first time that there is an inverse correlation between the levels of miR-101 and expression of EP4 receptor protein in human colon cancers. These data strongly suggest that EP4 receptor expression increases as a result of loss of miR-101.

We observed an increase in EP4 receptor expression in malignant colon cancer (i.e. primary colon cancer and metastasis) compared to normal colon mucosa and adenomas. This suggests a predominant role for PGE$_2$-EP4 receptor signaling at later stages of colon carcinogenesis. Moreover, PGE$_2$-EP4 receptor signaling promotes tumorigenic behavior (proliferation, resistance to apoptosis, motility, and invasion) (Wang and Dubois 2010). Previous studies report that miR-101 promotes apoptosis and inhibits cell proliferation, cell motility, and invasion (Varambally, Cao et al. 2008). Based on our present study showing that tumor suppressive effects of miR-101 can be overcome by concomitant ectopic expression of EP4, it is interesting to speculate that loss of miR-101 contributes to colon cancer progression at least in part by increased EP4 expression. However, since miR-101 has multiple targets that play a role in cancer (i.e. fos, EZH2, Cox-2, N-Myc, Mcl-1), and it is likely that other post-transcriptional targets of miR-101 also play a role in colon carcinogenesis.
Inhibition of PGE\textsubscript{2} biosynthesis by NSAIDs and COX-2 inhibitors (Coxibs) represents an effective therapy to treat colon cancers and other diseases (Flower 2003). Unfortunately, their clinical utility is limited by their potential to cause either gastrointestinal toxicity (by NSAIDs) (Bombardier, Laine et al. 2000) or cardiovascular (CV) side effects (by both NSAIDs and Coxib) (Solomon, McMurray et al. 2005). Therefore, there is a vast unmet medical need to discover alternatives for treating cancer and other chronic inflammatory conditions.

The adverse CV effects associated with NSAIDs and Coxibs are not completely understood however it is postulated that the pro-thrombotic and hypertensive effects are caused by inhibition of prostacyclin biosynthesis (for a recent review see (Funk and FitzGerald 2007)). It is plausible that a selective EP4 antagonist may be efficacious in the treatment of colon cancer without the potential CV side effects observed with NSAIDs and COX-2 inhibitors since they should not interfere with the biosynthesis of other prostanoids such as prostacyclins and thromboxanes. Indeed, several groups have reported the discovery of potent and selective EP4 receptor antagonists (Burch, Farand et al.; Chen, Muramoto et al.; Gauvreau, Dolman et al.; Elias, Akuffo et al. 2005). However, the effectiveness of this new generation of EP4 antagonists against malignant lesions warrants further investigation.

In conclusion, our present study indicates that the pro-tumorigenic EP4 prostanoid receptor is regulated at the post-transcriptional level by miR-101 via a specific target
motif at nucleotides +18 to +27 of the EP4-3’-UTR. Furthermore, miR-101 inhibits colon cancer cell proliferation and invasion. Our findings in clinically resected colonic tissues from colon cancer patients together with previously reported studies of miR-101 in several cancers support the notion that inhibitory pharmacological strategies against miR-101 target genes such as COX-2/EP4 and Histone-lysine N-methyltransferase (EZH2) (Smits, Mir et al.) could have a strong rationale for therapeutic applications in cancer in the future.
Figure 3.7: Proposed model of PGE\textsubscript{2}/EP4/miR-101 signaling events in colon cancer
The model illustrates miR-101 regulation of PGE\textsubscript{2}/EP4 signaling through negative regulation of EP4 protein expression in colon cancer cells. Inhibition of EP4 expression leads to the suppression of PGE\textsubscript{2}/EP4 downstream signals and subsequently attenuation of colon tumor progression.
CHAPTER 4:
S100P/RAGE SIGNALING POSITIVELY REGULATES MICRORNA-155
EXPRESSION VIA AP-1 ACTIVATION IN COLON CANCER

Introduction

Our group has publication demonstrating that PGE$_2$/EP4 signaling positively regulates S100P expression through cAMP response element-binding protein (CREB) activation in colon cancer cells (Chandramouli, Mercado-Pimentel et al. 2010). This suggests that constitutive signaling of PGE$_2$/EP4 due to loss of miR-101 would lead to up-regulation of S100P, consequently resulting to increase RAGE receptor signaling. S100P activation of RAGE stimulates growth, invasion and migration in colon tumor cells. Expression of S100P is up-regulated in a number of cancers like pancreatic (Fukushima, Sato et al. 2004), breast (Russo, Hu et al. 2001), lung (Bulk, Hascher et al. 2008), prostate (Basu, Azorsa et al. 2008), and colon (Fuentes, Nigavekar et al. 2007). However, the downstream signaling events in the S100P/RAGE signaling axis remain to be identified. Hence, we investigated whether activation of RAGE receptor by S100P leads to the stimulation of miR-155. Indications from previous reports have demonstrated that the activation of S100P/RAGE signaling leads to stimulation of NF-kappa B and MAP-kinase pathways (Arumugam, Simeone et al. 2004). Fascinatingly, these two pathways have been implicated in miR-155 regulation (Yin, Wang et al. 2008; Thompson, Herscovitch et al. 2011). These and other supporting evidences have led up to investigate whether miR-155 is regulated by S100P/RAGE signaling.
Results

S100P positively regulates the expression of miR-155

Activation of RAGE receptor signaling by S100P is known to stimulate both ERK and NF-kappa B signaling (Arunugam, Simeone et al. 2004; Fuentes, Nigavekar et al. 2007). In addition, miR-155 can be up-regulated by NF-kappa B, by adrenaline in colon cancer cells (Pu, Bai et al. 2012), and by ERK signaling in human B cells (Yin, Wang et al. 2008). Previous studies demonstrated that SW480 cells express the RAGE receptor but not S100P proteins (Fuentes, Nigavekar et al. 2007; Liang, Zhong et al. 2011). In addition, SW480 cells express a low level of miR-155 (He, Sheng et al. 2012). Furthermore, ectopic S100P confers a migratory phenotype to SW480 cells (Chandramouli, Mercado-Pimentel et al. 2010). Therefore, we investigated whether S100P/RAGE receptor signaling could up-regulate miR-155 expression. In order to investigate the relationship between miR-155 and S100P expressions, we evaluated whether changes in the S100P expression could alter miR-155 levels in colon cancer cells. S100P or empty vector was stably transfected in SW480 colon cancer cells and S100P transcript levels in the cells were analyzed using qRT-PCR (Figure 4.1A). The ectopic expression of S100P resulted in significant elevation of miR-155 (Figure 4.1B). To validate this result, S100P expression in LS174T colon cancer cells was knocked down using a stably transfected shRNA construct which targets S100P mRNA (ShS100P). A previous study by our group showed that knockdown of S100P using ShS100P significantly decreased S100P protein level in LS174T cells (Chandramouli, Mercado-Pimentel et al. 2010). The knockdown was once again confirmed using the
qRT-PCR (Figure 4.1C). The knockdown of S100P led to reduced miR-155 levels in the LS174T cells (Figure 4.1D). The results indicate that miR-155 expression is sensitive to altered S100P levels. Altogether, these results suggest that S100P positively regulates miR-155 expression in human colon cancer cells.
Figure 4.1: S100P positively regulates miR-155 expression levels in colon cancer cells
(A) SW480 cells with stable S100P expression shows increase S100P transcript. (B) Elevated miR-155 expression in S100P stably transfected SW480 cells. (C) Knockdown of S100P in LS174T cells with shRNA which targets S100P mRNA. (D) A marked decrease of miR-155 level in cells with shS100P knockdown. The results were normalized against the empty vector control. The vertical bar is the SEM. ** indicates p < 0.01, and *** also indicates p < 0.001.
**S100P positive regulation of miR-155 is RAGE dependent**

Since S100P is a secreted protein and ligand for the RAGE receptor, we evaluated whether S100P regulation of miR-155 expression is receptor mediated. SW480 cells were treated with purified human recombinant S100P for different time periods and analyzed for changes in miR-155 expression levels using the qRT-PCR. The miR-155 levels were increased 4 fold in 20 minutes, 11 fold in 1 hour, 1.5 fold in 2 hours and returned to baseline by 24 hours (Figure 4.2A). The return to baseline levels by miR-155 appears to be due to S100P no longer stimulating the RAGE receptor. We observed a similar pattern of miR-155 induction in LS174T cells (data not shown); however, the induction of miR-155 was markedly elevated at 4 hours and returned to baseline by 24 hours (data not shown). Treatment of LS174T with purified S100P showed a 2-fold increase in miR-155 expression at 4 hours (Figure 4.2B). Next, the dependence of miR-155 induction by S100P on the RAGE receptor was evaluated. Cells were pre-treated with blocking anti-RAGE antibody and then treated with recombinant S100P for different time periods followed by qRT-PCR to evaluate miR-155 induction. Analysis of the results indicates that pre-treatment with anti-RAGE antibody suppressed S100P induction of miR-155 (Figure 4.2C). Overall, the results indicate that induction of miR-155 by S100P is RAGE dependent.
Figure 4.2: S100P induces miR-155 expression through RAGE receptors

(A) Exogenous S100P treatment induced miR-155 in SW480 cells in time dependent response. Vehicle control was used for normalization. (B) miR-155 expression exhibited greatest induction at 4 hours in LS174T cells following exogenous S100P treatment. (C) Blockage of RAGE with anti-RAGE antibody suppressed miR-155 induction by exogenous S100P treatment of SW480 cells. The value of the results are presented as the mean ±SEM (**p < 0.01, ***p < 0.001).
S100P controls miR-155 expression through AP-1 in colon cancer cells

S100P is known to stimulate AP-1 and NF-kappa B activation via RAGE engagement. First, activation of AP-1 and NF-kappa B by exogenous S100P treatment was confirmed using Western blot (Figure 4.3A). Subsequently, we examined whether S100P/RAGE positive regulation of miR-155 occurs via AP-1 or NF-kappa B pathways. Colon cancer cells were pre-treated with either U0126 (a MEK/MAPK kinase inhibitor) or CAY10512 (a NF-kappa B inhibitor) prior to stimulation of RAGE receptor signaling with exogenous S100P. Both U0126 and CAY10512 suppressed S100P induction of miR-155 (Figure 4.3). These data suggest that both AP-1 and NF-kappa B may be involved in the induction of miR-155 by S100P.
Figure 4.3: S100P stimulates miR-155 via the activations of MAPK kinase and NF-kappa B pathways
(A) Exogenous S100P treatment induces the activation and nuclear translocation of AP-1 family (c-jun and c-fos) and NF-kappa B p65 in SW480 cells in a time dependent fashion. (B) Pre-treatment with MEK or NF-kappa B inhibitors (U0126 and CAY10512) blocks miR-155 induction by exogenous S100P treatments in SW480 colon cancer cells. The normalization unit is the value of the vehicle treated control. The data are means ±SEM of the experiments (**p < 0.01, ***p < 0.001).
To further elucidate the MIR155HG involvement of AP-1 and NF-kappa B in S100P-mediated miR-155 stimulation, we monitored the effects of pharmacological blockade of AP-1 or NF-kappa B on MIR155HG promoter activities in the presence of S100P using luciferase assay. Blockage of AP-1 and not NF-kappa B suppressed MIR155HG promoter luciferase activities in the presence of exogenous S100P (Figure 4A and 4B). Inhibition of AP-1 activation with MEK inhibitor – U0126, resulted in decreased luciferase activities; whereas inhibition of NF-kappa B activation with NF-kappa B inhibitor – CAY10512, resulted in no appreciable changes in the MIR155HG promoter luciferase activity. The AP-1 family consists of Jun members (c-Jun, JunB, and JunD) and Fos members (c-Fos, FosB, Fra-1 and Fra-2) (reviewed in (Verde, Casalino et al. 2007)). Because, c-jun has been shown to modulate gene expression in colon cancer cells, we chose to inhibit c-Jun with a dominant negative c-Jun construct (TAM67). Furthermore, genetic inhibition of the AP-1 family member c-Jun with TAM67 attenuated miR-155 induction by exogenous S100P (Figure 4.5). Combined, these results suggest that AP-1 may be involved in the transcriptional regulation of miR-155.
Figure 4.4: Inhibition of MAPK kinase suppresses MIR155HG promoter activities

(A, B) One million SW480 or LS174T cells were seeded and incubated overnight at 37 ºC. Next, the cells were transiently transfected overnight with MIR155HG-luciferase reporter gene or vehicle constructs. The cells were pre-treated with MEK inhibitor (U0126) or NF-kappa B inhibitor (CAY10512) for 4 hours and then treated with purified human recombinant S100P for 1 hour. Cells were harvested and analyzed. Pre-treatment with MEK inhibitor and not NF-kappa B inhibitor blocked MIR155HG luciferase activities. Data is the mean ±SEM (**p < 0.01, ***p < 0.001).
Figure 4.5: Genetic blockage of AP-1 activation represses miR-155 induction
(A) TAM67 suppresses miR-155 induction by S100P. TAM67 or empty vector was transiently transfected overnight into SW480 cells. Cells were treated with S100P for 1 hour and harvested for miR-155 qRT-PCR analysis. The results were normalized against the empty vector (control). (B) Schematic illustration of the mechanism of TAM67 action. Upon activation wild-types c-fos and c-Jun heterodimerized bound on AP-1 response element to drive gene expression. Blocking occur when the expression level of TAM67 is high enough to enable TAM67 homodimers to bound on the AP-1 site. In quenching TAM67 forms heterodimer with endogenous c-Jun or c-Fos and their binding leads inactive transcription state. Value of the results are shown as mean ±SEM (**p < 0.01, ***p < 0.001). (Han, Rorke et al. 2012)
Next, ChIP assays were performed to confirm that AP-1 positively regulates the miR-155 expression upon activation of S100P/RAGE receptor signaling. Results indicate that S100P treatment enhanced c-Fos occupancy on MIR155HG promoter region in two different colon cancer cell lines (Figure 4.6A and 4.6B). The c-Fos occupancy of the MIR155HG promoter was investigated following the interaction and activation of RAGE by exogenous S100P using chromatin immunoprecipitation assay analysis. The antibodies specific for RNA polymerase II were used as a positive control. The immunoprecipitated chromatins were analyzed using primers for the GAPDH active locus. Relative levels of the chromatin were the same and unaffected by the exogenous S100P treatment. Non-immune mouse IgGs were used as a negative control. We observed increased chromatin precipitates using c-Fos antibodies in the exogenous S100P treated cells compare to the vehicle control, indicating c-Fos binding to the MIR155HG promoter. Non-immunoprecipitates were used as the input control for the experiments. Overall, these results demonstrate that S100P/RAGE signaling pathway can stimulate miR-155 expression via AP-1 in colon cancer cells.
Figure 4.6: S100P prompts c-Fos enrichment at AP-1 site in MIR155HG promoter

(A, B) SW480 or LS174T cells were treated with exogenous S100P for one hour. Following this, the cells were fixed with 1% formaldehyde for 10 minutes at room temperature. The chromatin was then fragmented by sonication. Next, c-Fos protein was immunoprecipitated with anti-human c-Fos antibody. Finally, the results were analyzed using real time PCR. The results show that c-Fos binds to the MIR155HG promoter following S100P treatment. The results were normalized against the vehicle treated cells (as control). Value of the results are represented as mean ±SEM (**p < 0.01, ***p < 0.001).
Down-regulation of miR-155 decreases cell growth, motility and invasion in human colon cancer cells

S100P is implicated in cancer cell proliferation, migration and invasion (Arumugam, Simeone et al. 2004; Arumugam, Simeone et al. 2005; Fuentes, Nigavekar et al. 2007). The significant elevation of both S100P and miR-155 expression in tumor specimens and our findings that S100P stimulates miR-155 expression prompted investigation into the functional importance of miR-155. The miRNA “sponge” approach provides a useful means of creating miRNA loss of function (Brown and Naldini 2009; Ebert and Sharp 2010; Kluiver, Slezak-Prochazka et al. 2012). The miR-155 sponge or empty vector was transduced into colon cancers. Using the confocal microscope, we observed the expression of Green Fluorescent Protein (GFP) gene (Data not shown) in the transduced cells indicating the successful transduction of the retroviral plasmids. Also, reduced miR-155 levels were observed by performing qRT-PCR analysis and comparing cells bearing the sponge to empty vector (Figure 4.7). Next, cell growth, colony formation and migratory assays were performed using the transduced (SW480/S100P, DLD-1, LS174T) cells. Similar to LS174T cells, DLD-1 cells express both S100P and miR-155 (Bakirtzi, Hatziapostolou et al. 2011; Jiang, Lai et al. 2011). Knockdown of miR-155 significantly decreased cell growth in all cell lines (Figure 4.8). In the colony formation assay, miR-155 knockdown decreased the ability of the cells to form colonies (Figure 4.9). In addition, knockdown of miR-155 significantly reduced colon cancer cell motility and ability to invade into the transwells (Figure 4.10 and 4.11). Altogether, these results indicate that loss of miR-155 levels suppresses tumorigenic characteristics associated with the metastatic phenotype of colon cancer cells. These
functions support our notion that the positive regulation of miR-155 by S100P/RAGE signaling is an important step by which cancer cells promote their growth and migration during carcinogenesis.
Figure 4.7: Decreased miR-155 levels in miR-155 sponge transduced cells

The miR-155 sponge (pMSCV-puro-GFP-miR-155 sponge) or empty vectors (pMSCV-puro-GFP) were transduced in (A) SW480/S100P, (B) DLD-1, and (C) LS174T cells. The effects of the knockdown on miR-155 expression were analyzed using qRT-PCR. The results were normalized using the empty vector (control). Cells expressing the sponge show decreased levels of miR-155 compared to the empty vector. The values of the results are shown as mean ±SEM (**p < 0.01, ***p < 0.001).
Figure 4.8: Knockdown of miR-155 suppresses cell growth. (A) SW480/S100P or (B) DLD-1 or (C) LS174T cells transduced with empty vector or miR-155 sponge were seeded in triplicate wells at $2.5 \times 10^5$ cells per well in six-well plates. The cells were incubated and then counted every two days using trypan blue to monitor cell growth. Values are shown as mean ±SEM (**p < 0.05, ***p < 0.01).
Figure 4.9: Down-regulation of miR-155 expression decreases cell colony numbers

Empty vector or sponge transduced A) SW480/S100P or (B) DLD-1 or (C) LS174T cells were seeded at 1,000 cells per 100 mm plate in triplicate and cultured for 12 days for DLD-1 cells or 21 days for SW480/S100P and LS174T cells. The cultured media were aspirated and colonies stained with methylene blue. The plates were carefully rinsed with water and the number of colonies counted. The upper panels are results of the colony formation assay. The low panels are the representative results in histogram. The values are shown as mean ±SEM (***p < 0.001).
Figure 4.10: Enforced miR-155 sponge expression reduced the number of motile cells
Fifty thousand A) SW480/S100P or (B) DLD-1 or (C) LS174T cells expressing the sponge or empty vector were seeded into the transwells in triplicates for 24 hours for cell motility assay. After 24 hours incubation, the cells were stained with crystal violet solution. The stained cells that were in-between the pores were counted using a light microscope. The representative results were then graphed. Values are shown as mean ±SEM (**p < 0.01, ***p < 0.001).
Figure 4.11: Down-regulation of miR-155 decreased the number of invaded cells

Transwell invasion assays were performed with empty vector or sponge expressing A) SW480/S100P or (B) DLD-1 or (C) LS174T. The each cell line was seeded in triplicate at a density of 50,000 or 10,000 cells per transwell, respectively, and cultured for 24 hours. Invaded cells were stained with crystal violet, rinsed, and counted using a light microscope. The values are presented as mean ±SEM (**p < 0.01).
Discussion

Elevated levels of S100P have been detected in a variety of human tumors, including human colon cancers (Amler, Agus et al. 2000; Russo, Hu et al. 2001; Fukushima, Sato et al. 2004; Arumugam, Simeone et al. 2005; Bulk, Hascher et al. 2008; Rehbein, Simm et al. 2008), and have proven to play an important role in the pathogenesis of cancer since its discovery in 1992. S100P has become of special interest because of its ability to contribute to tumor invasion and metastasis and it is associated with a poor prognosis in cancer outcomes. In the present study we further investigated the molecular mechanism by which S100P and RAGE receptor signaling contribute to metastasis.

We have shown for the first time that oncogenic miR-155 can be regulated by the S100P/RAGE pathway. Enforced S100P expression up-regulates miR-155 levels in colon cancer cells. In addition, exogenous S100P protein stimulates miR-155 expression and this stimulation is RAGE dependent. The blockage of RAGE with anti-RAGE antibodies suppresses miR-155 induction by exogenous S100P protein. The positive regulation of miR-155 is MAPK kinase, and to a lesser extent NF-kappa B dependent. These data also show that S100P/RAGE mediated regulation of miR-155 is AP-1 dependent. Thus, these studies provide a molecular mechanism linking inflammation and oncogenic events important in colon cancer progression and metastasis.
To determine the biological relevance of miR-155 induction by S100P in colon cancer cells, we selectively inhibited miR-155 function using miR-155 sponge. The sponge RNAs contain complementary binding sites to a miRNA of interest and are produced from transgenes within cells (Brown and Naldini 2009; Ebert and Sharp 2010; Kluiver, Slezak-Prochazka et al. 2012). Therefore, miRNA sponges have proven to be very useful tools for understanding miRNA functions in several experimental systems. We observed a decrease in cell growth, colony formation, motility and invasion in colon cancer cells expressing the miR-155 sponge as compared to the empty vector control cells. This observation is in agreement with previous studies investigating miR-155 function in cancer cells (Han, Chen et al. 2012; Li, Nie et al. 2012; Li, Chen et al. 2012; Mattiske, Suetani et al. 2012).

Although we have been able to establish that S100P/RAGE signaling can regulate the expression of miR-155, the targets of miR-155 important in colon cancer progression and metastasis are not well defined. There have been many miR-155 targets identified using both genomic and proteomic approaches (Kong, Yang et al. 2008; Yin, McBride et al. 2008; Jiang, Zhang et al. 2010; Valeri, Gasparini et al. 2010). Most notably, suppressor of cytokine signaling 1 (SOCS1) (Jiang, Zhang et al. 2010) and RhoA (Kong, Yang et al. 2008) are two miR-155 targets implicated in migration and invasion in other cancers. Thus, the target genes controlled by the S100P/RAGE/miR-155 signaling axis remain to be identified.
To our knowledge, this is the first time it has been shown that S100P/RAGE receptor signaling positively regulates the expression of oncogenic miR-155. Previous reports have shown that inhibition of S100P, RAGE, or miR-155 suppressed colon cancer growth and metastasis (Arumugam, Ramachandran et al. 2006; Kim, Jung et al. 2009; Arumugam, Ramachandran et al. 2012; Li, Chen et al. 2012). Our results support these observations and also suggest that the newly identified S100P/RAGE/miR-155 pathway may help open up new avenues for understanding the relationship between inflammation and metastasis in colon cancer as well as aid efforts in the progression of effective therapeutic strategies for the treatment of late stage colon cancers.
Figure 4.12: Proposed model of S100P/RAGE/miR-155 mediated colon cancer
Activation of RAGE receptor signaling by S100P results in the stimulation of miR-155 through ERK and AP-1. RAGE signaling activation promotes tumor progression and metastasis through the miR-155. Depletion of matured miR-155 expression attenuates tumor progression and metastasis.
CHAPTER 5:
IDENTIFICATION OF NOVEL DOWNSTREAM TARGETS OF THE S100P/RAGE/MIR-155 SIGNALING PATHWAY

Introduction

In chapter 4, we demonstrated that miR-155 can be up-regulated through the stimulation of RAGE by S100P. Also, we showed that the positive regulation of miR-155 in this pathway occurs via AP-1. Alongside other studies, our laboratory group has shown that S100P/RAGE receptor signaling plays a functional role in colon cancer growth and metastasis (Chandramouli, Mercado-Pimentel et al. 2010; Ding, Chang et al. 2011; Jiang, Lai et al. 2011). These results, therefore link miR-155 to the S100P/RAGE pathway and demonstrated that miR-155 is downstream in this pathway. Separately, both miR-155 and S100P/RAGE have been shown to promote cancer progression. However, the downstream mediators within the S100P/RAGE/miR-155 pathway have not been elucidated. Exactly how miR-155 targets contribute to the pathogenesis of colon cancer is not known. The identification of miR-155 targets is necessary for understanding how this pathway promotes tumor progression and migration (Figure 5.1). This set of experiments was conducted to identify gene targets controlled by the induction of miR-155 upon stimulation by S100P/RAGE signaling that may contribute to the metastatic phenotype.
The activation of RAGE by S100P leads to the phosphorylation of ERK and its downstream targets. Phosphorylated ERK activates the AP-1 family members (c-Jun and c-Fos) by phosphorylation and thus causes them to translocate to the nucleus. In the nucleus c-Jun and c-Jun form a heterodimer and bind at the MIRHG promoter to activate MIRHG transcriptional activation.

Figure 5.1: Downstream targets genes of S100P/RAGE/miR-155 signaling pathway
Results

Downstream target genes of S100P/RAGE signaling pathway

To identify targets genes which are regulated by miR-155 in response to S100P/RAGE signaling, we performed transcriptome microarray analysis with S100P overexpressed SW480 cells and empty vector control cells. The experimental approach for these experiments is shown in Figure 5.2. Previous characterization of these cells showed that miR-155 levels were elevated (see Chapter 4). The results shown are from three independent experiments. There was an excess of down-regulated genes over up-regulated genes (Figure 5.3). Also, there was a strong abundance of genes without annotation (data not shown). Bio-informatics analysis of the differentially expressed genes from the microarray results identified 7 candidate genes containing miR-155 seed sequences (Table 5.1). Among these genes, five genes that includes: chromodomain helicase DNA binding protein 9 (CHD9), protein kinase inhibitor beta (PKIB), phospholipase C, eta 1 (PLCH1), IQ motif containing H, and coiled-coil domain containing 111 (CCDC) were significantly up-regulated. There were two other genes that were significantly down regulated that also contained miR-155 seed sequence. These two genes are WNK lysine deficient protein kinase 1 (WNK1) and zinc finger protein 493 (ZNF493). Since, miRNAs negatively regulate gene expression (i.e. reduce levels of mRNA/proteins) (Cano, Perez-Moreno et al. 2000; Calin and Croce 2006; Bartel 2009), we focused our studies on WNK1 and ZNF493.
Figure 5.2: Schematic representation of transcriptome microarray approach

SW480 cells stably transfected with S100P or empty vector were seeded at 2 million cells per 35mM plate and incubated overnight. RNA was isolated from the harvested cells and differentially labeled for Affymetrix 1.0 ST hybridization arrays. Bioinformatics analysis was used to identify miR-155 targets that were differentially expressed.
Figure 5.3: Identifying targets of miR-155 via microarray approach
(A) A “volcano plot” showing the mean expression difference log₂-transformed fold-change versus the log₁₀-transformed p-value for the fold-change for every gene detected above background in the microarray. Differentially expressed genes with significant fold change and high statistical significance are located at the upper left and right. Down-regulated genes are labeled with green and up-regulated genes with red color. Genes with greater fold change differences and variations are located at the lower left and right and colored with black. Finally, genes with no significant expression difference in the experiment are located in the lower center.
(B) Bar chart depiction of significantly differentiated genes.
Table 5.1: Differentially expressed genes with predicted miR-155 seed sequence

<table>
<thead>
<tr>
<th>ID</th>
<th>SYMBOL</th>
<th>GENENAME</th>
<th>ENTREZID</th>
<th>RESULT</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>8001402</td>
<td>CHD9</td>
<td>Chromodomain helicase DNA binding protein 9</td>
<td>80205</td>
<td>Positive</td>
<td>Unknown</td>
</tr>
<tr>
<td>8121768</td>
<td>PKIB</td>
<td>Protein kinase (cAMP-dependent, catalytic) inhibitor beta</td>
<td>5570</td>
<td>Positive</td>
<td>Regulates PKA/AKT</td>
</tr>
<tr>
<td>8091600</td>
<td>PLCH1</td>
<td>Phospholipase C, eta 1</td>
<td>23007</td>
<td>Positive</td>
<td>Unknown</td>
</tr>
<tr>
<td>7952986</td>
<td>WNK1</td>
<td>WNK lysine deficient protein kinase 1</td>
<td>65125</td>
<td>Negative</td>
<td>Regulates TGF-β</td>
</tr>
<tr>
<td>7984380</td>
<td>IQCH</td>
<td>IQ motif containing H</td>
<td>64799</td>
<td>Positive</td>
<td>Unknown</td>
</tr>
<tr>
<td>8180200</td>
<td>ZNF493</td>
<td>Zinc finger protein 493</td>
<td>284443</td>
<td>Negative</td>
<td>Unknown</td>
</tr>
<tr>
<td>8098556</td>
<td>CCDC111</td>
<td>Coiled-coil domain containing 111</td>
<td>201973</td>
<td>Positive</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
Figure 5.4: SW480 with ectopic S100P or S100P knockdown LS174T cells were seeded at 2 million cells per well in six-well plates and incubated for 24 hours. The cells were harvested and total RNA extracted. The RNAs were then subjected to qRT-PCR analysis for miR-155 target gene levels. (A) WNK1 and ZNF493 transcript levels decreased in the ectopic S100P SW480 cells as compare to empty vector (control) cells. (B) Elevation of WNK1 and ZNF493 transcripts levels in S100P knockdown LS174T cells compared to the control.
We first confirmed the down regulation of WNK1 and ZNF493 in SW480/S100P and control cells using the qRT-PCR. The result shows that the level of WNK1 and ZNF493 were decreased in ectopic S100P cells compared to the control cells (Figure 5.4A). Since down regulation of S100P by shRNA leads to a decrease miR-155 levels (Figure 5.1D), we asked whether WNK1 and ZNF493 expression levels would be elevated in S100P shRNA colon cancer cells. We found that WNK1 and ZNF493 expressions were up-regulated when S100P levels were depleted by shRNA using qRT-PCR analysis (Figure 5.4B). Altogether, these data suggest that WNK1 and ZNF493 are downstream of S100P/RAGE signaling pathways.

**WNK1 and ZNF493 are negatively regulated by miR-155 in colon cancer cells**

Next, we asked whether specific knockdown of miR-155 levels could lead to up-regulation of WNK1 or ZNF493. Interestingly, we observed that expression of both WNK1 and ZNF493 mRNAs were elevated in the miR-155 knockdown cells as compared to the control cells (Figure 5.5). WNK1 levels were elevated by about 1.4 fold; whereas, ZNF493 expression increased by 5 fold. Overall, our data suggests that both WNK1 and ZNF493 are novel targets of miR-155.
Figure 5.5: Elevation of WNK1 and ZNF493 expressions in miR-155 depleted cells
Oncogenic miR-155 were knockdown in SW480 cells that are stably expression S100P and previously shown to have elevated miR-155 expression. Knockdown of miR-155 was carried out with miR-155 sponge. WNK1 and ZNF493 expression levels were determined by performing qRT-PCR. Expression of WNK1 (A) and ZNF493 (B) were elevated.
WNK1 and ZNF493 are negatively correlated with S100P and miR-155 expressions in colon tissue specimens

In order to gain insights into the relevance of our findings to human disease, we evaluated the correlation of WNK1, ZNF493, S100P, and miR-155 in a subset of colon cancer patient. In a cohort of colon tumor specimens, WNK1 expression was significantly down regulated in 9 of 17 colon cancer cases (Figure 5.5A). ZNF493 expression was also significantly down regulated in 14 of the 17 colon tumor cases as compared with normal colon tissue (Figure 5.5B). Conversely, S100P levels were elevated significantly in 12 of the 17 cases relative to the normal tissue (Figure 5.5C). We also found significant elevation of miR-155 levels in the colon tumor specimen compared to the matched normal (Figure 5.6). These results suggest an inverse relationship between the expression of S100P or miR-155 and WNK1 or ZNF493 in colon cancer. These data also support that WNK1 and ZNF493 may be targets of miR-155.
Figure 5.6: WNK1 and ZNF493 levels are inversely correlated to S100P expression

qRT-PCR analysis of WNK1, ZNF493 and S100P expressions in matched normal and colon tumor specimen. The results show decreased expression of WNK1 (A) and ZNF493 (B) in colon tumor specimens as compared to normal specimens. (C) qRT-PCR analysis indicates that S100P transcript levels are elevated in colon tumor specimens as compared to normal colonic mucosa.
Figure 5.7: Elevated levels of miR-155 in colon tumor specimens
Characterization of miR-155 expression levels in colon tissue specimens using qRT-PCR indicates up regulation of miR-155 in tumor specimens as compared to the match normal.
Discussion

Our previous studies demonstrate that S100P/RAGE signaling leads to the induction of oncogenic miR-155 via AP-1. Here, we provide mechanistic insights into S100P/RAGE induced miR-155 through genome-wide transcriptome analysis and bioinformatics studies using colon cancer cells. We found two previously un-identified miR-155 targets WNK1 and ZNF493 in the S100P/RAGE signaling pathways.

Very little is known about the function of ZNF493. Studies in chickens have shown that ZNF493 is involved in the regulation of toll-like receptors in leukocytes (Gou, Liu et al. 2012). In humans, ZNF493 is among eleven genes that were identified in whole genome RNA microarrays of iodine-131 induced thyroid cancer (Abend, Pfeiffer et al. 2012). Down regulation of ZNF493 was observed in patients with thyroid cancer following large exposure of iodine-131 during the Chernobyl nuclear accident. Owing to its name, the ZNF493 is likely a member of the zinc finger family and may be involved in transcription. Based on this, we conclude that ZNF493 may be a transcription factor whose down regulation contributes to carcinogenesis.

There is more information regarding the structure and biological functions of WNK1. With-no-lysine kinase 1 (WNK1) is a member of the WNK family of proteins. The human WNK family is composed of four members, WNK1, WNK2, WNK3, and WNK4 (Xu, English et al. 2000; Verissimo and Jordan 2001) (Figure 5.8), and are characterized by their lack of a typical highly conserved lysine kinase residue in the
Figure 5.8: Schematic representation of WNKs family showing the domains and sites of mutation in cancer
The mutations on WNKs are shown in regard to the catalytic, autoinhibitory and coiled-coil domains. Location of the missense mutations are depicted as black arrow heads and frameshift are denoted by red signposts. Silent mutations are marked by blue forks (Moniz and Jordan 2010).
subdomain II, which is essential for ATP binding and catalyzing phosphoryl transfer (Hanks, Quinn et al. 1988). In place of the lysine, the WNK family has serine/threonine kinase residues (Hanks, Quinn et al. 1988; Xu, English et al. 2000). This variation set the WNKs apart from other protein kinase and are likely responsible for their unique properties (Moniz and Jordan 2010). WNK proteins are known to undergo autophosphorylation within the kinase domain and at least one serine residue has to undergo phosphorylation for the kinase to become active (Xu, Min et al. 2002; Zagorska, Pozo-Guisado et al. 2007). WNKs play essential roles in regulation of ion balance (Xu, Stippec et al. 2005), cell signaling (Xu, Stippec et al. 2004; Lee, Chen et al. 2007), survival (Verissimo, Silva et al. 2006), proliferation (Lee, Chen et al. 2007), mitosis (Tu, Bugde et al. 2011), neuronal differentiation (Zhang, Xu et al. 2009) and organ progression (Delaloy, Lu et al. 2003; O'Reilly, Marshall et al. 2003; Zambrowicz, Abuin et al. 2003; Huang, Cha et al. 2007; Xie, Yoon et al. 2013). Aberrant WNK1 protein expression is linked to several diseases such as neuropathy, hypertension and cancer (Reviewed in (Moniz and Jordan 2010)). Several studies have implicated a functional role for the WNK subfamily in neoplasia (Moniz, Verissimo et al. 2007; Moniz and Jordan 2010; Haas, Cuddapah et al. 2011; Moniz, Martinho et al. 2013). WNK kinases have been implicated in cell cycle progression, metabolic adaptation of tumors in the microenvironment and evasion of apoptosis (reviewed in (Moniz and Jordan 2010)).

Interestingly the WNK1 protein kinase has been implicated in invasion and metastasis (Moniz and Jordan). It is thought that many epithelial tumors cells switch to a
highly motile fibroblastoid or mesenchymal phenotype, a process called epithelial-mesenchymal transitions (EMT) (Thiery 2003). EMT is an early event in metastasis (Thiery 2003). It is well accepted that an inducer of EMT is TGFβ signaling which utilizes Smad-mediated gene expression to induce the transcription factors Snail and Slug (Padua and Massague 2009). Both Snail and Slug repress expression of E-cadherin gene which are required for epithelial cell adhesion, a hallmark phenotype of EMT (Heldin, Vanlandewijck et al.). WNK1 has been shown to phosphorylate SMAD2 in vitro and appears to negatively control SMAD2/3 dependent transcriptional responses and TGFβ signaling (Lee, Chen et al. 2007), this suggests that loss of expression or inactivation WNK1 mutations could promote EMT of colon cancer cells (Moniz and Jordan). Also miR-155 is regulated by the TGFβ/SMAD pathway (Kong, Yang et al. 2008). Taken together, our pilot observations and data from other studies suggest a model whereby S100P/RAGE signaling leads to the induction of miR-155 expression which results in loss of WNK1 expression (Figure 7.4). The loss of WNK1 could lead to stimulation of TGFβ and increases intra-nuclear phospho-SMAD levels in Hela cells indicating that WNK1 is a negative regulator of SMAD2/TGFβ signaling (Lee, Chen et al. 2007). WNK1 selectively activates ERK5 via MEKK2/3-dependent mechanism (Xu, Stippec et al. 2004). ERK5, which is a member of the MAPK family, has been reported to promote invasion in prostate cancer (Ramsay, McCracken et al. 2011) and cell proliferation in colon cancer cells (Nishimoto and Nishida 2006). Moreover, the catalytic domain of WNK1 has 50% homology to MEKK, Raf and PAK, members of the MAPKs protein family that have been link to oncogenic signaling (Verissimo and Jordan 2001).
In summary, we provide indirect evidence that WNK1 and ZNF493 are downstream targets in the S100P/RAGE/miR-155 signaling axis. Future studies using 3’UTR luciferase construct for these genes is warranted. Nevertheless, the proposed studies imply that down regulation of WNK1 and ZNF493 may contribute to colon cancer progression and metastasis.
Figure 5.9: Proposed model linking miR-155 target genes in the S100P/RAGE pathway to colon cancer pathogenesis

The activation of RAGE signaling by S100P results to downstream signaling cascades important for miR-155 stimulation. Stimulated miR-155 negatively regulates the expressions of WNK1 and ZNF493 thus promoting colon tumor progression and metastasis.
CHAPTER 6
METHYL-2-ACETAMIDOACRYLATE, AN ETHYL PYROVATE ANALOG, ATTENUATES S100P/RAGE SIGNALING AND COLON TUMOR GROWTH AND METASTASIS

Colon cancer treatment has improved substantially in recent years, nonetheless it remains the second most common cause of cancer deaths in men and women in the United States (Gallagher and Kemeny 2010; Siegel, Naishadham et al. 2012). Our understanding of colon cancer pathogenesis has significantly improved and remarkable advancements in the diagnosis and treatment of colon cancer have been made over the last three decades. This has led to an increased 5-year survival rate for patients with early stage cancers, but the survival rate drops for with lymph node involvement (Reviewed in (Kantha, Senger et al. 2012)). Regardless of all these improvements, the incidence of colon cancer colon is still on the rise. Approximately half of the patients diagnosed with colon cancer still develop the metastatic disease, which is the major cause of cancer related death in patients (Sheth and Clary 2005). Metastasis is largely considered incurable due to the lack of an effective therapy other than hepatic resection (Elias, Liberale et al. 2005; Jemal, Siegel et al. 2010). Hence, there is growing need to find an effective treatment for metastatic colon disease. Patients with colon cancer are commonly administered 5-flourouracil (5-FU), leucovorin, capecitabine, oxaliplatin, and irinotecan, as first line chemotherapy to suppress growth and induce apoptosis (Rougier, Van Cutsem et al. 1998; Chau and Cunningham 2002; Meyerhardt and Mayer 2005).
However, the efficacies of 5-FU and other chemotherapeutic agents are to some extent limited in colon cancer patients due to toxicity and acquired resistance. Therefore, identifying new pharmacologic strategies continues to be an important need.

Ethyl pyruvate (EP) is a derivative of an endogenous anti-oxidant pyruvate (Sappington, Cruz et al. 2005). EP can scavenge reactive oxygen species and can down regulate pro-inflammatory cytokines both in vitro and in vivo (Yu, Noh et al. 2010; Cook, Holcombe et al. 2011; Jacobs, Holcombe et al. 2012). EP inhibits the RAGE receptor ligand high mobility group box-1 (HMGB1) (Li, Wang et al. 2012) and decrease the activation of p38 mitogen-activated protein kinase (MAPK) (Chen, Bai et al. 2013) and NF-Kappa B (Papageorgiou and Patsalis; Luan, Zhang et al. 2010). EP also preserves mucosal histology and permeability after mesenteric ischemia-reperfusion injury (Alison, Russell et al. 1978), survival and intestinal damage after hemorrhagic shock (Wagner), and reduces injury associated with alcoholic hepatitis (Yang, Han et al. 2003; Yang, Uchiyama et al. 2004) and coronary ischemia-reperfusion (Woo, Taylor et al. 2004), as well as renal sepsis in mice (Miyaji, Hu et al. 2003). EP has also been shown to inhibit lung tumor growth (Park, Yi et al. 2011) and decrease the growth of colon liver metastasis (Liang, Chavez et al. 2009). In gastric cancers, EP suppresses tumor growth and EP tumor growth inhibition is associated with HMGB1-RAGE and Akt pathways (Zhang, Zhu et al. 2012). However, because of its chemical instability in aqueous solution, EP needs to be prepared immediately prior to injection in Ringer’s solution and used shortly after dilution, which limits its utility as a therapeutic agent. EP analogs,
including methyl-2-acetamidoacrylate (M2AA), also have anti-inflammatory properties and inhibit LPS-induced nitric oxide, TNF-α, NF-κB, and amelioration of the increase of epithelial permeability, and decrease sepsis induced acute kidney injury in mice (Leelahavanichkul, Yasuda et al. 2008), but with the advantage of more stable chemical properties. Based on these observations, we hypothesized that M2AA may be efficacious against colon cancer through inhibition of AP-1 and NF-kappa B.
Results

EP and M2AA decrease the number of viable colon cancer cells

One of the traditional properties of anti-cancer agents is their ability to kill cancer cell growth. As a result, we first investigated the ability of EP and M2AA to decrease the number of viable LS174T and SW480 human colon cancer cells at different concentrations. LS174T and SW480 cells were treated with EP or M2AA using different concentration, daily for 3 days. At the end of the treatment, viable cells were harvested and counted with trypan blue to determine the effect of EP or M2AA treatment on cells viability (Figure 6.1). Our data showed that both EP and M2AA decreased colon cancer cell viability in a dose-dependent manner. M2AA was also at least 100-fold more potent than EP in inhibiting tumor growth. The IC\textsubscript{50} values for EP were 9 mM and 13 mM for SW480 and LS174T cells, respectively. The M2AA IC\textsubscript{50} for SW480 and LS174T were 30 µM and 140 µM, respectively. These data are in agreement with an earlier report which indicated that M2AA is more potent than EP in action (Sappington, Cruz et al. 2005). This is the first data with M2AA in colon cancer cells.
Figure 6.1: M2AA is more potent than EP in decreasing number of viable colon cancer cells
One million LS174T or SW480 cells were seeded into a 6-well plated and incubated overnight. Next, the cells were treated with different concentration of EP (A), or M2AA (B) or vehicle daily for 3 days. Afterward, treated and control cells were counted using trypan blue to determine the effects of EP or M2AA treatment.
**M2AA inhibits primary tumor growth and metastasis in vivo in the CAM assay**

Next, we evaluated the effects of M2AA on in vivo tumor growth using the chick chorioallantoic membrane assay (CAM assay). Being naturally immunodeficient, the chick embryo is a good model system to study several aspects of tumor biology and has been employed to study tumor cell invatrassation and dissemination of many different human carcinomas including breast, prostate and colon carcinomas (Subauste, Kupriyanova et al. 2009). Other animal models such as mice or rats require immunodeficient model systems for the implantation of tumor cells (Morikawa, Walker et al. 1988; Kuo, Kubota et al. 1995; Warren, Yuan et al. 1995; Subauste, Kupriyanova et al. 2009).

We transplanted 1 million LS174T colon tumor cells on the chorioallantoic membrane of 9 day old chick embryo and allowed the cells to grow for 3 days. The LS174T is highly malignant compared to SW480 cells. On day 4, the tumors were treated with 140 µM M2AA or vehicle daily for 5 days. At the end of the treatment, the embryo was terminated and tumor weight obtained. We observed significant reduction of the tumor weight in the chick embryos that were treated with M2AA compare to the vehicle treated embryo (Figure 6.2A) and therefore, the result confirms our in vitro findings and show that M2AA can inhibit tumor growth in vivo.

To analyze the effects of M2AA tumor cells metastasis, the lungs were harvested and analyzed by Alu qPCR to determine relative level of disseminated human cells into
the host lung tissues. The Alu elements at present in human and other primates and are lacking in chickens (Subauste, Kupriyanova et al. 2009). The chicken embryonic lung organs provide the most ideal environment for survival and metastatic growth of human malignant colon cells (Subauste, Kupriyanova et al. 2009). The results demonstrate that fewer tumor cells metastasize in the M2AA treated embryo compared to vehicle treated embryos (Figure 6.2B). This preliminary finding demonstrates that M2AA may be efficacious in the treatment of metastatic colon cancer.
Figure 6.2: M2AA suppresses colon tumor growth and metastasis *in vivo* in CAM

One million LS174T cells were planted on the CAM of 9 day old egg embryo. The eggs were incubated for 3 days, after which they were treated with 140 µM M2AA or vehicle daily for 5 days. (A) The embryos were sacrificed and the tumor weight measured. Results show that tumors treated with M2AA have smaller weight compared with the control. pValue = 0.11945. (B) Anti-metastasis potential of M2AA was analyzed by human Alu qPCR to determine the relative level of CAM tumor metastasis to the chick embryos’ lungs. The results show relative low levels of Alu DNA in the M2AA treated as compared to the control. pValue = 0.02968
M2AA suppresses activation of RAGE signaling by S100P in colon cancer cells

To elucidate the mechanism associated with M2AA mediated anti-cancer effect, we asked whether M2AA treatment can inhibit S100P/RAGE signaling activation. As previously shown in chapter 4, exogenous S100P treatment activates RAGE signaling which leads to the stimulation of both the MAPK kinase and NF-κB pathways. The inhibition of S100P and/or RAGE signaling has been accomplished using several approaches including expression of a dominant negative truncated RAGE receptor (Taguchi, Blood et al. 2000), treatment with a RAGE blocking antibody (Abe, Shimizu et al. 2004), treatment with a short amino-acid antagonist peptide derived from HMGB1 (Huttunen, Fages et al. 2002), treatment with cromolyn (Arumugam, Ramachandran et al. 2006) gene deletion of RAGE (DiNorcia, Moroziewicz et al. 2010; DiNorcia, Lee et al. 2012), and gene silencing of S100P (Jiang, Lai et al. 2011), treatment with a S100P-derived RAGE antagonistic peptide (Arumugam, Ramachandran et al. 2012), and treatment with ethyl pyruvate (Zhang, Zhu et al. 2012). However, to date, none of these approaches has become clinically available.

To evaluate whether M2AA could inhibit S100P/RAGE signaling, SW480 cells were pre-treated with different concentrations of M2AA (25–100 µM) for 1 hour and then followed by S100P treatment for another 1 hour. Analysis of the results showed that pre-treatment with M2AA caused a concentration-dependent inhibition of S100P/RAGE signal activation of ERK and IκBα (Figure 6.3) starting at 50–100 µM M2AA concentration compare to controls. Next, we analyzed the nuclear protein for activated
AP-1 family members (c-Jun and c-Fos), as well as activated NF-κB. Once again, we observed that pre-treatment with M2AA inhibited the activation of components of both of AP-1 and NF-κB proteins including their subsequent translocation to the nucleus (Figure 6.4). Since both AP-1 and NF-κB are involved in inflammatory pathways that are linked to tumor cell growth, these data demonstrate the anti-inflammatory properties of M2AA and are in agreement with other reports (Leelahavanichkul, Yasuda et al. 2008).
Figure 6.3: M2AA attenuates the activation of ERK by exogenous S100P
SW480 cells were pre-treated with different concentration of M2AA for 1 hour and then with exogenous S100P for another 1 hour. The cells were harvested and total protein was extracted and analyzed with Western blot for M2AA effects.
Figure 6.4: M2AA suppresses S100P/RAGE signaling and miR-155 expression
SW480 cells were pre-treated with different concentration of M2AA for 1 hour and then with exogenous S100P for another 1 hour. The cells were harvested and their nuclear protein extracted and analyzed with Western blot for M2AA effects. The result indicated that M2AA suppresses S100P activation of both NF-κB, c-Jun, and c-Fos activation in concentration dependent fashion.
M2AA attenuates miR-155 induction by exogenous S100P

In chapter 4, we demonstrated that activation of S100P/RAGE signaling leads to the induction of miR-155. Next, we asked whether M2AA could inhibit miR-155 induction by S100P/RAGE signaling. We found a significant reduction of miR-155 expression in SW480 colon cancer cells pre-treated with M2AA and stimulated with recombinant S100P.
Figure 6.5: M2AA blocks miR-155 induction by exogenous S100P
SW480 cells were pre-treated with 100uM of M2AA or vehicle for 1 hour. Following this, the cells were treated for another 1 hour with purified S100P or vehicle. The cells were harvested and small RNAs isolated. The effects of the treatment on miR-155 induction were analyzed using qRT-PCR.
Discussion

For the first time, we have demonstrated the anti-cancer properties of M2AA. We began by showing that M2AA decreased the number of viable colon cancer cells. Then, we investigated M2AA effects on primary colon tumor growth and metastasis in vivo using the CAM assay. We then evaluated the molecular mechanisms responsible for M2AA tumor growth inhibition. We show, for the first time, that M2AA suppresses ERK, which is upstream of the AP-1 transcription factor complex. We also demonstrate that M2AA attenuates IκBα activation a critical component of NF-κB regulation. Finally, we showed that M2AA inhibits miR-155 stimulation by S100P/RAGE signaling in colon cancer cells.

M2AA expectedly exerts beneficial effects that have not been previous characterized as have the anti-inflammatory, anti-growth, anti-metastatic properties in cancer. Here, we demonstrated that the anti-inflammation properties of M2AA are associated with the inhibition of inflammatory mediators, NF-kappa B and AP-1, by S100P/RAGE signaling. It is also important to note that the anti-cancer growth and anti-metastatic properties of M2AA may be in part due to its effects on both the NF-kappa and AP-1 that have been shown to be elevated in many cancers by numerous studies. However, more studies need to be performed in this area to determine the full mechanism of cancer cell killing by M2AA. It is likely that the suppression of miR-155 stimulation by M2AA is attributed to inhibitory effects on NF-kappa B and AP-1 activation. Both NF-kappa and AP-1 have been shown by our group (See chapter 4) and others (Kluiver,
van den Berg et al. 2007; Yin, Wang et al. 2008) to regulate miR-155 expression. The M2AA effect on cell viability is not sufficiently due to direct cytotoxicity, since the in vivo CAM assay results does not support this notion. Our data, suggests that M2AA be effective in the treatment of metastatic colon disease.

In summary, we show that M2AA can inhibit colon cancer cell growth and metastasis. The tumor growth inhibitory effects of M2AA are associated with the suppression of S100P/RAGE receptor signaling events. The ability of M2AA to inhibit colon tumor growth and metastasis in a mouse model needs to be investigated.
Figure 6.6: Proposed model of M2AA suppression of S100P/RAGE signaling in colon cancer

The diagram shows that M2AA treatment inhibits S100P/RAGE signaling activation of ERK1/2 and subsequent downstream signaling leading to tumor progression and metastasis.
CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

These studies provide insights into the molecular mechanisms leading to the progression of metastasis in colon cancer. We linked inflammatory pathways and miRNAs changes to colon cancer progression. We also identified novel downstream target genes of oncogenic miR-155. Finally, we evaluated the properties of a novel pharmacological agent, M2AA in colon cancer cells.

In chapter 1, we addressed the importance of tumor suppressor miRNA, miR-101 in the negative regulation of PGE$_2$/EP4 signaling. The PGE$_2$/EP4 is an inflammatory pathway that has a strong association with tumor promotion. We demonstrated that the up-regulation of EP4 is due to the loss of miR-101 expression. Interestingly, miR-101 has been shown to also regulate other tumor promoters such as COX-2 (Strillacci, Griffoni et al. 2009) and β-catenin (Strillacci, Valerii et al. 2013) in colon cancer. Also important, enforced miR-101 has been shown to sensitized tumor cells to radiation (Yan, Ng et al. 2010). Taken together, these results demonstrated that miR-101 is a potent tumor suppressor and could serve as a diagnostic biomarker for colon cancer. The results also show the prognostic values of miR-101 leading to the notion that therapeutic strategies that targets restoration of miR-101 might be efficacious in the treatment of metastatic colon cancer.
Our study and others suggest that loss of miR-101 expression occurs frequently in many cancers including prostate (Varambally, Cao et al. 2008), lung (Thu, Chari et al. 2011), breast, gastric (He, Shao et al. 2012), colon (Varambally, Cao et al. 2008; Strillacci, Griffoni et al. 2009; Schee, Boye et al. 2012; Strillacci, Valerii et al. 2013), hepatocellular Carcinoma ((Su, Yang et al. 2009). However, the molecular mechanism by which loss of miR-101 expression occurs in colon cancers remains to be determined. Two other reports have shown that the loss of miR-101 expression in prostate (Varambally, Cao et al. 2008) and non-small cell lung (Thu, Chari et al. 2011) cancers is due to genomic loss. However, our pilot study suggests differently, providing evidence that the loss of miR-101 expression may be due to epigenetic modification of miR-101 host genes. Expression of miR-101 was restored following 5-aza-2'deoxyctydine (5-azaDC) treatment of colon cancer cells (Figure 7.1). The 5-azaDC is an inhibitor of DNA methyltransferase (DNMT), which is important for DNA methylation. Over methylation of cytosine residues of CpG islands, commonly located near the promoters, alters the chromatin structure leading gene silencing (Crea, Giovannetti et al. 2009). The 5-azaDC has been shown to reverse these epigenetic alterations by reactivating silenced genes (Yoo and Jones 2006; Crea, Giovannetti et al. 2009). This suggests that loss of miR-101 expression may be due to hypermethylation of pri-miR-101 promoter in colon cancers. To investigate this hypothesis, we have designed a set of specific experiments that will clearly help to achieve this goal. First, we will identify which pri-miR-101 genes give rise to matured miR-101 in colon cancer cells since the pri-miR-101 gene is encoded at two different locations in the genome. The RNase protection assay (RPA)
and qRT-PCR methods will be utilized in this methodology. The RPA approach determines the amounts of small RNA presently by measuring the amount that non-convalently hybridized to the small RNA. The qRT-PCR approach will be used to evaluate the expressions of pri-miR-101 by the two different loci using a normal colon cells (CRL-1831) and compare to LS174T colon cancer cell lines that expressed very low miR-101 level. In a second series of experiments, we will determine whether hypermethylation of the miR-101 promoter is responsible for loss of miR-101 expression in human colon cancer specimens. DNA will be isolated from a cohort (n=30) of matched normal and colon cancer cases. Next, the DNAs are bisulfite treated and subjected to pyrosequencing. The outcome from these results will show that loss of miR-101 expression is due to epigenetic silencing through hypermethylation of miR-101 promoters in colon cancer. These studies will clarify the molecular mechanism for loss of miR-101 expression in colon cancers.
Figure 7.1: Expression of miR-101 is rescued by 5-azaDC

One million LS174T colon cancer cells were seeded in 35 mM plates and incubated overnight. Next, cells were treated with 5-azaDC (5μM) or vehicle daily for 3 days. Cells were harvested at 48hs or 72hs following treatment and the total RNA were isolated. The status of miR-101 in 5-azaDC and vehicle treated were analyzed with the qRT-PCR.
In Chapter 4, we demonstrated the mechanism by which oncogenic miR-155 is regulated by the S100P/RAGE signaling pathway. We established that miR-155 is important for mediating S100P/RAGE biological effects on colon cancer cells *in vitro*. The deregulation of miR-155 could lead to an increase in MSI that contributes to the progression of colon cancer. Future *in vivo* studies are warranted.

Although, we were able to confirm that NF-kappa B was activated by S100P/RAGE signaling, our ChIP studies indicate that NF-kappa B may not be involved in regulating miR-155 expression at the transcriptional level. Therefore, the downstream targets controlled by NF-kappa B within the S100P/RAGE pathway remain to be established. As a result of this, we plan to analyze NF-kappa B target gene expressions using the mouse NF-kappa B signaling pathway PCR array which is capable of profiling 84 key genes responsive to NF-kappa B signal transduction. PCR array of total RNA isolated from tumor cells treated and control samples in each experimental condition will be performed according to the manufacturer’s instructions. We expect to identify NF-kappa B regulated-genes that will provide insight on the role of NF-kappa B in the S100P/RAGE pathway.

In Chapter 5, we identified downstream target genes in the S100P/RAGE pathway that are targets of miR-155. In these experiments, we provided evidence that WNK1 and ZNF493 are miR-155 target genes that are downstream of the S100P/RAGE pathway.
However, how WNK1 and ZNF493 contribute to colon cancer progression is not currently known. In regards to WNK1, other studies suggest that loss of WNK1 could regulate the SMAD2/TGFβ pathway (Lee, Chen et al. 2007). In addition, preliminary studies from our lab group indicate that S100P/RAGE activation results in the up-regulation of SNAIL and SLUG (Figure 7.2). Also, the pre-treatment of colon cancer cells with anti-RAGE antibody appears to abrogate the induction of snail and slug in a RAGE receptor dependent manner (data not shown). Furthermore, S100P treatment promotes invasion of LS174T colon cancer cells (Figure 7.3). Therefore, we postulate that S100P/RAGE/miR-155 induction contributes to colon cancer progression by the down-regulation of WNK1, which results in the activation of the SMAD2/TGFβ pathway (See Figure 7.4).

We have designed set of experiments to test this hypothesis. We will utilize the luciferase assay to show that WNK1 is a direct target of miR-155. The human wildtype-WNK1-3’UTR and mutant-wnk1-3’UTR luciferase constructs will be generated and cloned into pMiR-report vector. The mutant-wnk-3’UTR constructs will be mutated at the miR-155 binding sites. Our positive control for the luciferase assay will be 3’-UTR-SOS1. Luciferase assays will be performed with these constructs. Next, we will knockdown of WNK1 using ShRNA to recapitulate the effects of S100P/RAGE/miR-155 induction in colon cancer cells and analyze alterations in cell proliferation, colon formation, and migration assays. Finally, we will determine whether activation of the S100P/RAGE signaling leads to SMAD activation. Colon cancer cells will be subjected
to recombinant S100P treatment as previously described at different time period (i.e. 0, 6hs, 12hs and 24hs). SMAD activation will be analyzed using Western blot. WNK1 knockdown cells will also be used for the experiment to collaborate the initial results. We expect the experiments to show that WNK1 is a direct target of miR-155 and that down-regulation of WNK1 recapitulates S100P/RAGE/miR-155 functions in colon cancer cells. Lastly, we will observe elevation of activated SMAD in WNK1 knockdown cells.

In regards to ZNF493, there is no reported function in the literature. However, we intend to carry out the same experiments as described above to determine the function of ZNF493 in colon cancer. Owing to its name, we expect that the ZNF493 is a transcription factor. Since the antibodies against the ZNF493 protein are relatively new and untested for ChIPs, we will use the high throughput systematic evolution of ligands by exponential (HTSELEX) enrichment method to determine the ZNF493 binding elements and regulated genes. HTSELEX technique is frequently used for characterizing binding specificity of transcription factors (Jagannathan, Roulet et al. 2006; Jolma, Yan et al. 2013). Some of advantages of using HTSELEX are that it can analyze the sequence around the binding sites much longer than 30 base pair and does not require the purification of the binding protein (Zhao, Granas et al. 2009). The result from SELEX will be validated with EMSA, which is commonly used to characterize transcription factors. After determining the ZNF493 binding elements and regulated genes, we plan to confirm the expression of the ZNF493-regulated genes using qRT-PCR and Western blot analysis and investigate their roles in metastasis. Our experimental results will show that
ZNF493 is a direct miR-155 target in S100P/RAGE pathway. Down regulation of ZNF493 will likely promote tumor cell growth and migration, thereby recapitulating S100P/RAGE/miR-155 effects. Our results will also provide evidence as to whether ZNF493 is a transcription factor. Finally, we anticipate to link ZNF493 regulated genes to cancer progression.
Figure 7.2: S100P/RAGE signaling regulates EMT markers in colon cancer cells
Analysis of transcription factors repressors of E-cadherin, SNAI1 (Snail) and SNAI2 (Slug) in SW480 cells treated with exogenous S100P shows up-regulation of Snail and Slug as well as E-cadherin. Blocking S100P/RAGE signaling with a specific RAGE monoclonal antibody significantly decreases the levels of all the EMT markers. The levels of the E-cadherin repressors are higher in proportion to E-cadherin expression levels during the S100P induction. $P<0.05$ (**).
Figure 7.3: Exogenous S100P increases cell invasion in LS174T colon cancer cells
Seeded the LS174T cells at 50,000 cells into a transwell chamber and incubate overnight. Treated cells daily with 200 nM and incubated for 48 hours. Stained transwell with crystal violet for 1 minute and carefully was three times. Cells were counted under the light microscopy. The upperpanel is the pictorial representation and bottom panel is the graphical representation the raw data.
In Chapter 6, our studies focused on determining if pharmacological blockade of S100P/RAGE signaling reduces colon cancer cell growth \textit{in vivo}. We clearly established that M2AA has anti-cancer and anti-inflammatory properties. Moreover, we demonstrated that M2AA can abrogate S100P/RAGE signaling in colon cancer cells in addition to suppressing S100P/RAGE-induced CAM tumor growth and metastasis. However, little is known about the efficacy of M2AA in a mouse model of colon cancer. Hence, our future study will be centered on determining the efficacy of M2AA in an orthotopic mouse model of colon cancer. We will use murine MC-38 cells for these experiments. The cells are syngeneic with C57BL/6 mice and have been previously shown to express RAGE and its ligands in the intestinal tissue of mice (DiNorcia, Moroziewicz et al.). Luciferase-transfected MC-38 cells (1 x 10^6 cells) in 50 uL of PBS are injected into the cecum using a 30-gauge needle and a calibrated push-button controlled dispensing device (Hamilton Syringe Company). To prevent leakage, a cotton swab is held for 1 minute over the site of injection. The abdomen would be closed in one-layer with wound clips (Brantree Scientific). We expect to observe suppression of tumor growth and metastasis by M2AA in our mouse model.

In summary, we demonstrated here the importance of inflammatory pathways and miRNA changes and their role in colon cancer progression. Both inflammation and miRNAs are involved in tumor progression. These data provide new important insights and potential research directions. The results from these studies may lead to new markers
of metastasis detection and novel approaches to treat metastatic colon cancer in the future.

Figure 7.4: Proposed model of S100P/RAGE/miR-155 mediated colon progression
Indications from our pilot studies and other studies suggest a model that S100P/RAGE signaling leads to the stimulation of miR-155 expression through the MAP-kinase and NF-kappa B pathways. Elevation miR-155 level causes decreased WNK1 and ZNF493 expressions. The loss of WNK1 could lead to stimulation of TGFβ regulated genes and subsequently promotes EMT.
Accomplishment and Contributions

We have contributed in this important field by providing evidences implicating inflammation and miRNAs changes in colon cancer progression. These evidences provide new insights on the roles of inflammation and miRNAs, as key player in colon cancer pathogenesis. The knowledge gained from our work will improve understanding on colon cancer and can lead to the development of therapeutic strategies for the treatment of metastatic colon disease.
APPENDIX A: LIST OF PUBLICATIONS

Manuscripts


2. Melania E. Mercado-Pimentel, **Benjamin Chidi Onyeagucha**, Qing Li, Brenda Trevizo, Shahad Alabagi, Angel C. Pimentel, Giridhard Mudduluru, Heike Allgayer, Mark A. Nelson. “S100P/RAGE signaling plays a role in EMT through miR-21/RECK regulation.” (Accepted for publication).


Poster Presentations


4. Melania Mercado-Pimentel, **Benjamin Onyeagucha**, Qing Li, Mark Nelson S100P/RAGE Signaling regulates RECK mediated by miR-21 in colon cancer. 103rd Annual American Association of Cancer Research, Chicago IL, April 2012.
APPENDIX B: PERMISSIONS

Not applicable
REFERENCES


