THE ROLE OF PROSTAGLANDIN E_2/EP4 PROSTANOID RECEPTOR SIGNALING IN COLORECTAL CARCINOGENESIS

by

Anupama Chandramouli

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As members of the Dissertation Committee, we certify that we have read the dissertation

prepared by Anupama Chandramouli

entitled The Role of Prostaglandin E_2/EP4 Prostanoid Receptor Signaling in Colorectal Carcinogenesis

and recommend that it be accepted as fulfilling the dissertation requirement for the

Degree of Doctor of Philosophy

_____ Date: 05/13/2009
Mark A. Nelson, Ph.D.

_____ Date: 05/13/2009
Jesse D. Martinez, Ph.D.

_____ Date: 05/13/2009
Margaret M. Briehl, Ph.D.

_____ Date: 05/13/2009
Timothy G. Bowden, Ph. D.

_____ Date: 05/13/2009
Eugene W. Gerner, Ph.D.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: 05/13/2009
Dissertation Director: Mark A. Nelson

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SIGNED: Anupama Chandramouli

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DEDICATION

In loving memories of my great grand parents, Padmawati and Subramania Iyer and grand aunt, Bharathi Moorthy, all of whom succumbed to cancer. It is my constant hope that ongoing research in the field of cancer biology will help to improve diagnosis and therapy.

This dissertation is also dedicated to my parents Rama and K.V. Chandramouli and my brother Ajay Chandramouli, who provided the love and support for me to accomplish this dream. Special dedication goes to my aunts and uncles Shashikala and M. K. Bhaskaran, who encouraged me every step of my way and Usha and Monagur Muralidharan who opened their home and hearts to me.

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LIST OF ABBREVIATIONS

15-PGDH = 15 – hydroxyprostaglandin dehydrogenase
5-FU = 5 – fluorouracil
ACF = aberrant crypt foci
AKT = Ak strain transforming
AMP = adenosine monophosphate
AOM = azoxymethane
APC = adenomatous polyposis coli
BCA = bicinchonic acid
BSA = bovine serum albumin
BUB1 = budding uninhibited by benzimidazole 1
CAC = colitis associated cancers
cAMP = cyclic AMP
CIN = chromosomal instability
CMV = cytomegalo virus
COX = cyclooxygenase
CRC = colorectal cancers
CRE = cyclization recombination
CRE = cAMP response element
CREB = cAMP response element binding
DAB = diamino benzidine
DEPC = diethyl polycarbonate
DMSO = dimethyl sulfoxide
DNA = deoxyribonucleic acid
DTT = dithiothreitol
ECL = electro chemiluminescence
EGF = epidermal growth factor
EGFR = epidermal growth factor receptor
EP = eicosanoid prostanoid
ERK = extra-cellular signal regulated kinase
FAP = familial adenomatous polyposis
FFPE = formalin fixed paraffin embedded
FOBT = fecal occult blood test
GFP = green fluorescence protein
GPCR = G protein coupled receptors
HIF-1 = hypoxia induced factor – 1
HNPCC = hereditary non-polyposis colorectal cancer
IBD = inflammatory bowel disease
IKK = IκB kinase
IL = interleukin
IκB = inhibitor of NFκB

JNK = c-jun NH_2-terminal kinase
LV = leucovorin
MAP = mitogen activated protein kinase
MIN = multiple intestinal neoplasias
MLH1 = MutL homolog 1
MMR = mismatch repair
MSH2 = MutS homolog 2
MSI = microsatellite instability
NFκB = nuclear factor κ B isoform
NIH = National Institutes of Health
NSAID = non-steroidal anti inflammatory drugs
OCT = optical coherence tomography
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate buffer saline
PGD_2 = prostaglandin D_2
PGE_2 = prostaglandin E_2
PGES = prostaglandin E synthase
PGG_2 = prostaglandin G_2
PGH_2 = prostaglandin H_2
PGT = prostaglandin transporter
PI3K = phosphoinositide 3-kinase
PMSF = phenyl methyl sulfonyl fluoride
PPAR = peroxisome proliferator activated receptor
PTEN = phosphate and tensin homolog
PVDF = polyvinylidine fluoride
RAGE = receptor for advanced glycation end products
RNA = ribonucleic acid
ROS = reactive oxygen species
SDS = sodium dodecyl sulfate
Sp-1 = specificity protein – 1
SRB = sulforhodamine B
TAM = tumor associated macrophages
TBS = tris buffer saline
TCA = trichloroacetic acid
TCF/LEF = T cell factor/ lymphoid enhancer factor
TGFβ = transforming growth factor receptor β
TME = total mesorectal excision
TNF = tumor necrosis factor
TSG = tumor suppressor genes
TXA_2 = thromboxane A_2
UV = ultraviolet
VEGF = vascular endothelial growth factor
Wnt = Wingless and Int

ABSTRACT

Colorectal cancer, among other tumors, is characterized by elevated levels of prostaglandins due to the up-regulation of cyclooxygenase -2 (COX-2), a key enzyme in the eicosanoid biosynthesis pathway. Prostaglandin E_2 (PGE_2) is an important prostaglandin that exerts its biological function via four transmembrane G protein coupled receptors (EP1-4), among which the EP4 receptor is the most important. The relevance of EP4 receptor to the carcinogenic process and the consequences of its interaction with PGE_2 were explored in this dissertation.

Despite the importance of the EP4 receptor in colon carcinogenesis, studies looking at the receptor expression during cancer progression have not been extensive. One study showed that the protein levels of EP4 receptor were elevated in colon cancer whereas another study indicated that mRNA levels were decreased in tumor compared to normal. We expanded these observations and now report that the elevated protein levels of EP4 receptor in cancer are due to increased translation of proteins.

In addition, we identified S100P as a novel downstream target of the PGE_2/EP4 receptor signaling pathway. S100P has been previously implicated in a number of gastro-intestinal cancers such as pancreatic, gastric and colon cancers. However, its regulation via the PGE_2/EP4 receptor signaling pathway has never been investigated.

Here, we show that PGE_2 via the EP4 receptor signaling leads to the transcriptional activation of S100P and that this activation happens exclusively in the presence of CREB. In summary, this dissertation brings to light novel therapeutic targets which could be used as potential markers to stratify colon cancer patients as well as avenues for clinical intervention for the management of colon carcinogenesis.

CHAPTER 1:

INTRODUCTION AND BACKGROUND

Colorectal Cancer: a Public Health Perspective

The colon and rectum are two organs that are prone to more neoplastic conversions than any other organ in the human body (Kumar et al., 2007). Various insults to the gut, such as diet, pathogens and stress, often trigger a proliferative event within the lumen of the colon whereby the cells lining the gut epithelium grow abnormally and form protrusions called "polyps". Colon polyps are by and large classified as either 'non-neoplastic' that arise from abnormal mucosal maturation, inflammation or architecture (e.g. hyperplastic polyps) or 'adenomatous' or 'neoplastic' that arise from epithelial proliferation and dysplasia (Kumar et al., 2007). Adenomatous polyps have a high propensity to transform into cancers. Although hyperplastic polyps generally do not progress to carcinomas, a vast majority of hyperplastic polyps, particularly on the right side of the colon, show significantly high incidences of cancer (Richman and Adlard, 2002). In addition to polypoid tumors, occasionally "flat adenomas" also give rise to cancer. Such adenomas have a distinct flat shape and are considered to be more aggressive with a high tendency to invade surrounding tissue (Hart et al., 1998; Wolber and Owen, 1991).

Depending on the location within the colon and rectum as well on extent of spread, the clinical presentation of cancers may be variable. For the most part, the colon is a uniform organ. However, certain differences are present and govern how cancers may progress. Right-sided lesions often present with abdominal pain, weakness due to anemia, lethargy and a distinct abdominal mass. Left-sided tumors on the other hand, show signs of rectal bleeding along with constipation. One study showed evidence of high microsatellite instability (a key feature of colorectal cancers) in at least 20% of right sided cancers as compared to 1% in left-sided cancers (Elsaleh et al., 2000).

Cancers of the colon and rectum, collectively referred to as colorectal cancer (CRC), are the third most common type of cancers reported in the United States (2004). It remains a major malignancy in the westernized world and is becoming more common in countries that are adopting such a lifestyle. From an epidemiological context, it was observed that in immigrants from countries with low prevalence of colorectal cancer, such as Southern Greece or Italy, the overall incidence rates tend to increase after moving to host countries with high incidence, such as Australia or the United States (Rozen et al., 2006). This suggested that Western lifestyle and diet are major predicators of colorectal cancer. In addition, irrespective of diet certain families are at a higher risk for the disease. Other risk factors such as age, gender, racial descent, body build, presence of colon polyps and history of smoking also influence the probability of a person to develop colorectal cancer. In general, a male of 50 years or over,

particularly of African descent, has a higher individual probability of showing signs of colorectal cancer as compared to the rest of the population.

Management of Colorectal Cancer

Historically, research on colorectal cancer has seen a multitude of efforts since the mid-1970s. As of 2004, incidence rates for colorectal cancer have decreased substantially by as much as 26% since 1984 (Ries et al., 2007). A number of advances made in the fields of detection, surgery, chemotherapy and chemoprevention have helped this statistic. However, in spite of such advances made in the field of colorectal cancers, there were an estimated 148,810 new cases and 49,960 deaths due to colorectal cancer reported in the United States in both men and women in 2008 (Ries et al., 2007). Colorectal cancer remains the second leading cause of cancer-related mortality urging the need for incorporating a massive change in various aspects of management of this disease. Systematic preventative policies are just beginning to be implemented in the United States and worldwide. In contrast to management of breast cancer, colorectal cancer has had limited emotional appeal and political support in recent times (Edwards, 2007). Following is a brief account of the progress made to date in the management of the disease.

Advances in Early Detection

The process of colorectal carcinogenesis includes a number of distinct steps, some of which are clinically recognizable at premalignant stages. As colorectal cancer takes many years to progress, early detection of such pre-neoplastic events provides an excellent opportunity to improve rate of cure (Cancer Advances in Focus 2004). By far the most simple and common test developed for colorectal cancer screening is fecal occult blood test (FOBT), which tests for the presence of blood in feces. It is an inexpensive and non-invasive test that uses immunohistochemical detection of human hemoglobin (Rozen et al., 2006). More recently, a multi-target analysis of fecal DNA was performed by the Colorectal Cancer Study Group. Although still under clinical investigation, the study claimed to detect a high proportion of mutation profiles in DNA from colorectal neoplasias (Imperiale et al., 2004).

To date however, the gold standard in colorectal cancer screening has been the use of flexible sigmoidoscopy and colonoscopy. Both these techniques allow physicians to clearly visualize either the lower part of, or the whole colon, respectively. Studies have shown that although highly expensive and invasive, colonoscopy is not only effective in detecting precancerous lesions but also permits resection of pre-neoplastic polyps which may further develop into carcinomas if ignored (Winawer et al., 2003; Winawer et al., 2000). Additionally, innovative developments in optical coherence tomography (OCT) have enabled 3D viewing of the colon and rectum. Pilot studies from such virtual colonoscopy have been deemed as a non-invasive screening

alternative and are presently under clinical trials (Adler et al., 2009; Kimberly et al., 2009; Lansdorp-Vogelaar et al., 2009).

Advances in Surgery

Surgery has remained the primary treatment modality for colorectal cancer for over 35 years. In fact, 90% of colorectal cancers can be surgically resected with favorable prognosis when diagnosed early. Gradual assessment and changes in the surgical procedures have been occurring for the past 25 years. Surgery typically used to involve removal of the primary tumor along with some regions of the adjacent normal bowel, in a procedure called total mesorectal excision (TME) (Ridgway and Darzi, 2003). However, by late 1980s, surgeons started recognizing the possibility of local recurrence after surgery. Many studies identified the presence of clumped tumor cells in the portal blood of patients who had undergone surgical resection of the colon. To address this issue, Turnbull and colleagues used a technique of lymphovascular ligation, more popularly known as the "no-touch isolation procedure" (Maurer, 2004; Wiggers et al., 1988). In this technique, the blood supply to the colon and bowel lumen is sealed off prior to surgery, in order to reduce the risk of metastases manipulation by the surgeon (Hayashi et al., 1999).

An avenue where surgery has not shown promise is the long recovery period and whole body discomfort which often requires narcotics and oral analgesics for pain management. In recent times, more minimally invasive laproscopic techniques have

started gaining prominence. Four clinical trials comparing the use of laproscopic vs. conventional open surgery are still in play. Preliminary results from two of the studies suggest that cancer-related survival is significantly enhanced with laproscopic-assisted surgery (Boller and Nelson, 2007).

A major issue in surgery as a preventative measure against the spread of colorectal cancer is the variable outcome with colon and rectal cancers. In general, both no-touch isolation and laproscopic surgeries have shown high efficacy, particularly in cancers of the colon. However, such unanimous effects are yet to be seen in terms of rectal cancers. One reason for this discrepancy is the limitation of rectal tissue. The rectum is a smaller organ than the colon. In addition, removal of large amounts of rectal tissue compromises sphincter function. To date, laproscopic surgery has been able to show only minimal benefit towards rectal cancer. Although a few studies have reported some positive outcomes (low morbidity and mortality), TME by a skilled surgeon remains the accepted procedure for surgical intervention in rectal cancers (Boller and Nelson, 2007). Another potential impediment in the management of colorectal cancer is the high frequency of liver metastases (approximately 60%). The liver is the most common site for spread. To date hepatic resection has been the only successful curative form of treatment for metastatic colorectal cancer (Cromheecke et al., 1999).

Advances in Chemotherapy

Despite the high rate of surgical resectability, at least 50% of patients with CRC show variable degrees of metastasis and die of the disease. Survival for patients with colon cancer depends heavily on the disease stage and clinical parameters (extent of spread) and ranges from 60-80% for stage II vs. only 30-60% for stage III disease (Wu et al., 2004). The main reason for impeded success with surgery is that many patients with locally advanced tumors also have subclinical micro metastases, wherein viable individual tumor cells metastasize elsewhere prior to surgical intervention. Such tumor cells reside in the adjacent lymph nodes or bone marrow and are responsible for the spread of the disease after surgery. In contrast to colon cancer, rectal cancers show local recurrence instead of distant metastases.

Management of colorectal cancer with systemic treatment is multi-faceted. In general, systemic therapy for stage III colon cancer has been effective. However for stage II disease treatment is still under investigation. Three major avenues in clinical therapeutic practices include **adjuvant therapy** (where local recurrence and metastatic spread is prevented after complete surgical resection), **palliative therapy** (where survival is prolonged by controlling symptoms of patients with metastatic disease) and **neo-adjuvant therapy** (which involves pre-operative systemic therapy in order to enable secondary curative resection and increase relapse free survival). In case of rectal cancers, pre-operative radiotherapy is advantageous, however combined chemo-

adjuvant therapy protocols are yet to be established in clinical trials (Andre and Schmiegel, 2005).

For about 40 years, the only effective drug against colon cancer was 5-FU (5-fluorouracil). This drug specifically inhibits the enzyme thymidylate synthase which is required for *de novo* synthesis of thymidine nucleotides during DNA synthesis. Subsequently leucovorin (LV), a reduced folate biomodulator that enhances the activity of 5-FU, started being widely used in the United States and Europe (O'Connell, 1989). Today, the accepted standard of care for colon cancer is the FOLFOX regimen. This regimen administers the third generation platinum derivative, Oxaliplatin, with infusional 5-FU and LV and was inspired by the multi-center, international MOSAIC trial. Oxaliplatin is an alkylating agent that works synergistically with 5-FU causing bulky adducts to form in DNA and induces cellular apoptosis. This study randomized 2,246 patients with stage II/III colon cancer to receive the FOLFOX treatment and noticed an improved 3-year disease-free survival of 78.2% in combination as compared with 72.9% without oxaliplatin. The only major side effect against this regimen was dose dependent neuropathy escalating to partial or complete loss of sensory function, which could be easily reversed by taking patients off oxaliplatin (Andre et al., 2004). To date, the inclusion of Irenotecan, a campothecin derivative that blocks DNA replication by inhibiting Topoisomerase II, along with 5-FU and LV (FOLFIRI regimen) has not been able to show any benefit to colon cancer patients (Andre et al., 2004).

Targeted therapies have also proved to be of utmost significance in the field of colorectal cancer. Such a therapy entails interruption of specific molecular targets that lead to proliferation, angiogenesis, tumor spread and escape from apoptosis. Cetuximab, a humanized monoclonal antibody against the epidermal growth factor receptor (EGFR), has shown promising activity against tumor growth. Interestingly, use of Irenotecan in combination with cetuximab has a synergistic effect even in tumors resistant to irenotecans (Baselga and Albanell, 2002). Vascular endothelial growth factor (VEGF), responsible for blood vessel formation at tumor sites, is another molecular target that is over expressed in a number of tumors. The humanized monoclonal antibody, Bevacizumab, exerts an anti-angiogenic effect by neutralization of circulating VEGF. Two randomized combination Phase III clinical trials, one with Irenotecan, 5-FU, LV and Bevacizumab and the other with 5-FU, LV and Bevacizumab, showed excellent response rates resulting in the inclusion of bevacizumab in the FOLFOX regimen (Emmanouilides et al., 2004; Hurwitz et al., 2004). Recently, one study showed that specific gene expression signatures could help oncologists predict chemotherapy response in primary colon cancer tissue (Del Rio et al., 2007). Such studies emphasize the complexity of management of colorectal cancers and bring to light the pressing need for individualized therapy.

Advances in Chemoprevention

Although a number of advances have been made in screening, surgery and chemotherapy, most patients presenting with symptoms of colorectal cancer already have advanced stage disease. Due to patient non-compliance and limited awareness it has been impossible to screen all individuals who are at high risk of developing cancer. However, the individuals who do get screened have benefitted by planning their treatment regimen ahead of time. Thus, chemoprevention seems to be a good precaution, particularly for healthy individuals who have a strong family history or those who are susceptible for other reasons (Wang and Dubois, 2006). One promising avenue for chemoprevention of CRC is the use of anti-inflammatory drugs.

A number of epidemiological studies and case-controlled as well as clinical trial studies have demonstrated the benefits of regular use of non-steroidal anti-inflammatory drugs (NSAIDs) for the prevention of colon cancer. They act primarily by rebutting pain symptoms simultaneously targeting the cyclooxygenase (COX) enzymes. COX enzymes convert 20C plasma membrane lipids like arachidonic acid into oxygenated lipid signaling molecules such as PGE_2, PGI_2 and TxA2 (for a detailed account please refer to the section "Cyclooxygenase-2 (COX-2) and Colorectal Carcinogenesis"). Consumption of NSAIDs, such as aspirin and sulindac, over a period of 10-15 years has shown a reduction in the relative risk of colorectal cancers by 40-50% and is beneficial particularly to FAP patients, who otherwise do not have any documented alternatives for management of their condition (Gupta and Dubois, 2001;

Janne and Mayer, 2000; Kune et al., 1988; Rosenberg et al., 1991; Smalley and DuBois, 1997). However, the prolonged use of NSAIDs presents multiple gastrointestinal side effects such as abdominal pain, gastritis, peptic ulcers, nausea and renal toxicity due to the elimination of beneficial prostaglandins that protect the GI lining from harsh acidic conditions.

Research in the prostaglandin field has indicated that most of these side effects are manifested chiefly due to the involvement of constitutive COX-1 enzymes, and not due to inflammation-induced COX-2 effects (detailed description is provided in section "Cyclooxygenase-2 (COX-2) and Colorectal Carcinogenesis" and Figure 1.3). Thus, selective COX-2 inhibitors (COXIBS), such as celecoxib and rofecoxib were developed as the second generation of chemopreventative agents against colorectal cancers. These drugs retained anti-inflammatory and anti-tumor effects while the COX-1 mediated gastrointestinal toxicity was minimized by as much as 50% (Wang and Dubois, 2006). In a large scale case-controlled study it was determined that celecoxib (Celebrex®) could significantly decrease new adenoma growth by 33-45%. However, unexpected and severe cardiac complications were shown to be associated with the prolonged inhibition of COX-2 (Bertagnolli et al., 2006; Solomon et al., 2005a; Solomon et al., 2006; Solomon et al., 2005b). Nevertheless, use of Celebrex® (a COX-2 selective inhibitor marketed by Pfizer) is the only FDA approved chemopreventative drug for FAP patients, although surgical removal of the colon is the standard of care (Steinbach et al., 2000). Realizing the importance of inhibition of COX-2 pathway in bringing

down the incidence rates of colorectal cancers, research is being devoted to identify alternative targets in this pathway as potential chemoprevention strategies (Cha and DuBois, 2007).

The Multistep Model of Colorectal Carcinogenesis

Similar to most cancers, CRC shows a definite progression over time. Changes in the colon architecture result from the accumulation of a number of genetic as well as epigenetic events. These changes are responsible for the transformation of the normal colonic epithelium into adenomas and further into adenocarcinomas which entail morphological features resulting from distinct underlying molecular events. Describing the genetic basis of this sequence of events, Fearon and Vogelstein proposed a model for colorectal carcinogenesis (Figure 1.1). They suggested that colorectal tumors arise as a result of the activation of oncogenes or due to the inactivation of tumor suppressor genes (TSG). Oncogenes can be activated by mutation or translocation, whereas TSG are inactivated by mutation, deletion, truncation or methylation. Although both changes are common, the inactivation of TSG seems to govern the process. In addition, some mutant TSG display haploinsufficiency whereby, even in a heterozygous state the normal allele fails to function adequately (Payne and Kemp, 2005; Santarosa and Ashworth, 2004; Smilenov, 2006). Lastly they suggested that even though a definite sequence of events is preferred during progression, the total accumulation of genetic changes rather than the order of events is important.

When such genetic changes accumulate in a subset of colonic cells, they propagate often at rate higher than the surrounding cells thereby giving rise to a protruding mass or a polyp. An individual can live many years without showing any symptoms until this polyp accumulates enough number of changes to result in aggressive tumor. The events of transformation of a benign polyp to tumor are yet to be completely understood. Thus, colorectal carcinogenesis involves the classical steps of initiation (accumulation of genetic alterations in a cell), promotion (clonal expansion of initiated cells) and progression (irreversible shift to aggressive cancer) (Fearon and Vogelstein, 1990).

Understanding the importance of the first step of carcinogenesis, a number of research efforts have focused on genes that trigger tumor initiation. These genes directly control cell proliferation and allow cells to propagate uncontrollably when inactivated. Subsequent observations suggested a complex process driven by multiple genetics alterations constantly occurring in the genome at various sites on DNA (Kinzler and Vogelstein, 1997; Lengauer et al., 1997). Under the multistep carcinogenesis model, the genome of the cell is protected by three distinct lines of defense: “caretakers” (genes that maintain genomic integrity), “gatekeepers” (genes that specifically inhibit growth or promote death) and “guardians” (genes that protect the genome).

According to this theory, loss of caretaker function, by mutation or deletion, indirectly promotes tumor initiation by causing genomic instability of all genes (including gatekeepers). There are two major types of defects responsible for the disruption of caretaker function: **microsatellite instability (MSI)** that results from subtle changes in DNA base sequences and **chromosomal instability (CIN)** which involves alterations in chromosome number. In general, such instability occurs with age. However, in some individuals, deregulated cell cycle checkpoint genes (e.g. BUB1) that control kinetochore function or sister chromatid cohesion result in the CIN phenotype. Alternatively, presence of dysfunctional DNA mismatch repair (MMR) genes (e.g. MSH2, MLH1) result in the MSI phenotype. Such alterations are inherited from the parent in a dominant fashion and show 5-50 fold increased risk of developing colorectal cancer as compared to the normal population. Hereditary cancers such as hereditary non-polyposis colorectal cancer (HNPCC), also called Lynch syndrome, are autonomic dominantly-inherited forms of colorectal cancers that result from mutations in one of the five mismatch repair genes.

On the other hand, defect in gatekeeper function is considered to be the rate-limiting step during initiation of carcinogenesis. Both maternally as well as paternally inherited genes need to be altered for tumor formation. Individuals who are predisposed to CRC inherit an already defective copy of a gatekeeper such that the chances of fostering a mutation in the other allele are approximately 1000 fold higher than the normal population. In addition, genomic instability (that ensues in the cell due to lack

of caretaker function) hastens the occurrence of such defects in gatekeepers, exponentially increasing the probability of developing cancer. The most studied gene of the gatekeeper family is the adenomatous polyposis coli (APC) gene. APC is a tumor suppressor gene and a central player in the Wnt signaling pathway, which maintains epithelial integrity in the colonic mucosal surface. Inactivating APC mutations are observed in 85% of colorectal cancers. Another gene, also in the Wnt signaling pathway, β-catena, is responsible for the remaining 15% of cases. A genetic disorder, Familial Adenomatous Polyposis (FAP), is a germline autosomal dominant APC mutation that predisposes individuals to colorectal carcinoma. Although mostly benign, these individuals develop numerous adenomatous polyps by 20 or 30 years of age, some of which are guaranteed to develop into cancer during their lifetime.

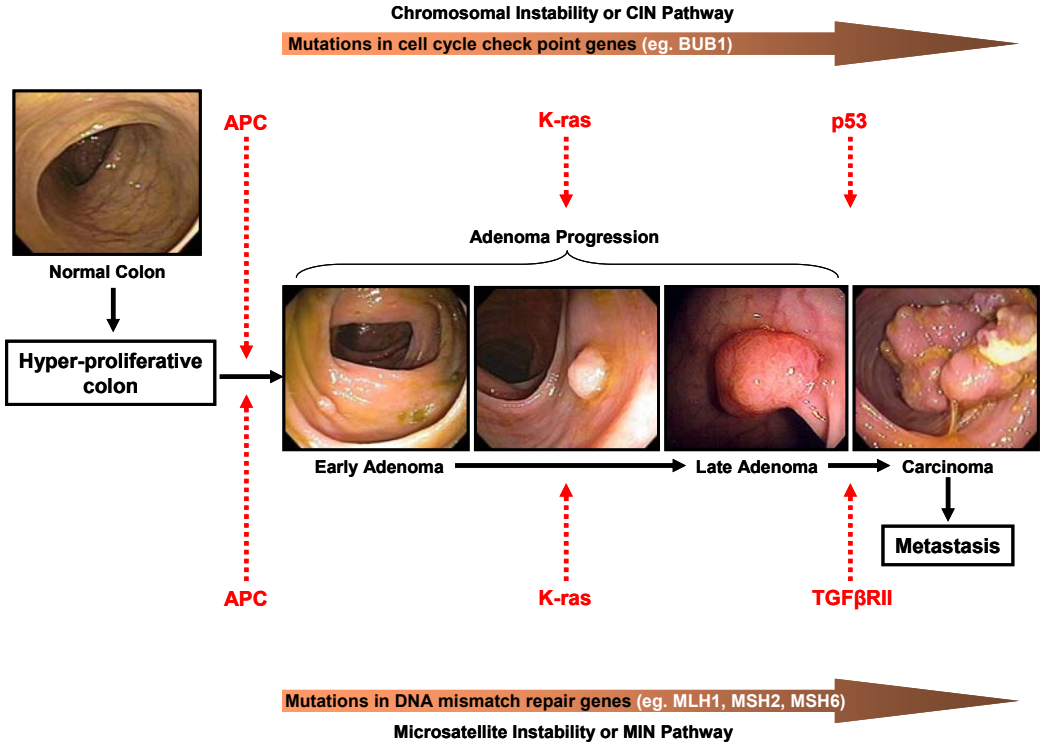

Figure 1.1: Molecular events leading to Colorectal Carcinogenesis Progression
Adapted from (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1997; Lengauer et al., 1997; Rozen et al., 2006).

The Role of Inflammation in Colorectal Cancer

Chronic inflammation, a term used to describe a collection of biological events orchestrated in response to external stimuli (such as pathogens, damaged cells or irritants), has long been known to trigger carcinogenesis (Balkwill and Mantovani, 2001; Rather, 1971). Fifteen percent of malignancies are caused by chronic inflammation (Kuper et al., 2000). This link between chronic inflammation and cancer involves a major interplay of the immune system. In the tumor microenvironment, release of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1, IL-6 and IL-8 in addition to reactive-oxygen radicals, growth factors and matrix disintegrating enzymes by the tumor cells, promote DNA damage (Coussens and Werb, 2002). Proliferation of tumor cells is also aided by tumor-associated macrophages (TAMs) which release immune factors and thereby provide a niche conducive for the tumors to flourish in (Sica et al., 2008). In a gastro-enteric setting, the condition called inflammatory bowel disease (IBD) has been most closely associated with cancer. Ulcerative colitis and Crohn's disease are examples of chronic inflammation of the gut tissue that increase the risk of developing colon cancer.

Nuclear Factor κ B (NFκB), an inflammation-induced transcription factor, is the most important protein in the symbiotic relationship between chronic inflammation and cancer (Karin, 2006; Marx, 2004). In general, in non-stimulated states, NFκB remains localized to the cytoplasm where it is inhibited by the Inhibitors of NFκB (IκB) proteins. Upon activation by pro-inflammatory cytokines during infection, IκB is

actively phosphorylated by IκB kinases (IKKs) and targeted for ubiquitin-mediated proteosomal degradation, thereby freeing NFκB subunits to translocate into the nucleus. Within the nucleus, NFκB transcriptionally activates a number of genes that signal to inhibit apoptosis, induce growth-promoting events and impart resistance to drug and radiation therapies (Marx, 2004; Vainer et al., 2008).

The role of NFκB in a gastrointestinal context is exemplified in cases of gastric and colitis-associated cancers (CAC). Ulcerative colitis results when the lining of the intestinal wall, particularly in the colon, becomes severely inflamed and develops into ulcers. It presents in individuals as early as 25-30 years of age. CAC results in such patients at a frequency of 8-43% and contributes to about 5% of all colorectal cancers (Greten et al., 2004). Epidemiological studies have shown that consumption of NSAIDs decreases the risk of developing CAC by at least 75-81% (Eaden et al., 2000). The downstream effects of NSAIDs, that are known to inhibit COX-2 and prostaglandin signaling, may also suppress NFκB signaling (Gupta and Dubois, 2001). It is of interest to note that COX-2 is a gene target of NFκB and the up regulation of COX-2 in tumor-associated macrophages is an early event in colon carcinogenesis (Hardwick et al., 2001; Janne and Mayer, 2000; Tsatsanis et al., 2006). In fact, the COX-2 gene promoter has a consensus sequence for the binding of NFκB proteins (Appleby et al., 1994). Recently it was shown that tissue-specific ablation of IKK in enterocytes and macrophages (two cell types documented to have activated NFκB expression during

colitis and CAC) drastically diminished the incidence and development of inflammation-associated cancer (Greten et al., 2004).

Cyclooxygenase-2 (COX-2) and Colorectal Carcinogenesis

Various inflammatory or immunological stimuli perturb cellular membranes by triggering the release of free fatty acids. Arachidonic acid is one such fatty acid that is released due to the action of phospholipases in response to hormones, ionophores, UV light, bee venom, tumor promoting agents and mechanical stress (Kuehl and Egan, 1980). Arachidonic acid is a 20-carbon fatty acid of the omega-6 family. In addition to its role as a lipid second messenger, it is a key inflammatory intermediate in the synthesis of oxygenated fatty acids called **prostaglandins**. Prostaglandins are hormone-like bioactive substances that mediate various patho-physiological processes in an autocrine and/or paracrine fashion (Konturek et al., 2005).

Cyclooxygenase (COX) enzymes are responsible for the oxygenation of arachidonic acid into prostaglandins. They convert arachidonic acid via a double dioxygenation reaction first into an endoperoxide derivative called Prostaglandin G_2 (PGG_2) and then to an alcohol called Prostaglandin H_2 (PGH_2). The two sequential reactions are distinct and for this reason COX enzymes are referred to as prostaglandin endoperoxide synthases or PGH synthases or PGG/H synthases. A unique property of the second oxidation reaction is peroxidase dependence, which is considered to trigger metabolic activation of carcinogens, providing a definite link between arachidonic acid

metabolism and DNA damage caused by reactive oxygen species (ROS). PGH_2 is the precursor for a family of bioactive lipids including prostaglandins, prostacyclins and thromboxanes (Figure 1.2). These lipid metabolites have distinct cell-type and tissue specific expression in the body. For instance, in the immune system PGE_2 is a key component of macrophages, PGD_2 is present on mast cells and TxA_2 is found in platelets. Primarily each prostanoid acts via its G-protein coupled receptor (GPCR) in order to trigger intracellular processes. Recently, signaling via nuclear hormone-receptors of the peroxisome proliferator activated receptor (PPAR) family has also been documented (Marnett and DuBois, 2002). Each prostanoid and its receptors have unique signaling roles in many physiological processes that are important in the human body. However, a discussion on each is beyond the scope of this dissertation. For relevance in colorectal carcinogenesis, this review will focus on the COX-2 and PGE_2 signaling mechanisms.

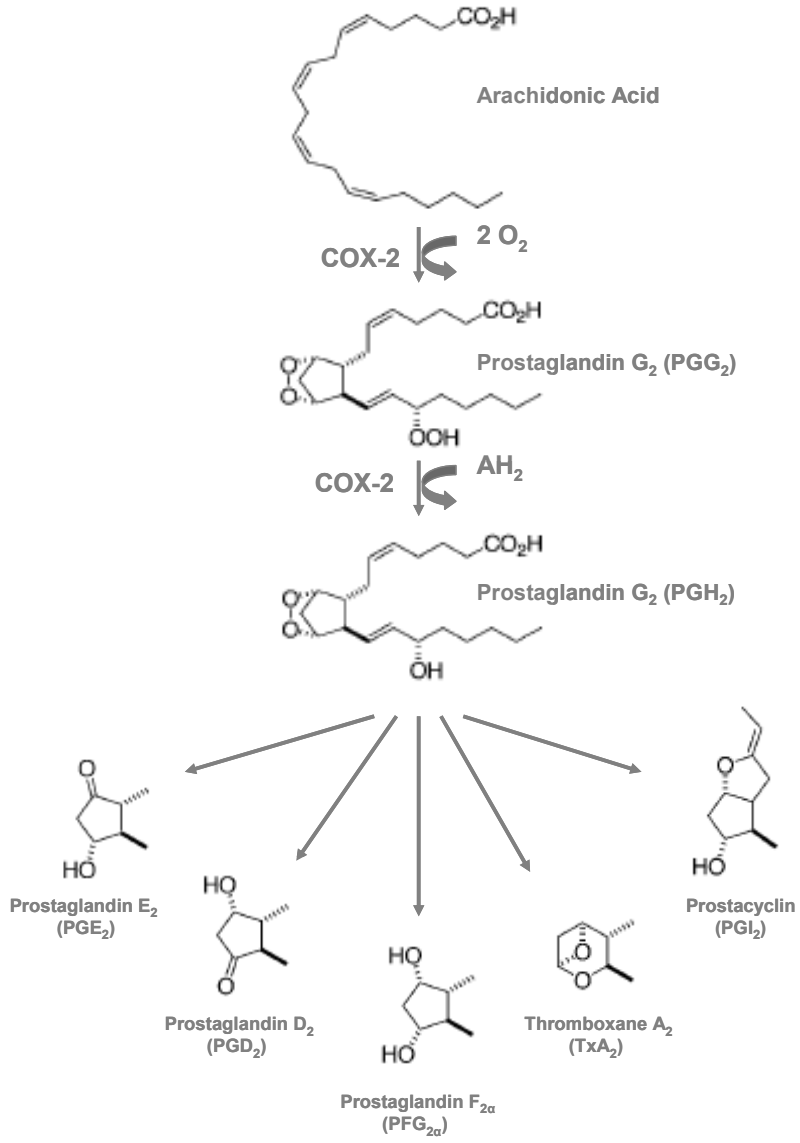

Figure 1.2: Eicosanoid Biosynthesis Pathway
20C Arachidonic acid is dioxygenated to result in PGG_2 and PGH_2 by the action of COX enzymes. PGH_2 is further converted by a range of enzymatic and non-enzymatic reactions into primary prostanoids: PGE_2, PGD_2, $PGF_{2\alpha}$, TxA_2 and PGI_2.

There are two main cyclooxygenase isoforms found in vertebrates: COX-1 (constitutive) and COX-2 (inducible). COX-1 is expressed in various tissues and the prostaglandins associated with it mediate cytoprotection of gastric mucosa, regulation of renal blood flow and platelet aggregation. COX-2, on the other hand, is induced only in response to specific stimuli such as growth factors, stress and inflammation, resulting in the synthesis of prostaglandins in inflamed and neoplastic tissues (Konturek et al., 2005). A yet unstudied isoform, sometimes referred to as COX-3, has also been recently reported. It is expressed in the brain and spinal cord of dogs where it mediates pain and fever and is believed to be an acetaminophen-sensitive isoform (Konturek et al., 2005). Some researchers suggest that the COX-3 isoform is a splice variant of the COX-1 enzyme (Berenbaum, 2004; Chandrasekharan et al., 2002; Kashfi and Rigas, 2005). Among these three isoforms COX-2 is the most studied. A number of agents including mitogens, inflammatory mediators, bile acids and growth factors induce the expression of COX-2 (Hardwick et al., 2001; Kim et al., 2006; Park et al., 2008; Song et al., 2007; Tsatsanis et al., 2006; Tucker et al., 2004).

Over the years, there has been strong evidence from multiple areas of research that have supported the pro-tumorigenic role of COX-2 enzyme. COX-2 expression was shown to be enhanced in azoxymethane (AOM)-induced intestinal adenomas in rats as well as $APC^{\Delta 716}$ mice (DuBois et al., 1996; Williams et al., 1996). AOM is a carcinogen, historically studied in Guamanian population associated with cycad flour consumption that induces O^6 and N^7 methylation changes in guanine nucleosides. The

$APC^{\Delta 716}$ mice on the other hand, have mutation in the APC tumor suppressor gene resulting in a truncated protein. These mice inherently posses multiple intestinal neoplasias (MIN) which frequently progress into intestinal cancers, much in the same way as humans with FAP (Rosenberg et al., 2009). Ectopic expression of COX-2 in mammary glands was shown to induce mammary hyperplasia, dysplasia and pro-metastatic transformation (Liu et al., 2001). In contrast, genetic depletion of COX-2 could demonstrate significant decrease in the number of intestinal polyps in $APC^{(MIN)}$ mice (Oshima et al., 1996). From an epidemiological standpoint specifically in colorectal cancers, COX-2 levels are known to be highly up regulated in at least 45% of colon adenomas and as much as 80% of colon carcinomas (Eberhart et al., 1994). This observation is also consistent with cancers of the breast, skin and pulmonary origins (Hwang et al., 1998; Prescott and Fitzpatrick, 2000; Sano et al., 1995; Sinicrope and Gill, 2004; Soslow et al., 2000; Taketo, 1998a; Taketo, 1998b). In addition, COX-2 mRNA levels were reportedly high and corresponded with endoscopic activity in patients with IBD indicative of a higher risk towards cancer progression (Hendel and Nielsen, 1997). Conversely, the administration of selective COX-2 inhibitors (Rofecoxib) in animal models as well as epidemiological studies could show a dramatic reduction in the number and size of intestinal polyps (Jacoby et al., 2000; Muller-Decker et al., 1999; Oshima et al., 2001; Steinbach et al., 2000).

COX-2 generates prostaglandins that regulate a number of cellular hallmarks of cancer such as growth and cell proliferation, tumor-associated angiogenesis, triggering

of the immune system, regulation of cell migration and invasion as well as evasion of apoptotic signals. Marnett and colleagues showed that the accumulation of a COX-2 derived byproduct, malondialdehyle, can directly form DNA-adducts resulting in frame shift mutations (VanderVeen et al., 2003). Such frame shift mutations, once established, particularly in mismatch repair genes, might lead to microsatellite instability and thereby initiate the process of carcinogenesis. Even though a number of cancer-related effects have been observed in conjunction with COX-2 over expression, the actual mechanism by which this happens is just emerging. Research has indicated that PGE_2 is a key metabolite often found in the tumor microenvironment that mediates the carcinogenic effects of COX-2 (Figure 1.3) (Cha and DuBois, 2007). Interestingly, the cardiac side effects associated with chronic administration of COX-2 selective inhibitors have been hypothesized to be attributed to prostaglandin mediators other than PGE_2, such as prostacyclins (PGI_2) and thromboxanes (TxA_2) (Marnett, 2008). One study showed that an imbalance in the levels of PGI_2 and TxA_2 results with the use of coxibs. Thus, inhibition of COX-2 could lead to PGI_2-mediated cardiovascular consequences (Fitzgerald, 2004; Murata et al., 1997). Taken together, these studies suggest that targeting the downstream COX-2/PGE_2 pathway could have higher potential for therapeutic intervention while avoiding the unwanted complications portrayed by the use of NSAIDs and coxibs (Cha and DuBois, 2007).

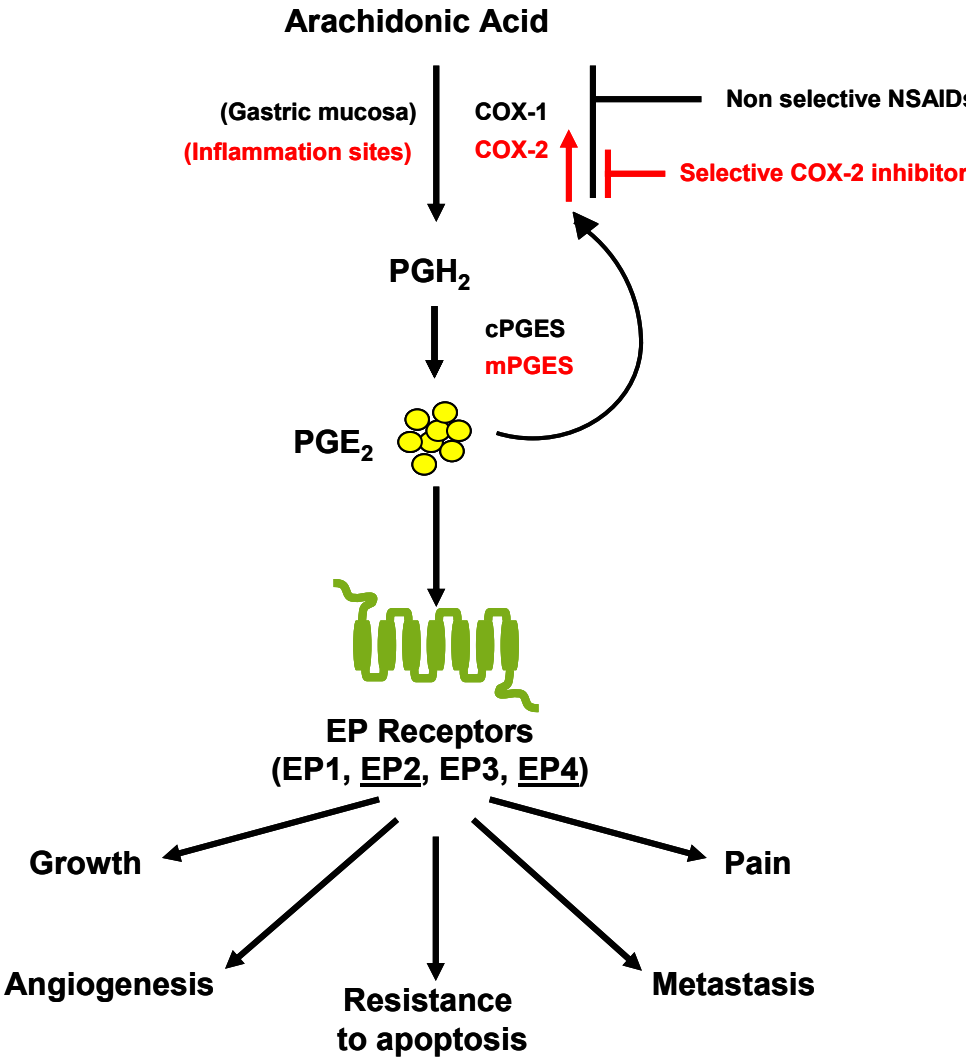

Figure 1.3: Schematic Representation of various Metabolites in the COX Pathway Cyclooxygenases (COX-1 and COX-2), are up regulated during colorectal carcinogenesis. These enzymes convert arachidonic acid into PGH_2 which is then enzymatically converted to PGE_2 by the action of PGE synthases (PGES). PGE_2 acts via four GPCRs among which EP2 and EP4 are implicated in colon cancer. A number of carcinogenic hallmarks are triggered via this response. In addition, PGE_2 can enhance the expression of COX-2 gene forming a feedback loop. COX enzymes have been therapeutically targeted by use of NSAIDs and selective COX-2 inhibitors.

Prostaglandin E_2 (PGE_2)

Prostaglandin E_2 is synthesized as a result of the isomerization of PGH_2 carried out by the prostaglandin E synthase (PGES) enzyme. Three distinct PGE synthases are known to date: cytosolic PGE synthase (cPGES) and microsomal PGE synthase-1 (mPGES-1) and -2 (mPGES-2). Among these, mPGES-1 is the only inducible form and seems to be colocalized, coregulated and metabolically coupled with COX-2. It is a membrane associated protein that is bound tightly to microsomal fractions during purification. A tight regulation exists between the activity and expression of COX-2 and mPGES-1 during colorectal cancers. Transcript as well as protein levels of both COX-2 and mPGES-1 are known to be elevated during colorectal carcinogenesis (Samuelsson et al., 2007; Shinji et al., 2005; Yoshimatsu et al., 2001). Recently, it was shown that a genetic deletion of mPGES-1 could suppress the appearance of intestinal tumors in $APC^{(MIN)}$ mice, providing a definite relevance for this enzyme in the COX-2/PGE_2 pathway (Nakanishi et al., 2008). In addition to synthesis, the steady state levels of PGE_2 in the tumor microenvironment are maintained by the enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) which degrades PGE_2 into an inactive 15-keto PGE_2 form. In colorectal cancers, loss of 15-PGDH correlates with tumor progression, indicating its clear tumor suppressive role. The 15-PGDH gene also happens to be a direct transcriptional target of the anti-proliferative TGF-β signaling pathway (Backlund et al., 2005; Cha and DuBois, 2007; Liu et al., 2008; Mann et al., 2006; Myung et al., 2006; Yan et al., 2004). In addition to the synthesis and degradation, a third level of control is maintained at the level of PGE_2 turnover. PGE_2

is generally perceived from the outside of the cell for signaling purposes. In order to get degraded by 15-PGDH, PGE_2 has to be pumped inside. While the efflux of synthesized PGE_2 occurs via simple diffusion, the influx of extracellular PGE_2 back into the cell requires the action of prostaglandin transporter (PGT) (Chi et al., 2006). It was recently shown that similar to 15-PGDH, the PGT expression is suppressed in human colorectal cancer as well as in APC $^{(MIN)}$ mice. This study also showed that ectopic expression of PGT maintains a high intracellular concentration of PGE_2 which is in turn converted into inactive 15-keto PGE_2 (Holla et al., 2008).

PGE_2 is considered to be the most important prostaglandin in colorectal carcinogenesis (Hull et al., 2004). PGE_2 itself is known to be elevated in FAP patients as well as in $APC^{(MIN)}$ mice in a polyp-size dependent manner (Kettunen et al., 2003). Exogenously administered PGE_2 enhances the growth of intestinal adenomas and worsens colorectal cancers (Kawamori et al., 2003; Wang et al., 2005; Wang et al., 2004a). It also protects $APC^{(MIN)}$ mice against NSAID-induced intestinal polyp reduction (Hansen-Petrik et al., 2002). In conjunction with COX-2, PGE_2 also plays a multitude of roles in colon cancer development by deregulating the various hallmarks of cancer (Greenhough et al., 2009; Hanahan and Weinberg, 2000; Mutoh et al., 2006). For instance, cells under the influence of PGE_2 often evade apoptosis when treated with highly selective COX-2 inhibitors. In HCA-7 and Caco-2 colorectal cancer cell lines, this occurs specifically due to induction of the anti-apoptotic Bcl-2 oncogenic protein via the Ras-MAPK/ERK or Ras-PI3K pathways respectively (Leone et al., 2007; Sheng

et al., 1998; Sheng et al., 2001). On the other hand, stimulation of cell growth, proliferation and survival by constitutively active Ras-MAPK pathway as a result of Kras and Braf mutations, or deregulated PI3K/AKT pathway from mutated PTEN and AKT proteins are common in colorectal cancers. In addition, PGE_2-mediated signaling represents a rescue effect wherever the aforementioned two signaling pathways have been inactivated. This provides self sufficiency of growth signals in cells that are committed to tumor development (Greenhough et al., 2009). Often times an additional impediment to cancer development are anti-growth signals that are maintained in healthy cells. This can occur at the level of cell cycle blockade or by the implementation of a "terminal differentiation state" on progenitor cells of the colonic crypts. Both these levels of control are broken in colorectal cancers by PGE_2-mediated deregulation of TGF-β signaling and abnormal activation of the APC/β-catenin pathways (Castellone et al., 2005; Markowitz et al., 1995; Massague, 2008; Siegel and Massague, 2003; van de Wetering et al., 2002).

In addition to facilitating cancer cell expansion and development, PGE_2 mediates crosstalk with other signaling pathways. One such example is the epidermal growth factor (EGF) signaling pathway which is either induced by PGE_2-mediated transcriptional amplification of Amphiregulin or by the transactivation of the EGF receptor (EGFR) (Pai et al., 2002; Shao et al., 2003). Sustained angiogenesis, another hallmark of cancer, is evident in studies showing stimulation of VEGF (vascular endothelial growth factor) expression, a growth factor that is known to induce the

formation of blood vessels in the vicinity of a tumor. Fukuda and colleagues showed that VEGF expression was stimulated by PGE_2 via the hypoxia induced factor-1 (HIF-1) protein, a transcription factor eminent under conditions of deprived oxygen such as a tumor microenvironment (Fukuda et al., 2003). COX-2/PGE_2 signaling also regulates an angiogenic switch representing a positive feedback (Wang and DuBois, 2004). Another unique property of cancer cells, not included in the original hallmarks, is the ability of tumors to evade immune surveillance (Tesniere et al., 2006; Zitvogel et al., 2006). One mechanism proposed by researchers involves a deceptive PGE_2-induced shift in cytokine production by antigen presenting cells which causes them to turn away from a "cell-killing" profile. This in turn reduces cytotoxicity against tumor cells which ultimately escape scrutiny by the immune system (Ahmadi et al., 2008; Harizi and Gualde, 2005; Harris et al., 2002). In another study, it was shown that PGE_2 could act as a pro-inflammatory signal, specifically in the gut, where it induces the expression of IL-8 (a pro-inflammatory cytokine) (Dey et al., 2009).

Intracellular Signaling via EP Receptors

The effects of PGE_2 on cellular responses appear to be mediated by its overall second messenger response. Intracellular signal transduction of PGE_2 occurs via four receptors namely EP1, EP2, EP3 and EP4. These receptors belong to three clusters within a family of membrane-spanning G-protein coupled receptors (GPCRs). Receptors EP2 and EP4 belong to one cluster and their activities are triggered via stimulatory $G_{\alpha s}$-mediated increase of intracellular second messenger cyclic AMP

(cAMP) levels. The other two clusters include the inhibitory EP3/$G_{\alpha i}$-coupled abrogation of cAMP levels and the EP1-G_q coupled accumulation of intracellular calcium levels (Cha and DuBois, 2007). In general, EP1, 2 and 4 receptors seem to contribute to pro-growth signals whereas EP3 receptor signaling represents anti-proliferative states.

Researchers have observed increased mRNA levels of EP1, EP2, and EP4 receptors, and decreased level of EP3 transcripts in murine carcinogen-induced colorectal cancers as compared to paired normal colonic mucosa (Mutoh et al., 2002; Shoji et al., 2004). In addition, the relevance of each receptor has been tested in studies using genetic knockouts in the murine intestinal tumor mouse models (Cha and DuBois, 2007; Kitamura et al., 2003; Regan, 2003; Ushikubi et al., 2000). For instance homozygous knockout of EP1, but not EP3 mice treated with AOM showed a 40% decrease in aberrant crypt foci (ACF) formation (Watanabe et al., 1999). Crossing APC $^{(MIN)}$ mice with $EP2^{-/-}$ mice showed a significant reduction in size and number of intestinal polyps. This reduction could not be observed in crosses with homozygous knockouts of EP1 and EP3 receptors (Sonoshita et al., 2001). Additionally, pharmacological inhibition by antagonists targeted either against individual EP receptors or the COX-2/PGE_2 pathway have shed light upon the importance of EP receptor signaling pathways in various cancers including those of colorectal origin (Mutoh et al., 2002). Although the above studies have provided a wealth of knowledge on the PGE_2/EP receptor signaling pathway, the role of individual receptor subtypes at

different stages of colorectal carcinogenesis is of utmost importance and has not been fully elucidated.

PGE_2 Signaling via the EP4 Receptor

There is a growing appreciation for the EP4 receptor as an important transducer of PGE_2 signals leading to cell invasion and motility during tumorigenesis. The EP4 receptor is over expressed in several different cancers including colon and rectal cancers (Cha and DuBois, 2007). Interestingly, constitutive expression of EP4 receptor promotes proliferation and anchorage-independent growth, demonstrating that the EP4 receptor may also be a key regulator of tumor progression (Chell et al., 2006). This receptor has been shown to have the highest affinity towards pro-tumorigenic PGE_2 ligand in ligand binding assays (Dey et al., 2009; Regan, 2003). EP4 receptor signaling also appears to be important for cell movement and motility during development. Studies in the zebra fish show that the EP4 receptor transduces PGE_2 signals to regulate appropriate speed of cell migration during gastrulation, demonstrating that regulation of cell motility by EP4 receptor signaling is evolutionarily conserved (Cha et al., 2006).

Signaling mediated through the EP4 receptor in colorectal carcinogenesis is constantly being appreciated. PGE_2 was shown to stimulate the proliferation and motility of LS174T adenocarcinoma cells through the EP4 receptor dependent activation of PI3K/AKT signaling (Sheng et al., 2001). Whereas PGE_2 inhibits apoptosis in human Caco-2 adenocarcinoma cells through an EP4 dependent pathway

(Leone et al., 2007). Furthermore, premalignant ACF formation in EP4 deficient mice following AOM treatment is suppressed as compared to those in EP4 wild type mice. This study also showed a reduction in colon adenomatous polyp formation in mice wild-type for the EP4 receptor but treated with the EP4 receptor antagonist ONO-AE2-227 (Mutoh et al., 2002). Treatment with another EP4 antagonist, ONO-AE3-208, decreased liver metastases after intrasplenic injection of MC26 colon cancer cells (Yang 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 in colon cancer cells (Cherukuri et al., 2007). Another avenue that PGE_2/EP4 signaling has been shown to contribute towards carcinogenesis is the Wnt signaling pathway. Interestingly, PGE_2 stimulated EP4 receptor can transcriptionally activate a transcription factor Tcf/Lef (T Cell Factor/Lymphoid Enhancer Factor) via a PI3K mediated pathway. This transcription factor regulates the expression of Cyclin D1 which is a key protein in cell cycle progression in colon carcinogenesis (Fujino et al., 2002; Tetsu and McCormick, 1999). Finally, a growing quest for the identification of drugs against colorectal and other cancers has encouraged pharmaceutical establishments to consider selective EP4 antagonists as novel therapeutic targets (Machwate et al., 2001; Mutoh et al., 2002; Omote et al., 2002; Shinomiya et al., 2001; Takayama et al., 2002; Yoshida et al., 2002). In addition, the EP4 receptor was determined to be a genetic risk factor in both ulcerative colitis as well as Crohn's disease in a study that used genome-wide associations to understand molecular pathways leading to IBD (Budarf et al., 2009). However, exactly how PGE_2/EP4

receptor activation contributes to colorectal cancer development *in vivo* still remains to be determined. Furthermore, the importance of downstream effector genes that are regulated via this pathway and that directly may affect carcinogenesis are recently being investigated. This dissertation will attempt to address these basic questions.

Statement of the Problem

Despite various advances in chemotherapy, detection, surgery and chemoprevention, colorectal cancer remains the third most commonly occurring cancer and the second leading cause of cancer related deaths in the United States (Seer Statistics Review, 2007). PGE_2/EP4 receptor-mediated events represent a key signaling pathway that is deregulated in colorectal cancers. A number of oncogenic hallmarks such as resistance to apoptosis, angiogenesis, cell proliferation and increased metastasis have been associated with PGE_2/EP4 receptor signaling pathway. Thus, studying and targeting this pathway for therapeutic intervention would be a positive step in the management of this disease. However, whether the EP4 receptor levels are aberrant in human colon cancers is not clear. Also, the cellular mechanisms by which PGE_2/EP4 receptor signaling contributes to colorectal carcinogenesis remain to be elucidated.

Overall Hypothesis:

It is hypothesized that the EP4 receptor expression is up-regulated during colon cancer development. Therefore, PGE_2/EP4 receptor signaling plays a functional role in the development of colon cancer.

Specific Aims:

1. To characterize the expression of EP4 receptor in human colon cancer specimens
2. To identify downstream targets of the PGE_2/EP4 receptor signaling pathway in colon cancer cells

CHAPTER 2:

MATERIALS AND METHODS

Chemicals and Antibiotics

PGE_2, PGE_1OH, GW627368, L-161,982 were purchased from Cayman Chemicals (Ann Arbor, MI). trichloroacetic acid (TCA), sulforhodamine B (SRB) dye was procured from Sigma-Aldrich (St. Louis, MO, USA). Tris, glycine and sodium dodecyl sulfate (SDS) were purchased from BioRad Pharmaceuticals (Philadelphia, PA, USA). All cell culture media were purchased from Invitrogen (Carlsbad, CA, USA). Graded alcohols and xylene for IHC studies were purchased from Sigma Chemicals (St. Louis, MO, USA). Hematoxylin and eosin solutions for viewing tissue sections were purchased from Richard Allan Scientific (Kalamazoo, MI, USA). Normal goat and horse sera for blocking during IHC were purchased from Invitrogen (Carlsbad, CA, USA) and Vector Laboratories (Burlingame, CA, USA) respectively.

Cell Culture and Maintenance of Cell Lines

LS174T, HCA 7, DLD 1, HCT 116, SW 480 and Panc 1 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in 1X Dulbecco's modified Eagle medium (DMEM) containing high glucose (4.5 mg/L), L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) and 5mg/mL penicillin-streptomycin. HEK 293 cells stably over expressing EP4 receptor were maintained in 1X DMEM supplemented with 10% FBS and 5mg/mL

penicillin streptomycin and containing 200 μg/mL geneticin (G418) for selection. MCF 7 cells were maintained in 1X Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and 5mg/mL penicillin-streptomycin. For PGE_2 exposure, a definite number of cells were plated either in 6 cm dishes (for RNA extraction), 10 cm dishes (for protein extraction), 6-well plates (for SRB assay) or 24-well plates (for dual luciferase assay). After propagation for 24 hours, cells were serum starved in OptiMEM® I Reduced Serum Medium for 20 hours before drug treatments. All media were purchased from Invitrogen (Carlsbad, CA, USA). All cell lines were grown at 37ºC under 5% CO_2 conditions.

Drug Treatments

Cell lines were plated in different sized dishes as indicated in previous section in medium containing 10% FBS. Subsequently, cells were serum starved in OptiMEM for 20 hours before treatment with indicated concentrations of PGE_2, PGE_1OH, GW627368X or L-161,982. All drugs were prepared in DMSO which was maintained in culture at concentrations < 0.1 %.

SRB Cell Survival Assay

Cell survival in response to different drug treatments was performed by the SRB assay as previously described (Skehan et al., 1990). Briefly, 8 X 10^4 cells were plated per well in a 6-well plate and allowed to grow for 24 hours. Cells were serum starved for 20 hours and subsequently stimulated with PGE_2, GW627368X or vehicle (DMSO) for

72 hours. For pre-treatment with GW627368X, cells were stimulated first for 2 hours with the antagonist and then treated with PGE_2 for 72 additional hours. Later, the viable cells were fixed in cold 50% TCA (to a final concentration of 10%) for 1 hour at 4ºC. Cells were then washed in deionized water and stained in 0.4% SRB dye (prepared in 1% glacial acetic acid) for 10 min at room temperature. Finally the cells were washed with 1% glacial acetic acid to remove excess SRB dye. SRB dye taken up by cells was released in solution by solubilizing them in 1M unbuffered Tris and incubated at room temperature with constant shaking for 10 min. Finally optical density measurements were taken using the BioMek Plate Reader at 540 nm. Percent survival was calculated by taking the ratio of cells treated with various drugs over vehicle (DMSO) treatment.

Methylene Blue Colony Formation Assay

Five hundred cells were seeded in 10 cm plates in triplicates with appropriate selection antibiotic (as indicated in relevant sections). Cells were incubated at 37ºC for 3 weeks to allow for colonies to form. Subsequently, media was removed and colonies were stained in Methylene Blue dye (0.5% dye dissolved in 50% methanol) at room temperature for 10 min. Plates were then gently rinsed in water and visible colonies were counted.

Immunofluorescence

Cells expressing GFP were grown on coverslips, washed twice with cold 1X PBS and then fixed in 4% paraformaldehyde (prepared in 1X PBS) for 20 min at room temperature. Cells were then rinsed two times in 1X PBS and placed upside down on glass slides containing a drop of DAKO mounting medium containing DAPI (Invitrogen, Carlsbad, CA, USA). Slides were incubated at 4ºC overnight for DAPI to penetrate the nuclei of cells and viewed by fluorescence microscopy.

Isolation and Purification of Plasmid DNA

The murine ptger4 cDNA was purchased from OpenBiosystems (Huntsville, AL, USA) as a bacterial stock stored in glycerol. The glycerol stock was streaked on LB-agar plates containing kanamycin (50 μg/mL) maintaining sterile conditions. Plates were incubated at 37ºC overnight. The following day, individual colonies were revived using sterile pipette tips and allowed to grow in 2 mL of liquid LB medium overnight. For knockdown of EP4 receptor, shRNA plasmids against control (shCtrl) and the EP4 receptor (shEP4) were purchased from SantaCruz Biotechnology (Santa Cruz, CA, USA) as packaged in Lentiviral particles. Plasmid DNAs were not isolated in this case for further cloning or purification purposes. Packaged viral particles were directly used for stable transfections (as described in the next section). For CREB knockdown experiments, empty vector (pCMV500) and dominant negative (pCMV500-ACREB) plasmids were a gift from Dr Charles Vinson, National Institutes of Health (Bethesda,

MD, USA). Promoter deletion constructs for s100p promoter (-236/+58, -124/+58 and -236/-14) were a gift from Dr. Silvia Pastorekova Institute of Virology, Slovak Academy of Sciences (Bratislava, Slovakia). Short hairpin RNA (shRNA) against scrambled sequence (shSCRM) and S100P protein (shS100P-1 and -2) were a gift from Dr. Carsten Müller-Tidow, Department of Medicine, Hematology and Oncology, University of Münster (Münster, Germany). Plasmid DNAs, obtained from the three above sources, were received as blotted on filter paper. The filter paper was carefully sliced into small pieces using a sterile surgical blade and solubilized using sterile distilled water. Five microliters of the DNA solution was transformed into chemically competent Top-10 bacteria (Invitrogen, Carlsbad, CA, USA). Briefly, chemically competent cells were incubated with DNA solution on ice for 20 min and heat-shocked in a 42ºC waterbath for 45 sec. Cells were snap cooled for 2 min on ice and then revived in SOC Medium (Invitrogen, Carlsbad, CA, USA) at 37ºC for 1 hour with regular shaking. Subsequently, the cells were spread on LB-agar plates containing the appropriate selection antibiotic overnight at 37ºC. The following day, bacterial colonies were revived using a sterile pipette tip into 2 mL of liquid LB medium (containing the appropriate antibiotic), grown overnight at 37ºC with constant agitation. Finally, plasmid DNAs were extracted using the Qiagen Mini Kit (Qiagen, Valencia, CA, USA). Bacteria containing plasmid DNAs were grown in 250 mL liquid LB medium overnight. Endotoxin-free plasmid DNAs were isolated from these liquid cultures using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). Purified plasmid DNA was quantified using a UV spectrophotometer (Eppendorf, Westbury, NY, USA).

Stable Transfection of Mammalian Cells

All transient transfections of plasmid DNA constructs were performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA). Briefly, an appropriate amount of cells was plated in cell culture dishes or plates. Equal volumes of DNA (diluted in OptiMEM medium) and Lipofectamine 2000 Reagent (diluted 1:25 in OptiMEM medium) were mixed and incubated in the dark at room temperature. The DNA: Lipofectamine mixture was gently poured over adherent cells in medium without antibiotic and incubated at 37ºC with 5% CO_2 for 6 hours. Subsequently, medium was replaced and cells were allowed to grow without selection for 16-20 hours. Finally medium was replaced again and cells were allowed to grow under appropriate selection for 72 hours. Transfection using Lentiviral particles (shCtrl and shEP4) were performed using Polybrene® (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells were plated at appropriate numbers according to manufacturer's directions for 24 hours. Medium was replaced with complete medium containing polybrene at a final concentration of 5 μg/mL. Lentiviral particles (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) were gently thawed at room temperature and 10 μL were used for infecting cells. The plates were swirled and incubated at 37ºC with 5% CO_2 overnight. The following day, medium was replaced with complete medium without Polybrene and allowed to grow for 24 hours. Finally, cells were selected under appropriate selection antibiotic for another 24 hours. For selection of stable clones, cells transfected with Lipofectamine (selected for 72 hours) or with Lentiviral particles (selected for 24 hours), were plated

at low cell density (500 cells/plate) in 10 cm plates with appropriate selection for 3 weeks. Individual colonies were cloned into separate wells of a 24-well plate and propagated with selection. Expression of protein in case of shRNA constructs was assessed by western blotting against targeted protein. In case of shSCRM and shS100P constructs, green fluorescence was observed for selecting stable transfectants.

Isolation of RNA from Mammalian Cell Lines

Cells that were treated appropriately were snap-frozen in liquid nitrogen and stored at –80ºC until RNA extraction. Cells were disrupted in RLT buffer and by passing them through Qiashredder columns. Total RNAs was extracted using the RNeasy Mini Kit and treated with DNase enzyme in order to avoid genomic DNA contamination before elution. The Qiashredder columns, RNeasy kit, and RNase-free DNase were purchased from Qiagen (Valencia, CA, USA). Amount of RNA in each sample was estimated in spectrophotometer (Eppendorf, Westbury, NY, USA).

Collection of Archival Tissue Specimen and IRB Approval

Archival tissue samples were collected from the Pathology Department, University of Arizona. IRB approval (Project No. 08-0681-04) was obtained from the Human Subjects Protection Program at the University of Arizona on 8 September, 2008 with an expiration date of 7 September, 2009 (Appendix 1). The requirement for obtaining informed consent was waived since the research involved no more than minimal risk to patients. Human colon tissue sections from surgically resected colorectal adenomas and

carcinomas, along with normal colonic mucosa (from healthy volunteers) were obtained. Normal tonsil and placental tissues have been used as controls for Ki67 and S100P respectively.

Isolation of RNA from Fresh Frozen Tissue Specimens

About 0.5 cm block was cut out of tissue specimens collected from the Pathology Archives. Each tissue sample was placed in 1 mL of Trizol and minced using a sterile tissue tearor. All instruments used for RNA extraction were washed thoroughly first in RNase-Away solution (Invitrogen, Carlsbad, CA) and then in RNase-free water. The tissue sonicator was washed first in RNase-Away solution and then in trizol reagent before mincing tissues. After tissues were thoroughly minced, 100 μL of chloroform was added and the samples were vortexed well to result in a homogenous mixture. Samples were spun at full speed at 4ºC for 10 min to pellet the cell debris. The aqueous solution was carefully recovered and mixed thoroughly with equal volume of 70% ethanol (prepared in DEPC-treated deionized autoclaved water) and immediately placed in RNeasy mini spin columns. Further steps in RNA extraction were performed as per manufacturer's directions. RNA yield was estimated by spectrophotometry (Eppendorf, Westbury, NY).

Reverse Transcription and Quantitative Realtime RT-PCR Analysis

Reverse transcription was performed using 1 μg of RNA from either frozen tissues or mammalian cell lines using the iScript cDNA synthesis kit (BioRad Pharmaceuticals,

Philadelphia, PA, USA). Reverse transcription was preformed in a thermocycler at 95ºC for 5 min, 42ºC for 2 hours and 85ºC for 10 min (Eppendorf, Westbury, NY, USA). Samples were stored at – 80ºC until further use in realtime RT-PCR analysis. For realtime analysis, 1 μL (50 ng) of the cDNA mix was added to a 25 μL reaction mix containing 1X Roche premix, 0.5 μM each of forward and reverse gene specific primers (Sequences detailed in the relevant section). Realtime RT-PCR was performed using Roche SyBr-Green reagents in the LC480 Light Cycler (Roche, San Diego, CA, USA). Relative quantification was performed using the $2^{-\Delta\Delta C}{}_{T}$ method (as described in (Livak and Schmittgen, 2001).

Isolation of Proteins from Mammalian Cell Lines

Cells treated appropriately were snap-frozen in liquid nitrogen and stored at – 80ºC until protein extraction. Cells were lysed first in cell lysis buffer (50mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Sodium deoxycholate and 1% Protease Inhibitor Cocktail, 1mM PMSF, 1 mM Sodium orthovanadate and Sodium Fluoride). Following lysis, the samples were centrifuged at 13,000 X g for 30 min at 4ºC. Protein yield was estimated by using the bicinchonic acid (BCA) assay (Pierce, Rockland, IL, USA).

Isolation of Proteins from Tissue Specimens

Approximately 0.5 cm block was cut out of tissue specimens into microcentrifuge tubes containing 500 μL of protein lysis buffer and minced using sterile tissue tearor (as described for RNA extraction). The tubes were centrifuged at 13,000 X g for 30 min at 4ºC to settle the cellular debris. Protein content was estimated using the BCA assay.

Western Blot Analysis and Antibodies

Equal amount of proteins isolated either from mammalian cell lines or from tissue specimens, were resolved by electrophoresis in a 10% SDS-polyacrylamide gel for EP2, EP4 or tubulin blots. For S100P blots, proteins were resolved by electrophoresis in a 15% SDS-polyacryamide gel. Proteins were then transferred onto PVDF (polyvinylidene difluoride) membrane by wet electric transfer at 30V for 90 min (and 30 min for S100P transfer). After transfer the membrane was then blocked in 5% non-fat dry milk prepared in 1X TBS-T buffer (Tris, NaCl, 0.01% Tween-20). Then the membrane was probed with appropriate primary antibody with gentle shaking for 3 hours, followed by gentle wash in 1X TBS-T solution. Membranes were finally probed with appropriate horseradish-peroxidase linked secondary antibodies, washed gently with 1X TBS-T and detected by electro chemiluminescence (ECL) method as per manufacturer's directions (Perkin Elmer, Waltham, MA, USA).

Processing of Formalin Fixed Paraffin Embedded Tissue Specimens

Surgically resected tissue specimens were fixed in 10% neutral buffered formalin solution (VWR, West Chester, PA, USA) for 24 hours and embedded in paraffin. Serial sections of 5 micron thickness were prepared from the paraffin embedded tissues. Tissues were dehydrated in graded alcohols and finally soaked in xylene. Paraffinized tissue sections were stained with hematoxylin and eosin (H&E) and subjected to review by a pathologist.

Immunohistochemistry (IHC)

Detection of EP receptors:

Paraffinized tissue sections were first de-paraffinized in xylene, followed by rehydration in a graded series of alcohols, ending with immersion in distilled deinozed water. Antigen retrieval was performed by microwave exposure in sodium (100 mM citrate buffer, pH 6.1). Endogenous peroxidase blocking was performed with 3% H_2O_2 in methanol and sections were blocked in 1.5% normal serum (goat serum for EP2 and EP4 IHC, horse serum for S100P IHC) diluted in 1X PBS-T solution (1X PBS: ; 0.1 % Tween-20). Sections were then incubated in primary antibody diluted appropriately in 1X PBS-T solution for 1 hour at room temperature. Following this, tissue sections were washed in 1X PBS solution and then incubated in secondary antibody appropriately diluted in 1X PBS-T solution. The sections were then treated with Vectastain Elite ABC Reagent, used according to manufacturer's instructions for 30 min at room temperature (Vector Laboratories, Burlingame, CA, USA). Finally, the slides were

soaked in diaminobenzidine (DAB) solution followed by Hematoxylin counterstain. Coverslips were placed on slides and sealed with mounting medium compatible with organic solutions.

Detection Ki67 nuclear antigen:

Cell proliferation was assessed by immuno-histochemistry staining for Ki67 antigen using anti-mouse KI-67 antibody (NovaCastro NCL ki67p raised in rabbit; at 1:500 dilution). IHC was developed using the biotin-streptavidin complex method developed by the TACMASS Core Facility by the GI-SPORE program at the University of Arizona, using the Discover® XT Automated IHC System (Ventana Molecular Discovery Systems Inc., Tucson, AZ, USA). To improve antigen detection, antigen retrieval was performed by microwave as described above.

Scoring of IHC Slides

For EP4 prostanoid receptor, staining was scored in two ways. First, intensity of staining was determined based on the following scale: no staining = 0, low intensity = 1, medium intensity = 2 and strong staining = 3. Second, the percentage of cells exhibiting the above staining intensities was calculated. Final score was determined as;

Score = intensity of staining X percentage of cells

Thus, each score ranges from 0 to 300 where zero represents no staining in 100% of cells to strong staining (intensity = 3) in 100% cells.

Antibodies for Western Analysis

Primary antibodies against EP4 and EP2 receptors, raised in rabbit, were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and used at a dilution of 1:5,000 for western blot analysis and 1:100 for IHC. Primary antibody detecting S100P protein, raised in goat, was purchased in R & D Systems (Minneapolis, MN, USA) and used at 1:1,000 dilution for western blot analysis and 1:50 for IHC. Anti-human tubulin antibody, raised in mouse, was procured from EMD (Gibbstown, NJ, USA), at 1:1,000 dilution for western blot analysis. Secondary antibodies anti-rabbit (raised in goat), anti-goat (raised in donkey) and anti-mouse (raised in goat) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). All secondary antibodies were probed at a concentration of 1:10,000 for western blot analysis. For IHC, secondary antibodies anti-rabbit (raised in goat) and anti-goat (raised in horse) were purchased from Vector Laboratories (Burlingame, CA, USA) and used as recommended by the manufacturer.

Site-Directed Mutagenesis of S100P promoter

To assess the promoter activity of s100p gene, pGL3 based deletion constructs were obtained from Dr. Silvia Pastorekova (Centre for Molecular Medicine, Institute of Virology, Slovak Academy of Sciences, Slovak Republic). Schematic representation of the sequences are detailed in Figure 4.14. The -236/+58 construct containing STAT and

CRE binding sites were mutated by mutagenesis using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). For this purpose, primers detailed below were purchased from Sigma Genosys (St. Louis, MO, USA):

MutSTAT-Fwd: 5'-GGGGAAAGGTgCCAcAAACGTCATCACAAC-3'

MutSTAT-Rev: 5'-GTTGTGATGACGTTTgTGGcACCTTTCCCC-3'

MutCRE-Fwd: 5'- GGGGAAAGGTTCCAGAAAgccCATCACAAC-3'

MutCRE-Rev: 5' GTTGTGATGggcTTTCTGGAACCTTTCCCC-3'

Bases indicated in lowercase and underlined were modified from the proximal sequence. These bases were shown to be important in multiple studies for promoter activities (Eresh et al., 1997; Yu et al., 1999; Zhang et al., 2005). Briefly, mutant strand synthesis was performed by thermal cycling with mutant primers by one cycle of denaturation at 95ºC for 2 min, 18 cycles of annealing at 95ºC for 20 sec, 60ºC for 10 sec and 68ºC for 8 min followed by 1 cycle of extension at 68ºC for 5 min. Parental methylated and hemi-methylated plasmids were subsequently digested with DpnI at 37ºC for 5 min and finally transformed into XL-10 Gold ultracompetent cells as per instructions provided. Colonies were finally revived under ampicillin (100 μg/mL), purified and propagated as described in previous sections for transient transfections.

Dual Luciferase Promoter Reporter Assay

Transient transfections were performed in LS174T cells. Briefly, 5 X 10^4 LS174T cells were plated in each well of a 24-well plate and transfected with the deletion and mutagenesis plasmids using Lipofectamine 2000 Reagent according to the

manufacturers's directions. After transfection, cells were serum starved in OptiMEM medium for 20 hours and treated with 1 μM PGE_2 for 24 hours. Subsequently, the activity of luciferase was assayed using the Dual Luciferase Reporter™ Assay (Promega, Madison, WI, USA) according to directions provided. Briefly, cells were first lysed in 1X Passive Lysis Buffer. In a sterile borosilicate tube, 50 μL of LARII reagent (firefly luciferase substrate) was added to 10 μL of the cell lysate. Luminescence was measured in the Sirius Luminometer (Berthold Detections Systems, Oak Ridge, TN, USA). Subsequently, 50 μL of Stop&Glo® reagent was added in order to simultaneously quench firefly fluorescence and introduce the Renilla luciferase substrate. Second measurement of luminescence represents the background luminescence of the samples. Promoter activity measured by this method is represented as a ratio between Firefly luciferase over Renilla Luciferase activities.

Statistical Correlations

All realtime quantitative analyses are represented as mean ± S.D. Significance was determined by the two sample Student t-test and considered significant at $p \leq 0.05$, $p \leq 0.01$ or $p \leq 0.001$ wherever indicated. Non-parametric and parametric analyses were utilized to study the EP4 receptor expression in human tissue specimen. Significance was determined by Wilcoxon Rank Sum test and considered significant at $p \leq 0.05$.

CHAPTER 3:

CHARACTERIZATION OF THE EP4 RECEPTOR EXPRESSION IN HUMAN COLON CANCER SPECIMENS

Introduction

The accumulation of PGE_2, an important metabolite in the prostaglandin signaling pathway, contributes to colorectal carcinogenesis. The intracellular signaling of PGE_2 through the EP4 receptor triggers multiple pathways leading to carcinogenesis. The protein expression of this receptor was shown to increase with progression from normal colonic epithelium to carcinoma, thereby providing strong evidence regarding the importance of the EP4 receptor in colon carcinogenesis (Chell et al., 2006). However, another group showed that EP4 receptor mRNA was decreased in tissues from colon cancer patients as compared to tissue from normal individuals. They also reported that that the EP4 receptor protein could not be detected in tumor cells (Gustafsson et al., 2007). Thus, there are conflicting data regarding the EP4 expression in colon cancers.

In order to clarify this controversy, we examined the protein expression of EP4 receptor in a subset of tissues from colon cancer patients and healthy volunteers. Immunohistochemistry (IHC) as well as western blot analyses showed elevated protein

expression of the EP4 receptor in adenoma and carcinoma as compared with normal tissue. By scoring the intensities of EP4 receptor staining, we have also determined that the difference in intensities between carcinomas and adjacent normal tissue taken from the same patient is significant.

Results

Investigation of EP4 Receptor Expression by IHC

To examine the protein expression of the EP4 receptor in colon cancer tissues, we obtained archival tissue samples from the Department of Pathology, at The University of Arizona with the appropriate IRB approval (Appendix). This collection of tissues contained surgically resected colon adenomas (polyps), carcinomas and metastatic tissues (lung, liver and lymph node). In addition, tissues from normal individuals were also obtained. Formalin fixed paraffin embedded (FFPE) tissue samples were processed and stained for the EP4 receptor as described in Materials and Methods. Figure 3.1 shows the tissue specific expression in normal individuals vs. carcinoma patients. These data show low or no staining in normal tissue whereas adenocarcinoma tissue stained strongly for the EP4 receptor. Lack of staining in the stromal tissue in both normal as well as cancer indicates specificity of staining to the colonic cells. In addition, there was increased staining in high grade carcinomas. We also performed EP4 receptor staining in a few adenomas and observed that these tissues express elevated levels of EP4 protein as compared to normal tissue (data not shown). Interestingly, in one case, colon cancer cells that had penetrated into the intravascular

space also showed elevated EP4 staining. Taken together these data indicate that expression of the EP4 receptor is enhanced progressively in colon tumors.

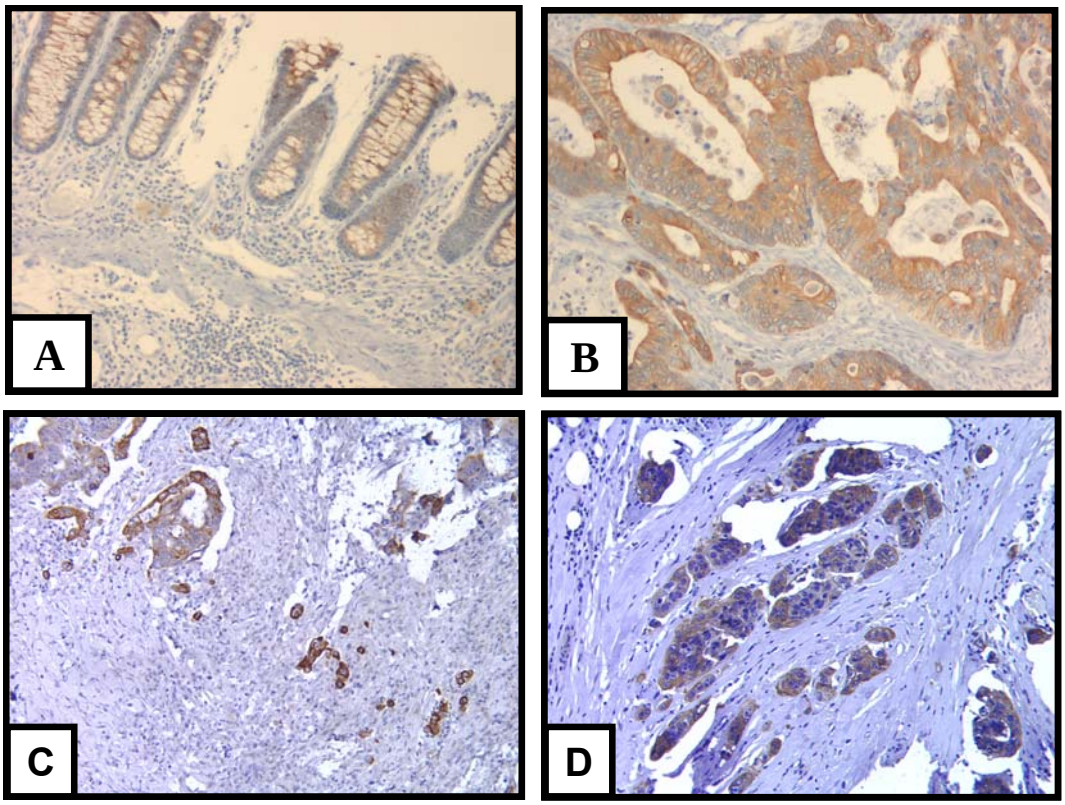

Figure 3.1: EP4 Receptor Expression in Human Colon Cancer Specimens
Immunohistochemical staining of EP4 receptor protein was performed in human tissue specimen (1:200 dilution). Tissues were counterstained with hematoxylin stain to view cellular detail. (A) Normal colonic mucosa. (B) Colon adenocarcinoma. (C) High grade colon adenocarcinoma. (D) Colon tumor cells localized within intravascular space. 200X Magnification.

Comparative Analysis of EP4 Receptor Expression during Cancer Progression

In order to determine whether the difference between EP4 staining in normal tissues vs. adenocarcinoma was significant, we performed pairwise comparisons. For each case, we first scored the intensity of EP4 receptor staining. Intensity of staining was determined visually by a pathologist and assigned a numerical value as follows: no staining = 0, low intensity = 1, medium intensity = 2 and high intensity = 3. Then, percentage of cells staining with a particular intensity was determined. Final scores were calculated as,

Final Score = Intensity of staining X Percentage of stained cells

Thus, final scores for each case ranged between 0 to 300 where zero represents no staining in cells and 300 represents strong staining in all cells. Finally, the mean score for normal tissue from a normal individual was determined to be 49 ± 38.22 (data not shown) whereas the score for normal tissue adjacent to a cancerous tissue was 83 ± 56.4 (Figure 3.2). By contrast, the mean score for EP4 receptor staining in cancerous tissue was 180 ± 71.1 (Figure 3.2). The mean score for adenoma was observed to be 50 ± 29.44 (data not shown). However, only four adenomas and four tissue from normal individuals were included in the collection giving rise to a high standard deviation. Thus, non-parametric analyses were used to determine whether the differences in scores between cancer and normal tissue next to tumors were relevant. Figure 3.2 shows box

plots for the EP4 staining scores indicating that EP4 receptor expression is significantly higher in cancer tissue than in adjacent normal areas ($p = 0.0026$).

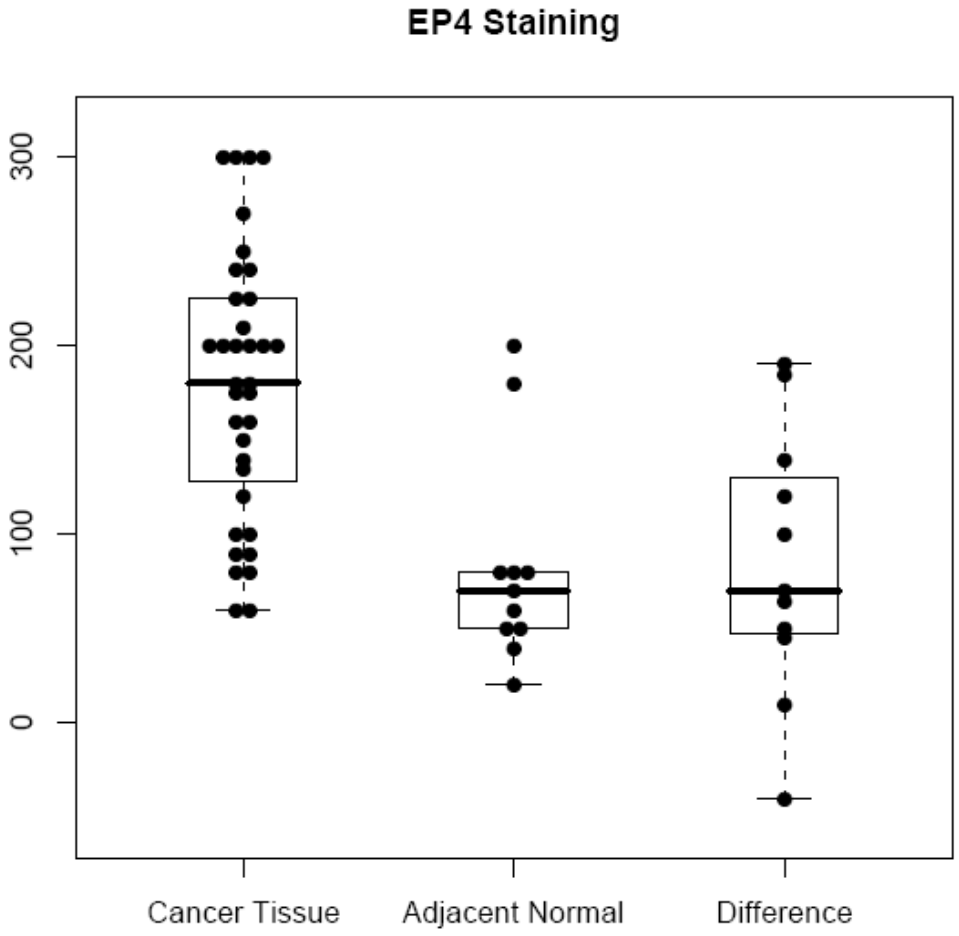

Figure 3.2: Pairwise Comparison of Differences in EP4 Staining Scores
Parametric and non-parametric analyses were used to analyze final EP4 scores in tumors vs. adjacent normal tissues. Raw scores for cancer tissue and adjacent normals are shown as a box and whisker plot. Difference between final scores (intensity X percentage) is also shown (extreme right) and determined to be significant using Wilcoxon Ram Sum test ($p = 0.0026$).

Analysis of EP4 Expression in Frozen Colon Cancer Specimens

Flash frozen surgically resected tissue specimens were also obtained from the Pathology Archives under proper IRB approval. RNA and proteins were extracted from these frozen tissues and used to perform realtime RT-PCR analysis as well as western analysis as described in Materials and Methods. Figure 3.3 shows that mRNA expression in tumor specimen was either reduced or similar to the levels in normal tissue. This is consistent with the data provided by *Gustafsson et al., 2007*. On the other hand, we observed a significant increase in protein expression of the EP4 receptor in tumors (T) as compared to matched normal (N) tissue from three patients (Figure 3.3). Equal loading was ensured by probing for α-tubulin. Taken together, the above data suggest that EP4 receptor protein is elevated in tissues from colon cancer patients and that the corresponding mRNA levels are not significantly altered.

Additionally, murine studies using genetic knockout of EP receptors have indicated that both EP4 as well as EP2 receptors are protumorigenic in response to PGE_2 signals. Biochemical studies also report that both receptors can bind to PGE_2 ligand. However, it was not clear which receptor plays a major role in colon carcinogenesis. Thus, the protein expression of the EP2 receptor was also investigated in the same set of tumors with matched normal tissue. Figure 3.3 indicates that protein levels of the EP2 receptor are not significantly different. These data clearly indicate that the up-regulation of EP4 receptor, rather than EP2 receptor, is prevalent in colon cancer specimens.

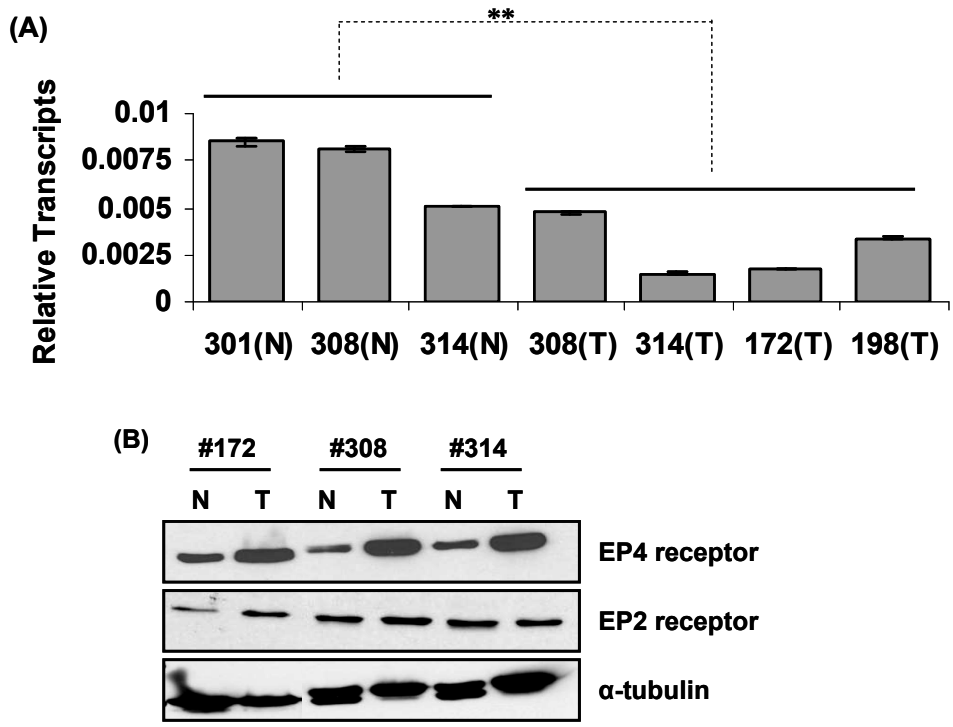

Figure 3.3: EP4 Receptor Expression in Frozen Colon Cancer Specimen
(A) RNA was extracted from frozen tissue specimen from patients with colon cancer (T). Adjacent normal tissues (N) were also utilized. Realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized to the β-actin gene. Relative quantification was performed. Differences in relative transcripts between normal and tumor samples are compared and represented as a bar graph. Values are shown as Mean ± SD and ** represents $p < 0.01$. (B) Protein was extracted from matched tumor (T) and normal (N) tissues of three patients with colon cancer. Western blot analysis was performed to detect EP4 and EP2 receptors. Equal loading was confirmed by probing with α-tubulin antibody.

Effect of Inhibition of EP4 receptor on Growth and Cell Survival

In order to study the relevance of the receptor *in vitro*, we decided to choose a colon cancer cell line that expresses adequate levels of EP4 receptor. Protein was extracted from HCA7, LS174T, DLD1, HCT116 and SW480 cells and western analysis was performed (Figure 3.4). Human kidney epithelial HEK 293 cells (ATCC # CRL-1573) that were stably transfected with the gene for EP4 receptor (PTGER4), (a gift from Dr John Regan, Department of Pharmacy) were used as a positive control. These data show that the expression of EP4 receptor in different cell lines is variable. LS174T, DLD1 and HCT116 cells showed high expression levels of the EP4 receptor. LS174T (ATCC # CL-188) is a highly aggressive colon epithelial cell line derived from a 58 yr-old Caucasian female who was diagnosed with Dukes' type B colorectal adenocarcinoma. Although both DLD1 and HCT116 cells showed expression levels similar to LS174T, we chose LS174T for our subsequent studies. This is because LS174T cells also have adequate levels of S100P (a protein which is discussed extensively in Chapter 4). DLD1 and HCT116 cells, on the other hand, do not express high levels of S100P protein (data not shown).

Furthermore, to understand whether inhibition of the EP4 receptor could affect cell survival, LS174T cells were treated either with DMSO (vehicle control), 2 μM PGE_2 (to stimulate EP4 receptor with high affinity) or 5 μM GW627368X (a selective EP4 receptor antagonist). Drug treatments were performed as detailed in Materials and Methods. Cells were grown in the presence of the drugs followed by SRB assay. The

experiment was performed in triplicates in 6-well cell culture plates and repeated at least three times for consistency.

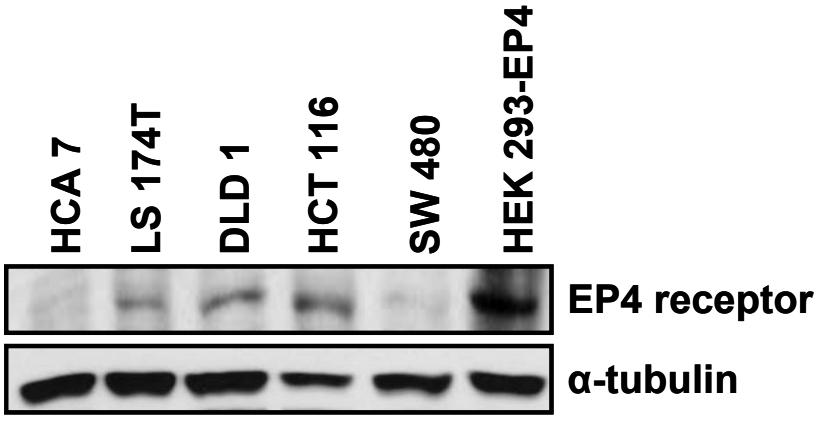

Figure 3.4: EP4 Receptor Expression in Colon Cancer Cell Lines
Various colon cancer cell lines harvested and western blot analysis was performed using antibody against the EP4 receptor. Equal loading was confirmed by probing with α-tubulin antibody.

Figure 3.5 is a representative summation of the results from one such assay. These data show that PGE_2 could effectively stimulate the proliferation of LS174T cells by 8% compared to vehicle control. On the other hand in the presence of EP4 antagonist, the proliferative potential was diminished by 45% compared to vehicle control suggesting that inhibition of the EP4 receptor diminished cell survival. Additionally, the reduction of cellular growth with use of EP4 antagonist was maintained even in the presence of 2 μM PGE_2, suggesting that growth was favored by the activation of the EP4 receptor by PGE_2.

In order to inhibit the EP4 receptor by a genetic approach, we stably transfected LS174T cells with Lentiviral particles containing shRNA plasmids targeted against EP4 receptor (shEP4). Cells transfected with shRNA targeted against a scrambled control sequence (shCtrl) were used as controls. Colony growth, assessed by counting the number of visible colonies stained with methylene blue, was drastically diminished in shEP4-transfected cells as compared to shCtrl-transfected cells (Figure 3.6). These data suggest that the genetic suppression of the EP4 receptor causes a significant decrease in colony number.

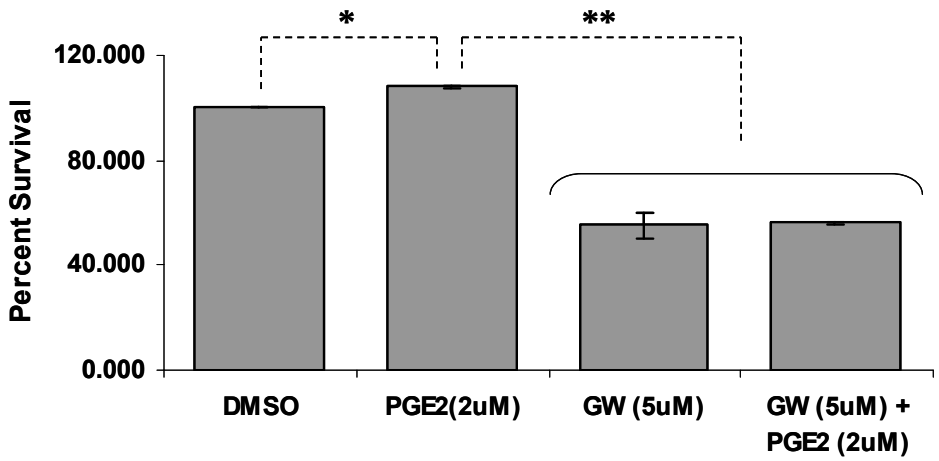

Figure 3.5: Effect of Pharmacological Inhibition of EP4 Receptor on Proliferation LS174T cells were treated with DMSO, PGE_2 (2μM), GW627368X (5μM) for 72 hrs or pre-treated with GW627368X (5μM) for 2 hours followed by PGE_2 (2μM) treatment for additional 72 hrs. Cell survival was measured by SRB Assay. Data is represented as percent survival compared to DMSO treatment, Mean ± S.D. * $p < 0.05$; ** $p < 0.01$.

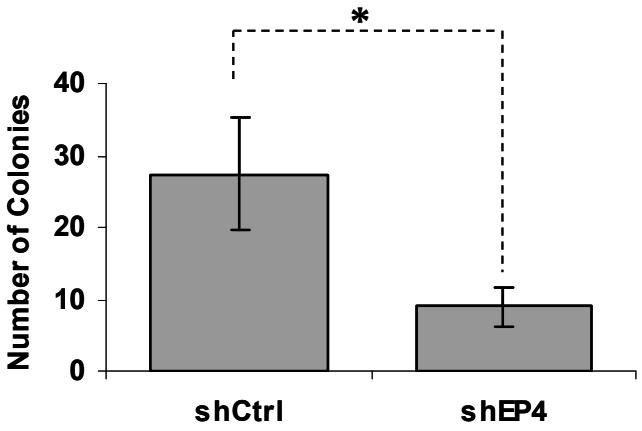

Figure 3.6: Effect of Genetic Knockdown of EP4 receptor on Colony Formation
LS174T cells stably transfected with shEP4 or shCtrl were seeded at 500 cells/plate in 100mm dishes. Cells were grown under 2µg/mL puromycin selection and incubated for 3 weeks to allow for colony formation. After 3 week, medium was aspirated and colonies were stained with methylene blue solution. Number of visible colonies was counted. Values are shown as Mean ± SD and * represents $p < 0.05$.

Discussion

This series of experiments was performed to clarify whether the EP4 receptor is up-regulated in human colon cancers. In our studies, we found that the EP4 receptor expression was markedly elevated in human colon cancers compared to adjacent normal tissue within the same patient. We also observed that the EP4 receptor expression in adenomas was slightly elevated. However more cases need to studied before a final conclusion can be made regarding the EP4 receptor expression in benign colon cancers. Nevertheless, our data suggest that elevated EP4 protein levels seen in our studies may correlate with late stage disease, because EP4 receptor over-expression was seen in both primary and metastatic colon cancer lesions.

Our immunohistochemistry findings are in agreement with the previous studies by *Chell et al., 2006* which demonstrated that the EP4 protein expression was elevated in primary adenocarcinomas. In addition, our results also agree, in part, with the observations made by Gustafsson et al., 2007. This group found that the EP4 receptor mRNA levels were higher in normal and stromal tissue compared to mRNA isolated from tumor cells. However, IHC studies performed by Gustafsson et al., 2007, show that EP4 receptor was not detected. One explanation for this disagreement could be variances in antibodies and conditions used for IHC. Utilizing western analysis on paired normal and tumor specimens, we were able to verify that the EP4 receptor protein levels are elevated in colon adenocarcinoma specimens. Our IHC findings suggest that the elevated EP4 receptor expression seen in our studies as well as the

studies performed by Chell et al., 2006 are due to increased translation of proteins. Thus, further studies are needed to investigate the mechanisms by which enhanced expression of EP4 receptor occurs during colon cancer progression.

Our EP4 receptor expression studies in human colon cancer specimens suggest that the EP4 receptor may be a therapeutic target for colon cancer. Other studies have also shown that administration of EP4 selective antagonists reduce colon polyp size and number in $APC^{(MIN)}$ mouse model in addition to murine liver metastases (Kitamura et al., 2003; Mutoh et al., 2002; Yang et al., 2006). Our cell culture studies using EP4 receptor antagonist and RNA interference based approaches as well as *in vivo* chick embryo assay (courtesy of Durga Cherukuri, Ph.D.; data not shown) support these observations. Therefore, further preclinical testing of the efficacy of EP4 receptor antagonists for malignant diseases is warranted.

CHAPTER 4:

IDENTIFICATION OF NOVEL DOWNSTREAM TARGETS OF THE PGE_2/EP4 RECEPTOR SIGNALING PATHWAY

Introduction

In Chapter 3 we have demonstrated that the EP4 receptor expression is aberrant in colon cancers. However, the downstream target of PGE_2/EP4 receptor interaction remains an active area of investigation (Fujino et al., 2002; Leone et al., 2007; Sheng et al., 1998; Sheng et al., 2001; Tetsu and McCormick, 1999). Previous studies in our laboratory have shown that PGE_2/EP4 receptor signaling activates the transcription factor CREB via ERK/MEK pathway (Cherukuri et al., 2007). Studies employing genetic as well as pharmacological inhibition of CREB have shown that it can suppress growth of cancer cell lines, including endometrial, ovarian as well as colon cancer (Catalano et al., 2009; Corona et al., 2007; Linnerth et al., 2008). However, the downstream target genes activated by PGE_2/EP4 → CREB sequence have not been identified. We wanted to investigate what downstream genes may be induced by the PGE_2/EP4/ERK/CREB pathway in colon cancer cells (Figure 4.1).

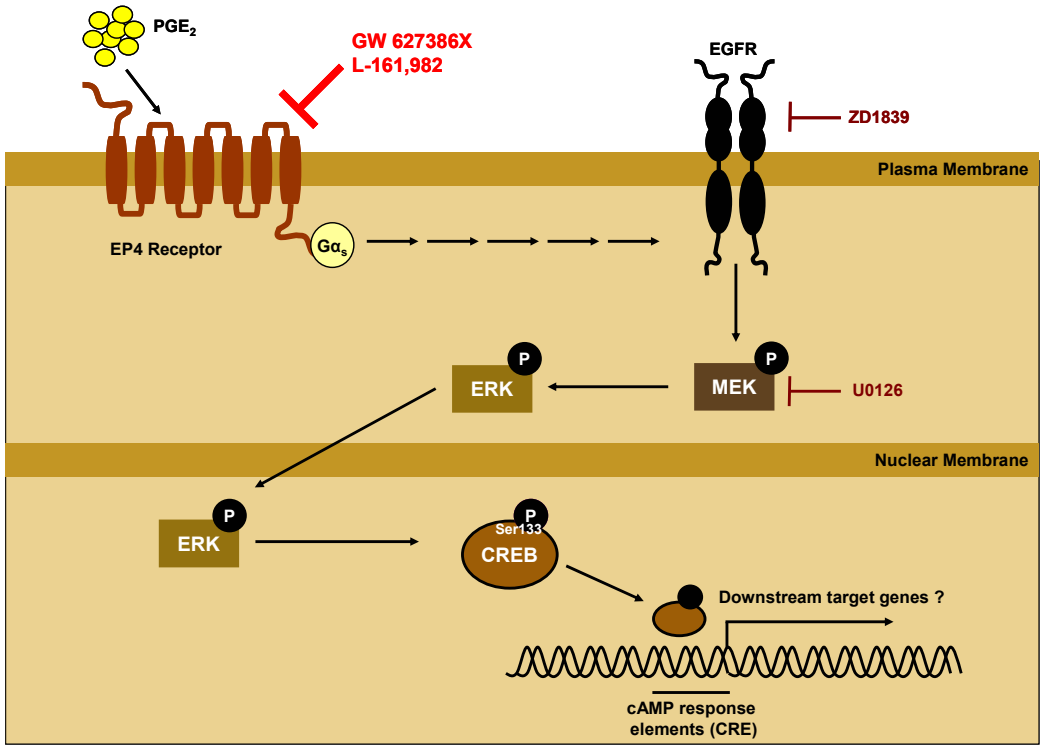

Figure 4.1: Downstream Target Genes of PGE_2/EP4/CREB Signaling Pathway
The activation of EP4 receptor by PGE_2 leads to the phosphorylation of ERK (via EGFR) and its downstream MEK pathway. Upon phosphorylation, ERK translocates to the nucleus and activates CREB by phosphorylation at the Ser^{133} residue. Compiled from (Cherukuri et al., 2007).

Results

Effect of CREB Mutant on Colon Cancer Cell Growth

In order to understand the function of CREB protein in colon cancers induced by PGE_2/EP4 receptor signaling, we asked the question whether the transcription factor could affect the growth of colon cancer cells. Two colon cancer cell lines (LS174T and HCA7) were stably transfected with vector control or dominant negative construct against CREB (a gift from Dr Charles Vinson, National Institutes of Health, Bethesda, MD). The dominant negative protein, termed ACREB, dimerizes with wildtype CREB protein and prevents it from binding DNA sequences. Colon cancer cells stably expressing either pCMV500 vector alone or pCMV500-ACREB construct were plated under neomycin (G418) selection and allowed to colonize for 3 weeks. Figure 4.2 shows that the number of methylene blue stained colonies were significantly decreased in cells transfected with non-functional CREB protein as compared to those transfected with vector alone. These data suggest that the knockdown of transcription factor CREB can significantly diminish colony growth.

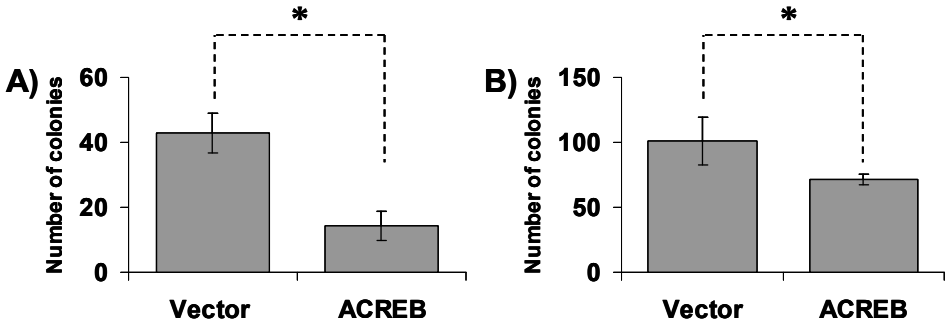

Figure 4.2: Effect of Dominant Negative CREB on Colony Growth
HCA 7 (A) or LS174T (B) cells stably transfected with pCMV500 (Vector) or pCM500-ACREB (ACREB) constructs were seeded at 500 cells/plate in 10 cm cell culture dishes. Cells were grown under 200 µg/mL Geneticin (G418) selection and incubated for 3 weeks to allow for colony formation. After 3 weeks, medium was aspirated and colonies were stained with methylene blue solution. The number of visible colonies was counted. Values are shown as Mean ± SD and * represents $p < 0.05$.

Downstream Target Genes of PGE_2/EP4 Receptor Signaling Pathway

In order to identify target genes which are triggered by the transcription factor CREB in response to PGE_2/EP4 receptor signaling, we analyzed a microarray data-set that was generated by Dr John Regan's laboratory (Department of Pharmacy). The microarray experiment was designed to identify genes that were differentially regulated in the presence of PGE_2 in HEK293 cells that have stable ectopic expression of EP4 receptor. Figure 4.3 shows the approach that was taken to analyze the microarray data. A total of 39 genes were significantly up regulated at least 3 fold in PGE_2-treated cells ($p < 0.001$). The data were a culmination of three independent experiments. Among these genes, a gene encoding for a calcium binding protein, S100P, was chosen for further validation and investigation based on the following criteria:

- S100P plays an important role in carcinogenesis
- S100P shows aberrant expression in gastrointestinal cancers including gastric and pancreatic cancers
- The promoter region of s100p gene contains a CREB Recognition Element (CRE) sequence

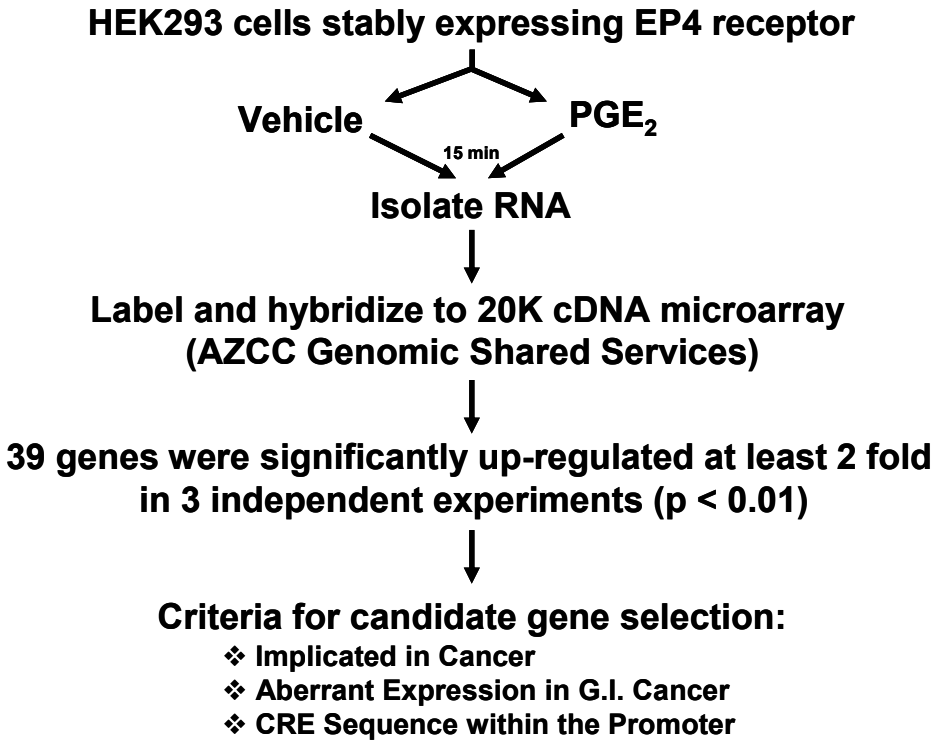

Figure 4.3: Schematic Representation of Microarray Approach
HEK 293 cells stably transfected with ptger4 were treated with vehicle (DMSO) or 2 μM PGE_2 for 15 min. RNA was isolated from harvested cells and differentially labeled for hybridization to 20K cDNA microarray. Gene for further study was selected based on the criteria indicated.

S100P Function and its Role in Colon Carcinogenesis

S100P is a member of the S100 family of calcium binding proteins, which share consensus EF-hand motifs. S100 proteins are exclusively found in vertebrates. Members of this family generally occur in the form of dimers and show immense sequence similarity among each other (Figure 4.4). Two EF-hand motifs and a hinge regions are highly conserved, indicating that these sequences are important for maintaining structural integrity. To date, twenty one S100 family members have been identified, among which S100P is one of the least studied proteins (Donato, 2001; Donato, 2003).

Dimerization appears to be a requirement for the proper activity of S100 proteins (Donato, 2003; Gribenko et al., 1998; Gribenko and Makhatadze, 1998; Zhang et al., 2003; Zhang et al., 2002). Every S100 protein can bind to Ca^{2+}, Mg^{2+} or Zn^{2+} ions. When bound to these divalent cations, S100 proteins can crosslink target proteins at either termini in order to facilitate various cellular functions (Gribenko et al., 2002). Cellular functions of this family of proteins include protein phosphorylation, enzyme activity, supervision of cytoskeletal integrity, maintenance of intracellular Ca^{2+} homeostasis and protection from oxidative cellular damage (Donato, 2001; Donato, 2003). A schematic representation of S100 dimer: target protein interaction is depicted in Figure 4.5.

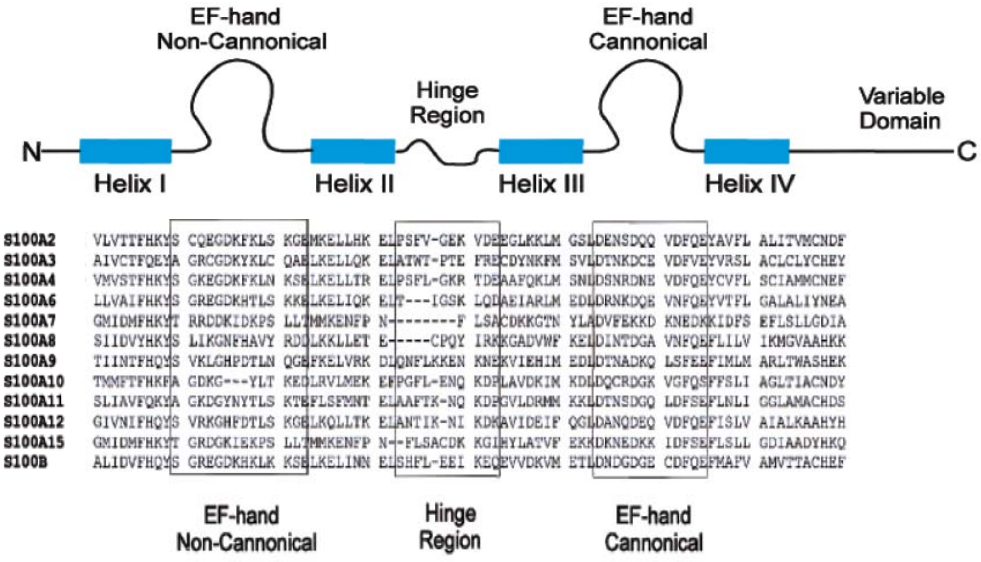

Figure 4.4: Sequence Similarity between S100 Proteins
Each S100 protein family member contains four α-helices, two calcium binding EF-hand motifs and a central hinge region. Each protein also contains variable lengths of C- and N-termini. The complete lengths of the termini are not shown. Amino acid sequences for the two EF hands and the Hinge regions are highly conserved among all S100 protein family members. Compiled from (Eckert et al., 2004).

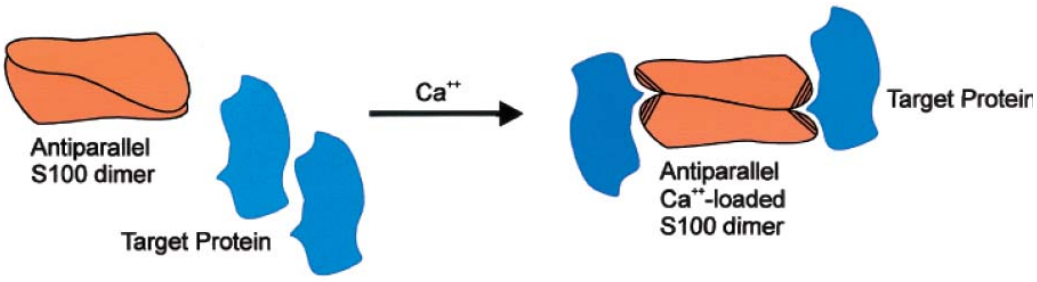

Figure 4.5: Model for S100 Protein: Target Protein Interaction
S100 proteins generally exist as anti-parallel dimers. An increase in calcium concentration within the cell results in a conformational change in the dimer that results in exposure of a cleft. This cleft region (cross-hatched) serve as binding sites for target protein. In the "calcium-loaded state", each S100 protein in the dimer is capable of interacting with a target protein at its C-terminal. Compiled from (Eckert et al., 2004).

The human S100P gene is located on chromosome 4p16 (Jin et al., 2003). This ~95 amino acid protein (11kDa) was first isolated from placenta (Becker et al., 1992; Schafer et al., 1995). The majority of studies have been performed in pancreatic cancers, where S100P promotes cell growth, survival and invasion by coupling with a receptor (RAGE) by downstream signaling via NFκB and ERK pathways (Logsdon et al., 2007). Disruption of this interaction could effectively antagonize proliferative signals within the cell (Arumugam et al., 2006; Arumugam et al., 2004; Arumugam et al., 2005). In addition, S100P has proven to be an ideal candidate as an early developmental biomarker for pancreatic cancers (Deng et al., 2008; Ohuchida et al., 2006). It was also shown that the artificial over-expression of S100P leads to disorganization of the actin cytoskeleton network (Whiteman et al., 2007). Yet another study indicated that S100P is a target for bone morphogenetic protein 4 (BMP4, a member of the TGFβ signaling pathway) and could induce cell migration in pancreatic cancer cell lines (Hamada et al., 2009).

An investigation of the promoter elements revealed the presence of STAT/CREB, SMAD and SP/KLF cis-acting sites, indicating that signaling mediated by TGFβ and/or cAMP secondary messengers are important for the transcriptional regulation of S100P gene (Gibadulinova et al., 2008). This study also demonstrated that the CREB/STAT binding sequence is critical for S100P transcription. In addition, another study showed that transcriptional regulation of S100P gene also depends on SMAD4, however this regulation is independent of SMAD4 binding site (Hamada et

al., 2009). In pancreatic cancers, the mechanism of up regulation of S100P message has been speculated to be due to hypomethylation of its promoter sequence (Sato et al., 2004). However, no studies have as yet investigated the molecular mechanisms that lead to the elevated levels of S100P in colorectal cancer.

A number of proteins are known to interact with Sl00P. S100P proteins can heterodimerize with S100A1 and S100Z proteins (Gribenko et al., 2001; Wang et al., 2004b). In the plasma-membrane: cytoskeleton interface, S100P interacts with Ezrin. Ezrin is a component of the ERM family of proteins, that promote metastatic spread of cancers (Curto and McClatchey, 2004). In fact, ezrin is required for the organization of the murine intestinal epithelium and morphogenesis of vili (Saotome et al., 2004). In resting cells, ezrin is present in an auto-inhibited conformation and requires Ca^{2+} dependent activation by S100P (Koltzscher et al., 2003). A recent study showed that S100P-mediated Ezrin activation is necessary for migratory potential of lung cancer (NSCLC) cells particularly in the trans-endothelial passages (Austermann et al., 2008). A novel protein known to interact with S100P is called S100PBPR (S100P Binding Protein Riken) that may be involved in early pancreatic cancer progression (Dowen et al., 2005). Calcyclin – Binding Protein/ Siah-1 – Interaction Protein (CacyBP/SIP) is another protein capable of interacting with S100P (Filipek et al., 2002). It is a component of the novel ubiquitin ligase complex, important in degradation of various cancer related proteins, particularly β-catenin, DCC, N-Cor, PHD1/3 and cMyb (Filipek et al., 2002). Interestingly, two studies have shown that CacyBP/SIP negatively

regulates cell proliferation, tumorigenicity and invasion in gastric and renal cancers (Ning et al., 2007; Sun et al., 2007). This may in part explain the discrepancy in a few studies where the expression of S100P was shown to be reduced in gastric cancers as compared to normal gastric mucosa (Ji et al., 2004; Jia et al., 2009; Zhi et al., 2003). Thus it is possible that S100P has pleiotropic functions depending on its cellular context and partners.

By far the most well-documented interacting partner for S100P is the Receptor for Advanced Glycation End products (RAGE). RAGE is a multi-ligand receptor known to interact with a variety of ligands such as advanced glycation end products (important in diabetes), S100 proteins (particularly B, A8, A9, A12 and P isoforms), amyloid proteins (with relevance in Alzheimer's disease) and amphoterin (protein that facilitates tumor adhesiveness and invasiveness) (Riuzzi et al., 2006; Stern et al., 2002). RAGE is over-expressed in a number of malignancies particularly in prostate, pancreatic and colon cancers (Logsdon et al., 2007). RAGE expression increases in colorectal cancer with stage of progression (Kuniyasu et al., 2003). Conversely, the administration of sRAGE (a decoy receptor that essentially inactivated intracellular RAGE signaling) could significantly reduce the number of polyps in the $APC^{(MIN)}$ mouse model (Huang et al., 2006). Interestingly, RAGE is also involved in the interface between inflammation and colon carcinogenesis. A mouse model of inflammation-induced enterocolitis (genetic deletion of cytokine IL-10 in mouse) showed a decrease in inflammation when sRAGE was administered (Berg et al., 1996; Hofmann et al.,

1999). The RAGE promoter contains NFκB binding sites and RAGE activation by S100P stimulates cell growth, migration and survival via an NFκB dependent pathway (Arumugam et al., 2004; Fuentes et al., 2007; Li and Schmidt, 1997).

The RAGE-S100P interaction represents an excellent therapeutic target for combination therapies specifically if S100P status of patients can be determined. A number of S100 proteins serve as biomarkers for various cancers such as S100B in malignant melanoma (Harpio and Einarsson, 2004; Salama et al., 2008). Cromolyn and amphoterin are two such examples that have effectively shown to disrupt RAGE-S100P interaction as well as inhibit cancer cell growth, survival, invasion and NFκB signaling. Cromolyn in a natural product derived from *Ammi visnaga* and is used as muscle relaxant and for asthma treatment. Amphoterin on the other hand is a peptide mimetic that resembles the RAGE ligand (Arumugam et al., 2006; Arumugam et al., 2004).

The expression of S100P has been shown to be up regulated in a number of cancers such as pancreatic (Arumugam et al., 2004; Arumugam et al., 2005; Crnogorac-Jurcevic et al., 2003; Fukushima et al., 2004; Hamada et al., 2009; Logsdon et al., 2003; Missiaglia et al., 2004; Sato et al., 2004; Whiteman et al., 2007), breast (Mackay et al., 2003; Russo et al., 2001; Wang et al., 2006), colon, prostate (Amler et al., 2000; Basu et al., 2008; Chuang et al., 2007; Hammacher et al., 2005; Mousses et al., 2002) and lung (Bulk et al., 2008; Diederichs et al., 2004; Kim et al., 2007; Rehbein et al., 2008). In colon cancer, numerous microarray studies on frozen tissue specimen from patients

have shown significant increase is the expression of S100P (Birkenkamp-Demtroder et al., 2005; Higgins et al., 2007; Kita et al., 2006). Immunohistochemical analyses also showed the tissue specific over expression of S100P in tumor vs. normal counterparts of colorectal, gastric, ovarian, pancreatic, breast and prostate (Parkkila et al., 2008). Interestingly, microarray profiling on frozen tumor specimen showed elevated S100P levels in colon cancer (Birkenkamp-Demtroder et al., 2005; Higgins et al., 2007; Kita et al., 2006). S100P levels were shown to be elevated at least 4 fold in a DNA microarray performed on inflamed colonic tissue from ulcerative colitis and Crohn's disease patients, indicating the relevance of this protein in inflammation-induced colorectal carcinogenesis (Lawrance et al., 2001). S100P and RAGE have both been implicated in chronic inflammation as well as colorectal carcinogenesis. Nevertheless a direct role of S100P in colorectal carcinogenesis has not been examined.

Analysis of S100P Expression in Frozen Human Colon Cancer Specimens

Flash frozen tissue specimens, preserved in RNALater, were obtained from the Pathology Archival Reserve abiding by the regulations set by Institutional Review Board (IRB). Total RNA was extracted and cDNA synthesis was performed (Materials and Methods). Figure 4.6 shows realtime RT-PCR quantification of s100p transcripts of tissues from patients with colon cancer. Gene specific primers against β-actin were used as control. The data shows that patients with colon cancer had significantly higher mRNA expression of s100p as compared to matched or unmatched normal patients.

Induction of S100P Expression after PGE_2 Exposure

In order to validate our microarray findings, human embryonic kidney HEK 293 cells stably expressing high levels of EP4 receptor, were treated with either DMSO (vehicle control) or with 1 μM PGE_2 for 1, 3, 6, 12 and 24 hours. Drug treatments were performed as indicated in Materials and Methods. After drug treatments, the cells were harvested for RNA extraction and qRT-PCR analysis was performed by SyBr Green amplification of s100p gene. Transcripts of s100p were normalized to β-actin gene using gene specific primers. Relative quantification revealed that the s100p message is significantly elevated in the presence of 1 μM PGE_2 at 12 and 24 hrs (9.5 and 29.4 fold induction compared to DMSO respectively) (Figure 4.7).

Second, in order to investigate PGE_2 dependent response in colon cancer cells, LS174T cells were treated with 1 μM PGE_2 for the same time points. These cells express high endogenous levels of EP4 receptor (Figure 3.4). Figure 4.8 shows that exposure of LS174T cells to 1 μM PGE_2 effectively induced the expression of the s100p gene by 3.3 fold after 24 hours. The induction began gradually and peaked at 24 hours. In addition, S100P protein levels were also induced to high levels after 24 hours (Figure 4.9). Thirdly, to ensure that the induction is universal and not cell-line specific, S100P induction was investigated in breast and pancreatic cell lines in the presence of PGE_2 as well as PGE_1OH (Figure 4.10)

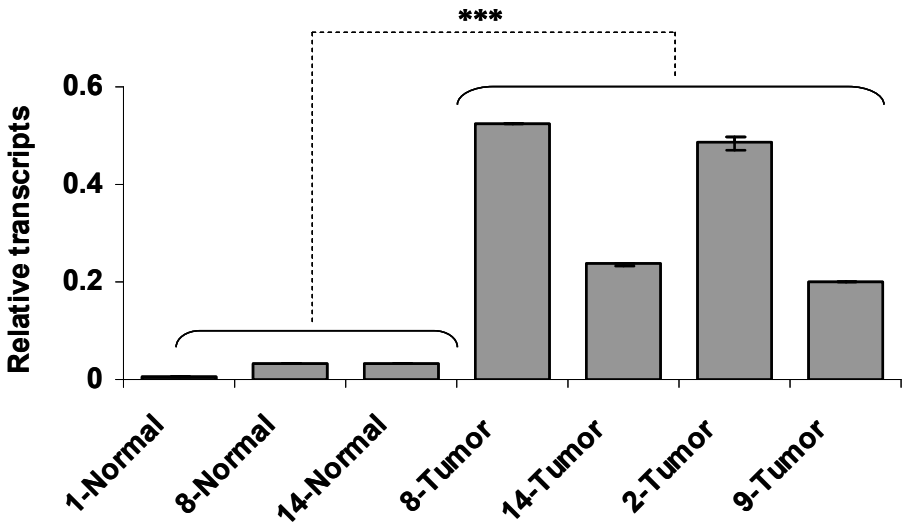

Figure 4.6: mRNA Expression of S100P in Human Colon Cancer Specimens
RNA was extracted from frozen tissue specimen from patients with colon cancer (8, 14, 2 and 9). Adjacent normal tissues (1, 8 and 14) were also utilized. Realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized to the β-actin gene. Relative quantification was performed. Differences in relative transcripts between normal and tumor samples are compared and represented as a bar graph. Values are shown as Mean ± SD and *** represents $p < 0.001$.

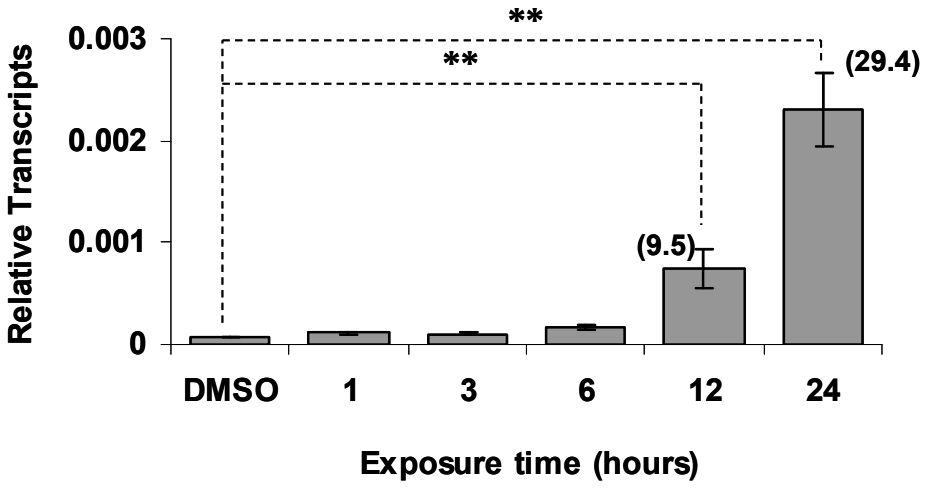

Figure 4.7: PGE_2-Induced S100P mRNA Expression in HEK 293 Cells
HEK 293 cells over-expressing the EP4 receptor were plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes, serum starved for 20 hrs and then treated with 1uM PGE_2 for the indicated time periods. Cells were harvested and realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized against β-actin gene. Relative quantification was performed. Differences in relative transcripts as compared to DMSO (vehicle control) treatment are represented as a bar graph. Fold changes over DMSO are indicated in parentheses for 12 and 24 hour exposure times. Values are shown as Mean ± SD and ** represents $p < 0.01$.

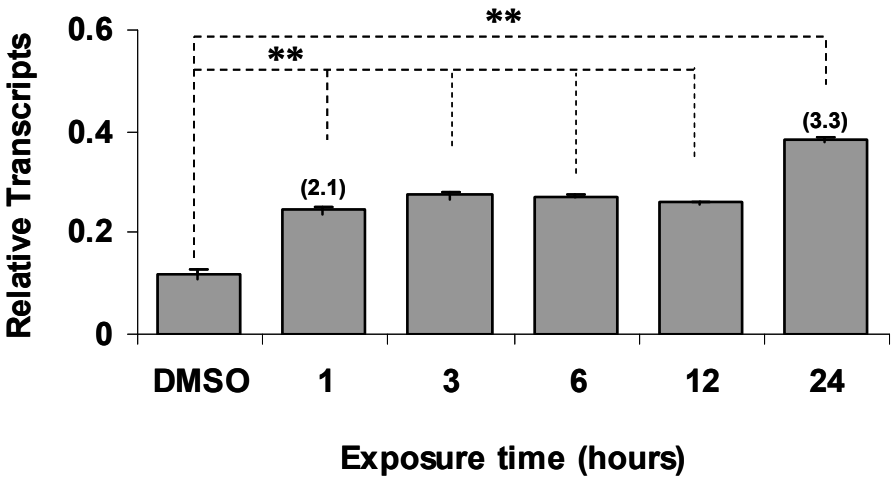

Figure 4.8: PGE_2-Induced S100P mRNA Expression in LS174T Cells
LS174T cells plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes, were serum starved for 20 hrs and then treated with 2 μM PGE_2 for the indicated time periods. Cells were harvested and realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized to β-actin gene. Relative quantification was performed. Differences in relative transcripts as compared to DMSO (vehicle control) treatment are represented as a bar graph. Fold changes over DMSO are indicated in parentheses. Values are shown as Mean ± SD and ** represents $p < 0.01$.

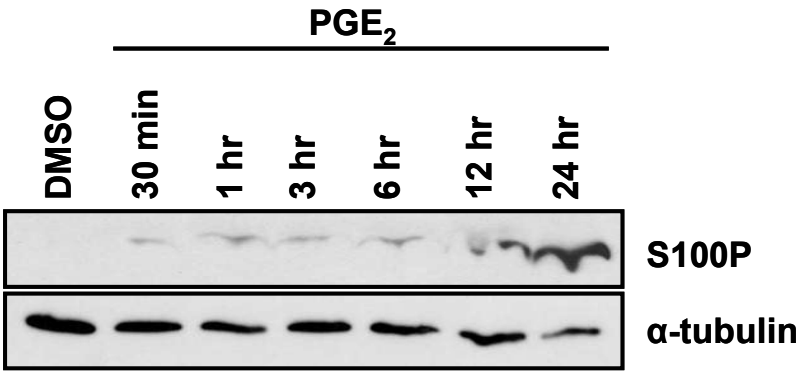

Figure 4.9: PGE_2-Induced S100P Protein Expression in LS174T Cells
LS174T cell lines, plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes, were serum starved for 20 hrs and then treated with 1uM PGE_2 for the indicated time points. Cells were then harvested and western blot analysis was performed using S100P antibody. α-tubulin is used as loading control.

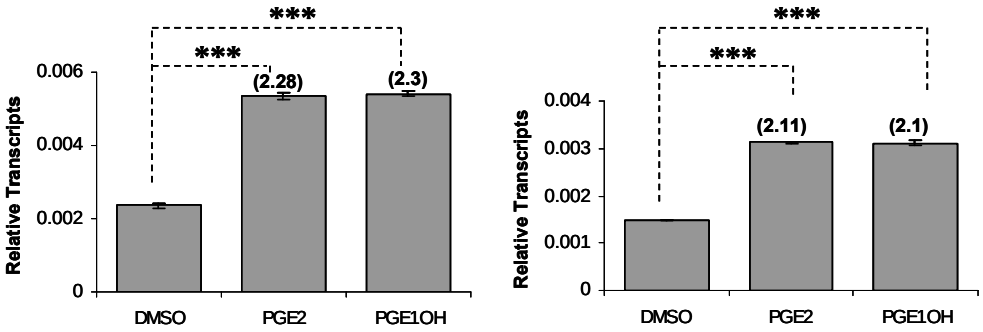

Figure 4.10: PGE_2-Induced S100P mRNA Expression in MCF7 and Panc1 Cells
MCF 7 (left) and Panc 1 (right) cell lines plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes were serum starved for 20 hrs and then treated either with 1uM PGE_2 or PGE_1OH for 24 hours. Cells were harvested and realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized to the β-actin gene. Relative quantification was performed. Differences in relative transcripts as compared to DMSO (vehicle control) treatment are represented as a bar graph. Fold changes over DMSO are indicated in parentheses. Values are shown as Mean ± SD and *** represents $p < 0.001$.

Suppression of S100P Induction by Inhibition of the EP4 Receptor

Next, we wanted to establish whether the EP4 receptor plays a role in PGE_2-mediated induction of S100P. To this effect, LS174T cells were first exposed to 1μM PGE_1OH. In addition, cells were exposed to two selective EP4 receptor antagonists: 1μM GW627368X and 2μM L-161,982. Exposure to PGE_1OH showed a 2.2 fold increase in the S100P relative transcript levels, similar to PGE_2 mediated induction, whereas treatment with EP4 receptor antagonists repressed the induction of S100P to levels comparable to vehicle control (Figure 4.11). Values are represented as Mean ± S.D.

On the other hand, when EP4 receptor was knocked down in LS174T cells (shEP4) exposure to 1 μM PGE_2 significantly diminished the induction of S100P (Figure 4.12). These levels were comparable to LS174T shEP4 cells treated with DMSO (vehicle control). By contrast, in LS174T shCrtl cells, S100P levels were induced to 1.8 fold when treated with 1 μM PGE_2 compared to treatment with DMSO. Taken together these two results suggest that PGE_2-dependent induction of S100P is mediated via the EP4 receptor.

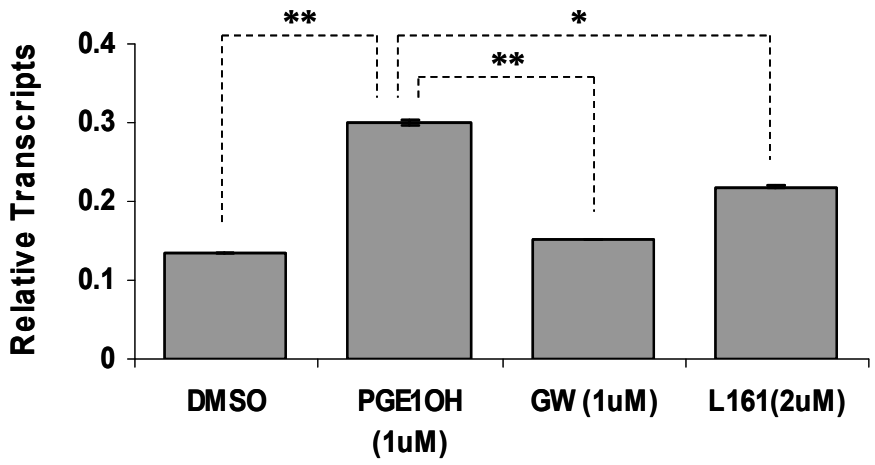

Figure 4.11: Effect of Pharmacological inhibition of EP4 receptor on S100P induction

LS174T cells plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes, were serum starved for 20 hrs and then treated either with 1uM PGE_1OH, GW627368X or 2 μM L-161,982 for 24 hours. Cells were harvested and realtime qRT-PCR analysis was performed using s100p gene-specific primers normalized to the β-actin gene. Relative quantification was performed. Differences in relative transcripts as compared to DMSO (vehicle control) treatment are represented as a bar graph. Fold changes over DMSO are indicated in parentheses. Values are shown as Mean ± SD and * represents $p \leq 0.05$; ** represents $p \leq 0.01$.

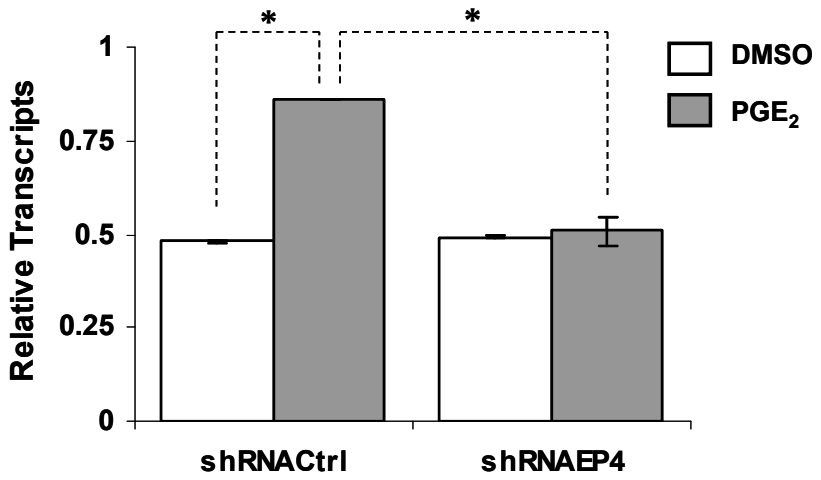

Figure 4.12: Effect of Genetic Inhibition of EP4 Receptor on S100P Induction
LS174T cells stably transfected either with shRNACtrl or shRNAEP4, were plated at 1 X 10^6 cells/plate in 10 cm cell culture dishes, serum starved for 20 hrs and then treated with 2uM PGE_2 for the indicated time periods. Cells were harvested and realtime qRT-PCR analysis was performed using egr1 gene-specific primers normalized against β-actin gene. Relative quantification was performed. Differences in relative transcripts as compared to DMSO (vehicle control) treatment are represented as a bar graph. Fold changes over DMSO are indicated in parentheses. Values are shown as Mean ± SD and * represents $p < 0.05$.

S100P Promoter Sequence

In order to identify specific sequence elements within the s100p promoter that are responsible for the induction of the gene, promoter reporter assays were conducted. Three cis-acting elements (SMAD, STAT and CRE) have been reported to be important for the transcription of the s100p gene. The sequence of the core s100p promoter is depicted in Figure 3.9. Functional analysis of the promoter was performed by transient transfection of three promoter deletion constructs (obtained from Dr Silvia Pastorekova, Institute of Virology, Slovak Academy of Sciences) that were cloned in pGL3-based reporter vector upstream of the firefly luciferase gene (Figure 4.13): (-236/+58) that contains SMAD, STAT as well as CRE sequences, (-124/+58) lacking all three sequences and (-236/-14) that contains SMAD, STAT and CRE sites, but lacks the proximal region of the s100p promoter.

In order to investigate whether SMAD, STAT or CRE sequences are involved in response to PGE_2 levels, constructs A, B and C were exposed to vehicle control (DMSO) or 1 μM PGE_2. Figure 4.14 shows that constructs (-236/+58) and (-236/-14) could significantly induce luciferase gene expression in the presence of PGE_2 (~ 2 folds). However, construct (-124/+58) could not induce the expression of luciferase gene. Taken together these results indicate that either SMAD, STAT or CRE sequences could be important for gene expression.

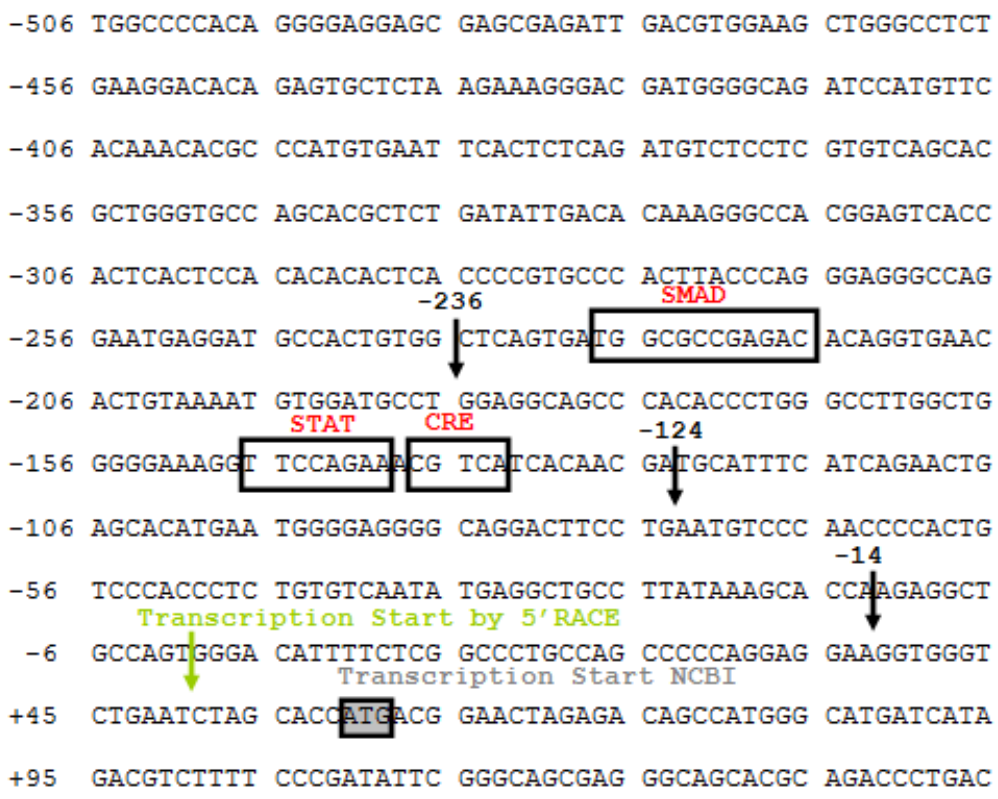

Figure 4.13: S100P Promoter Sequence
The S100P promoter sequence contains SMAD, STAT and CRE binding sites (boxed). Black arrows represent the positions of promoter deletions. Green arrow represents transcription start site as determined by 5'RACE. ATG (grey box) represents the NCBI predicted transcription start site. Compiled from (Gibadulinova et al., 2008).

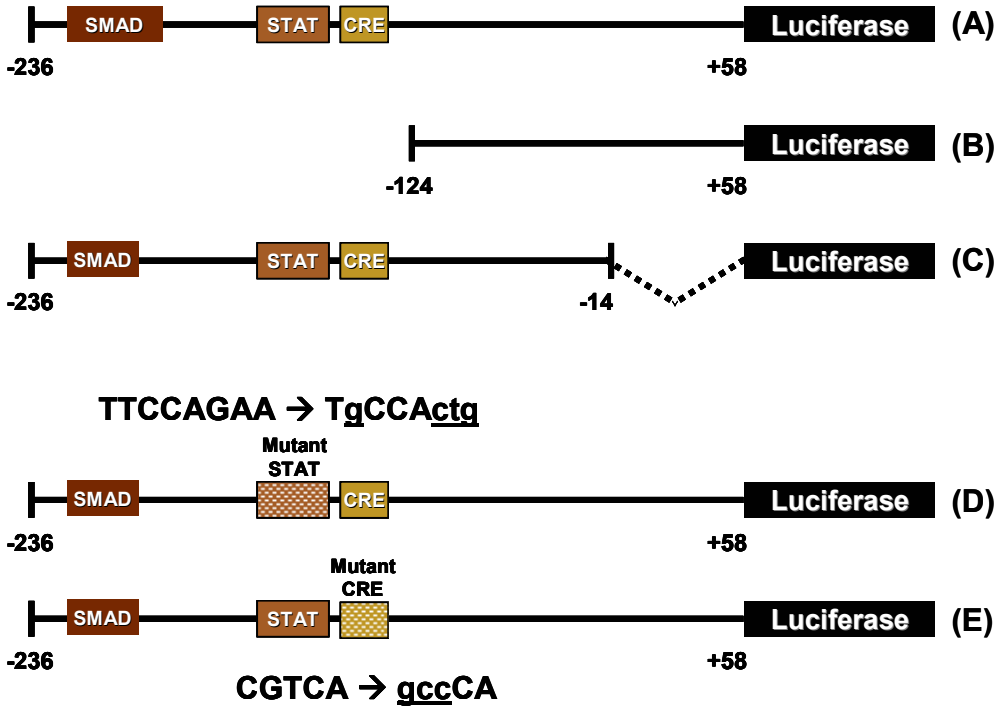

Figure 4.14: S100P Promoter Deletion and Mutagenesis Constructs
Three promoter deletion (A, B and C) and two mutant (E and F) constructs are indicated. Filled boxes indicate the positions of SMAD, STAT and CRE binding sites within the S100P promoter sequence. Hashed boxes represent mutant sequences (STAT: TgCCActg and CRE: gccCA). Bases in lower cases have been modified. Numbers on the constructs represent positions of nucleic acid with respect to the transcription start site (as determined by 5'RACE). All constructs were cloned into pGL3-Basic vector with deletion and mutant constructs fused in frame to the Luciferase coding sequence. Mutagenesis was performed based on previous data (Eresh et al., 1997; Yu et al., 1999; Zhang et al., 2005).

Mutagenesis of STAT and CRE Sequences

Previous studies by Dr Silvia Pastorekova's laboratory group have shown that the S100P promoter activation is primarily mediated by the STAT/CRE and the SMAD binding sites. As the PGE_2/EP4 receptor-mediated signaling has been shown to activate CREB, we hypothesized that the disruption of the CRE-binding site would abolish luciferase activity. As the two sequences are juxtaposed and we wanted to clarify which sequence is responsible for driving S100P transcription, STAT and CRE binding sites were mutated by site directed mutagenesis (TTCCAGAA:T**g**CC**actg** for STAT and CGTCA:**gcc**CA for CRE). The above-mentioned mutated sequences have been shown to be important for the binding of STAT and CREB transcription factor in mammalian, drosophila and yeast systems (Eresh et al., 1997; Yu et al., 1999; Zhang et al., 2005). Data shows that while mutation of the STAT sequence did not affect S100P induction mutation of the CRE sequence decreased both basal as well as PGE-induced S100P expression (Figure 4.15). It is also important to note here that although SMAD-binding site was not mutated in any of the constructs, mutation of CRE site alone could completely abolish luciferase activity. Taken together, these data indicate that PGE_2 enhances the promoter activity of S100P via the CRE binding site and that SMAD-binding site may not be important for its activity.

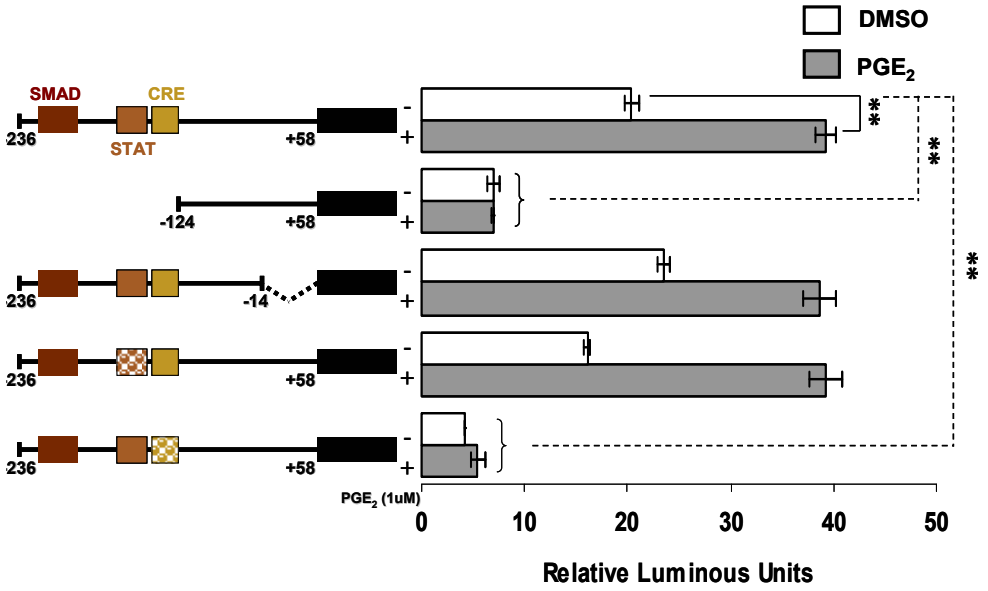

Figure 4.15: S100P Promoter Reporter Analysis
LS174T cells plated at 5 X 10^4 cells/plate in 24-well plates, were first transfected with S100P promoter deletion or mutant (hashed boxed) constructs. After 24 hours of transient transfection, cells were serum starved for 20 hrs and then treated with 1uM PGE_2 for 24 additional hours. Cells were lysed subjected to firefly and Renilla luciferase measurements. Experiment was performed in triplicates. Relative Luminous Units represents the ratio between luminescence generated by Firefly Luciferase over Renilla Luciferase. Values are shown as Mean ± SD and ** represents $p < 0.01$.

Measurement of Colon Cancer Cell Growth after Genetic Knockdown of S100P

In order to assess the effect of S100P knockdown on cellular growth, S100P protein was suppressed in LS174T cells by stable transfection with shRNA. Protein was isolated and western analysis was performed. Figure 4.16A shows decrease in S100P protein in the presence of shRNA against S100P. Furthermore, colony growth was measured by counting the number of methylene blue stained colonies after 3 weeks of growth. The number of visible colonies were counted and found to be significantly reduced when S100P was knocked down (shS100P-2) as compared to control (shSCRM) (Figure 4.15B). These data suggest that S100P protein is important for colony growth.

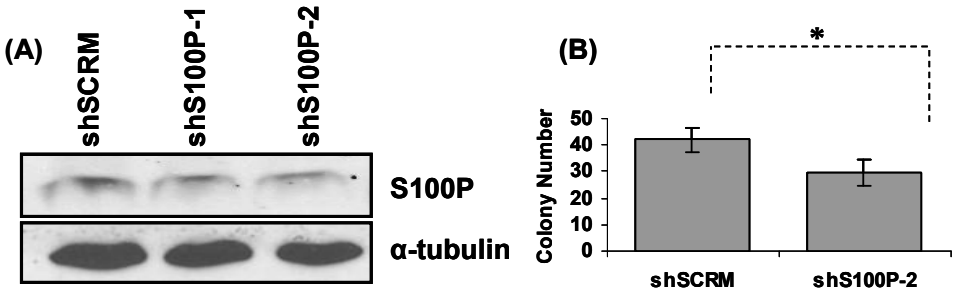

Figure 4.16: Effect of Genetic Knockdown of S100P on colony formation
(A) LS174T cells were stably transfected with either shSCRM or two shS100P constructs (-1 and -2) under 200 μg/mL geneticin (G418) selection. S100P knockdown was confirmed probing with antibody against S100P. Equal loading was confirmed by probing against α-tubulin. (B) LS174T cells stably expressing shSCRM and shS100P-2 were seeded at 500 cells/plate in 100mm dishes and incubated for 3 weeks to allow for colony formation. After the 3 week period, medium was aspirated and colonies were stained with methylene blue solution. Number of visible colonies was counted. Values are shown as Mean ± SD and * represents $p < 0.05$.

Discussion

Previous studies from our laboratory suggest that PGE_2/EP4 receptor, via ERK, leads to the activation of transcription factor CREB. However, whether or not CREB activation contributes to colon cancer cell growth has not been previously investigated. We have shown new evidence that suppression of CREB activity, by a well-characterized dominant negative mutant construct, suppresses colon cancer cell growth.

In the present study, we focused on identifying the downstream target genes of the PGE_2/EP4 receptor in colon cancer cells. We used microarray analysis to find novel PGE_2/EP4 receptor target gene. The calcium binding protein, S100P gene was identified as being significantly up-regulated by PGE_2. This finding is the first of its kind and sheds light on the regulatory mechanisms of this pro-tumorigenic pathway.

Altered expression of S100P has been observed in a wide variety of human cancers (Arumugam et al., 2004; Arumugam et al., 2005; Basu et al., 2008; Crnogorac-Jurcevic et al., 2003; Logsdon et al., 2003; Mousses et al., 2002; Wang et al., 2006). Consistent with this literature, we also observed an increase in S100P expression levels in human colon cancers compared to normal specimen. Despite this evidence, the upstream regulator of S100P expression, in colon cancer, had not been clarified until now. Our present results reveal that the PGE_2/EP4/CREB pathway can up-regulate S100P expression in colon cancer cells as well as other cancer cells (i.e. breast and pancreatic cancer) and that this may be relavant in colon carcinogenesis.

However, we observed only a modest suppression on cell growth in our shS100P-2 cells compared to shSCRM controls. This suggests that the induction of S100P by the PGE_2/EP4 receptor signaling pathway may affect other characteristics of the tumorigenic phenotype. In fact, several reports indicate that S100P plays an important role in cancer cell migration and metastasis in breast and prostate cancer patients (Arumugam et al., 2005; Basu et al., 2008; Wang et al., 2006). Particularly in colon cancers, S100P has stimulates, growth, migration and NFκB and ERK mediated cell signaling pathways (Fuentes et al., 2007). Thus, the contribution of the PGE_2/EP4/CREB/S100P pathway to colon cancer progression needs to be clarified by further analyses.

CHAPTER 5:

DISCUSSION AND FUTURE PROSPECTS

In chapter 3 we have discussed that the EP4 receptor is up regulated in colon cancer. This was exemplified by dramatic increase in EP4 receptor expression in metastatic lesions. This suggests that EP4 receptor signaling may be causal to late stage disease. However, presently, there are no good animal models that recapitulate human colon cancer late stage disease. Towards this goal, we have developed a transgenic vector capable of tissue specific over-expression in mouse model systems (Figure 5.1). This construct ($PTGER4^{TG}$) brings in tissue specific expression of the EP4 receptor. In addition, it provides utility in screening and imaging *in vivo* as well as *in vitro*. A 2.4 kb murine PTGER4 cDNA fragment encoding EP4 protein is placed under the control of the 1.6 kb chicken β-actin protein. Two loxP sites, flanking multiple poly A signal sequence, are placed between the promoter and the PTGER4 coding region. The coding region for the PTGER4 is connected with the venus GFP coding region by an internal ribosome entry site (IRES) to avoid unwanted effects of a fusion protein. The presence of the 3' polyA sequence prevents PTGER4 and GFP expression. The bicistronic transgene is silent until activated by Cre recombinase, which in turn, leads to the excision of the poly A stop signal thereby allowing transgene expression. As transcription ensues a polycistronic message containing both the EP4 receptor and the Venus GFP is produced.

To test the localization and functionality of the EP4 transgene, we transiently introduced the $PTGER4^{TG}$ vector into murine NIH3T3 cells. We found that the bicistronic transgene was expressed in NIH3T3 cells only upon exposure to Cre recombinase (Figure 5.2). Thus, these results indicate that our targeting vector is functional. This vector will be utilized to develop a transgenic mouse. Subsequent breeding of this transgenic mouse to a Villin-Cre expressing mouse would result in the deletion of the floxed stop signal bringing PTGER4 expression specifically in the small intestines and colon (el Marjou et al., 2004). Further studies employing known models of colon carcinogenesis such as AOM/DCA or $Apc^{(MIN)}$ models are needed to assess the contribution of EP4 receptor to colon carcinogenesis.

In chapter 4, we have identified S100P as a downstream target of the PGE_2/EP4/CREB signaling pathway. Here, we showed that PGE_2-dependent induction of S100P occurs in the presence of a functional CRE-binding sequence in the promoter region of S100P. However, how S100P contributes to colon cancer development will require further work. It is known that RAGE receptor activation, by S100P, stimulates cell growth, migration and survival via NFκB dependent pathway (Arumugam et al., 2004; Fuentes et al., 2007; Li and Schmidt, 1997). Interestingly the COX-2 gene promoter contains NFκB and CRE binding sites (Appleby et al., 1994; Tang et al., 2001). Moreover, S100B (a different S100 family member), could stabilize the COX-2 transcripts within monocytes by a dual mechanism involving RNA binding proteins and microRNAs (Shanmugam et al., 2008). We postulate that the induction of S100P by

PGE_2/EP4 receptor signaling may be part of a positive feedback loop that leads to the up-regulation of COX-2 gene expression (Figure 5.3). In this direction, we will analyze COX-2 expression in colon cancer cell lines by using recombinant S100P protein. In addition, we will utilize COX-2 promoter constructs to ask if S100P regulates COX-2 promoter and identify potential transcription factors.

Thus, in summary, this dissertation investigated the clinical ramifications of the EP4 receptor signaling in colon cancers and has contributed to the knowledge of how PGE_2/EP4 receptor signaling is important to colon carcinogenesis. It also identified a novel downstream target of the PGE_2/EP4 signaling pathway. Collectively, these findings indicate that the EP4 receptor and S100P may play a role in the patho-physiology of colon cancer. Thus, EP4 receptor and/or S100P expression can be used as potential prognostic markers to help identify patients who may develop metastatic disease. Conversly, combinatorial strategies targeting the RAGE (the receptor for S100P) and EP4 receptors could be used as therapy against late stage disease (Figure 5.3). It would be interesting to study the effect of therapeutic blockade of either one or both of these proteins in conjunction with Cetuximab, which inactivates a well-known receptor (EGFR) in the PGE_2/EP4/CREB signaling pathway.

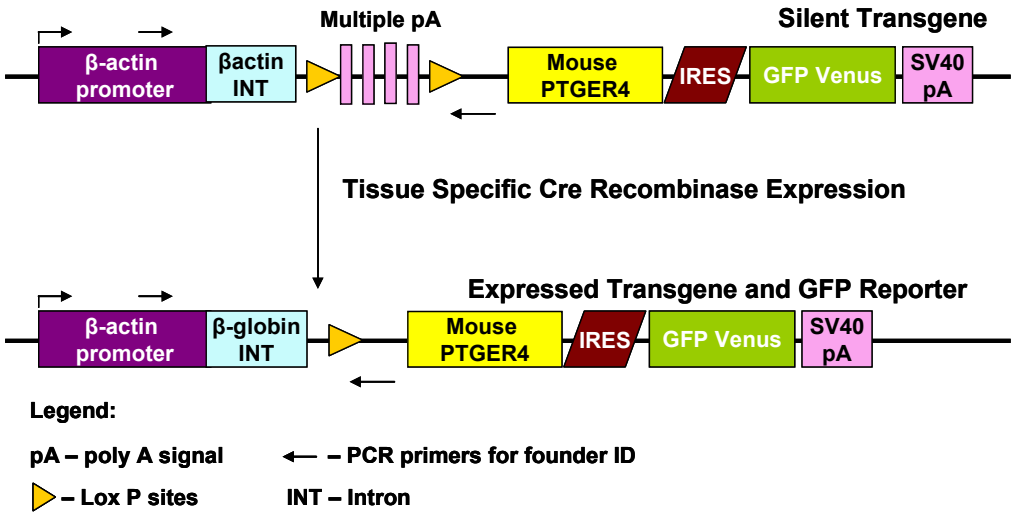

Figure 5.1: Schematic Representation of the EP4 Receptor Transgenic Vector
A potent ubiquitous β-actin promoter was used to drive a series of cassettes, including a floxed poly A sequence, the open reading frame of murine ptger4 cDNA, and the Venus GFP (top). When Cre-mediated recombination occurs the floxed Poly A sequence is excised and the downstream polycistronic transcript is activated. Black arrows represent position of primers for the identification of founder mice. Yellow triangles represent Lox P sites capable of recombination in the presence of Cre recombinase.

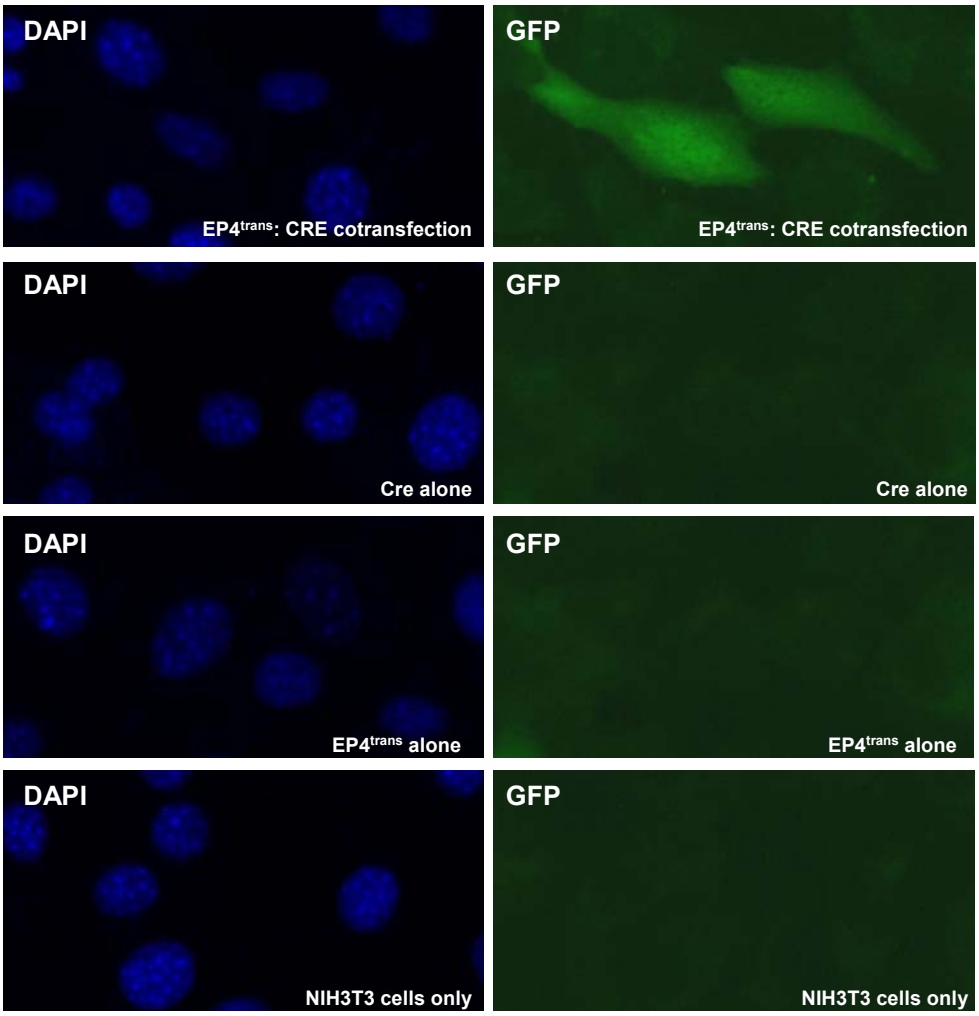

Figure 5.2: Validation of the Conditional EP4 Transgene in Cultured Cells
The EP4 conditional transgenic construct and pCMV-CRE recombinase plasmid were co-transfected into NIH 3T3 cells. After 24 hours the cells were stained with DAPI and GFP expression visualized by immunofluorescence microscopy. A) Cells co-transfected with EP4 transgene and pCMV-CRE recombinase plasmid. B) Cells transfected with pCMV-CRE plasmid only. C) Cells transfected with EP4 conditional transgene only. D) Cells alone.

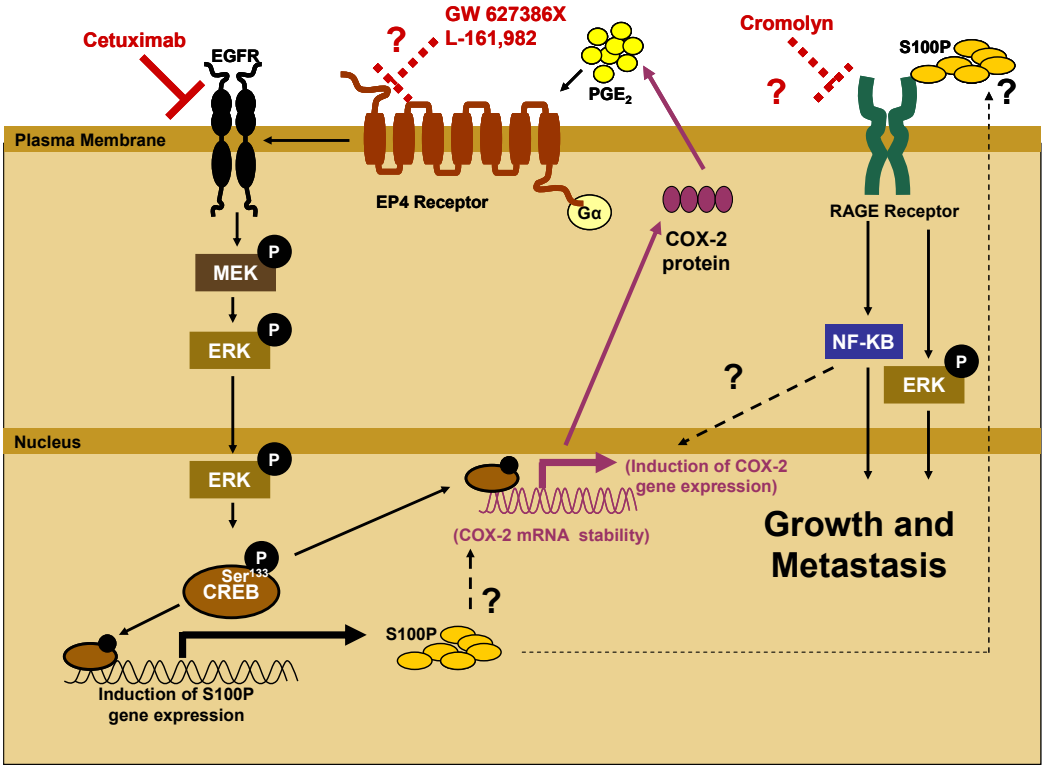

Figure 5.3: Proposed Model of PGE_2/ EP4/ CREB/ S100P-Mediated Colon Carcinogenesis

EP4 receptor status within colon cancer patients and PGE_2-mediated S100P up-regulation was determined in this dissertation. Known pathways and therapeutic intervention strategies are shown as bold arrows. Avenues for future research and therapeutic targets are represented as dotted arrows. Adapted from (Appleby et al., 1994; Arumugam et al., 2006; Cha and DuBois, 2007; Cherukuri et al., 2007; Shanmugam et al., 2008).

CHAPTER 6:

APPENDIX

Approval Letter from Institutional Review Board (IRB)

THE UNIVERSITY OF ARIZONA.

Human Subjects
Protection Program

1235 N. Mountain Ave.
P.O. Box 245137
Tucson, AZ 85724-5137
Tel: (520) 626-6721
http://irb.arizona.edu

8 September 2008

Anupama Chandramouli, Graduate Student
Advisor: Mark Nelson, PhD
Pathology
College of Medicine
PO Box 245043

RE: PROEJCT NO. 08-0681-04 STUDYING THE ROLE OF PGE2/EP RECEPTORS SIGNALING IN COLORECTAL CARCINOMA

Dear Anupama:

We received your research proposal as cited above. The procedures to be followed in this study pose no more than minimal risk to participating subjects and have been reviewed by the Institutional Review Board (IRB) through an Expedited Review procedure as cited in the regulations issued by the U.S. Department of Health and Human Services [45 CFR Part 46.110(b)(1)] based on their inclusion under ***research category 5***. The requirement for ***obtaining informed consent*** has been waived for this study since the research involves no more than minimal risk, the waiver will not adversely affect subjects' rights and welfare, the research could not practicably be carried out without the waiver [and whenever appropriate, the subjects will be provided with additional pertinent information after participation], as allowed by 45 CFR46.116(d). NOTE: Since no Protected Health Information (PHI) is being collected a PHI authorization form is not required.

Although full Committee review is not required, notification of the study is submitted to the Committee for their endorsement and/or comment, if any, after administrative approval is granted. This project is approved with an **expiration date of 7 September 2009.**

The Institutional Review Board (IRB) of the University of Arizona has a current ***Federalwide Assurance*** of compliance, ***FWA00004218***, which is on file with the Department of Health and Human Services and covers this activity.

Approval is granted with the understanding that no further changes or additions will be made to the procedures followed without the knowledge and approval of the Human Subjects Committee (IRB) and your College or Departmental Review Committee. Any research related physical or psychological harm to any subject must also be reported to each committee.

A university policy requires that all signed subject consent forms be kept in a permanent file in an area designated for that purpose by the Department Head or comparable authority. This will assure their accessibility in the event that university officials require the information and the principal investigator is unavailable for some reason.

Sincerely,

Brenda J Witman

Brenda J Witman, MD, MPH
Chair, Biomedical Committee
UA Institutional Review Board (IRB)

BJW:mm
cc: Department/College Review Committee

List of Publications

Research Articles:

1. **Chandramouli A.**, Hutchinson A., Shañas R., Davenport J., Gibadulinová A., Bhattacharyya AK., Watts, G., Regan JW. and Nelson MA. (2009). Regulation of S100P and its contribution to colon carcinogenesis via the PGE_2/EP4 receptor signaling pathway. *Cancer Research*, (manuscript in preparation).

2. **Chandramouli A.**, Stankova L., Shañas R., Davenport J., Owens J., Bhattacharyya AK., LaFleur B., and Nelson MA. (2009). Analysis of the EP4 receptor expression during colon carcinogenesis. *Clinical Cancer Research,* (manuscript in preparation).

3. Doldan A., **Chandramouli A.**, Shañas R., Bhattacharyya A., Leong S.P., Nelson M.A. and Shi J. (2008). Loss of eukaryotic initiation factor 3f in melanoma. *Molecular Carcinogenesis*, 47:806-813.

4. Nigam N., Singh A., Sahi C., **Chandramouli A.** and Grover A. (2008). SUMO-conjugating enzyme (Sce) and FK506-binding protein (FKBP) encoding rice (Oryza sativa L.) genes: genome-wide analysis, expression studies and evidence for their involvement in abiotic stress response. *Molecular Genetics and Genomics***,** 279: 371-383.

5. Doldan A., **Chandramouli A**., Shañas R., Bhattacharyya A., Cunningham J.T., Nelson M.A. and Shi J. (2008). Loss of the eukaryotic initiation factor 3f in pancreatic cancer. *Molecular Carcinogenesis*, 47: 235-244.

6. **Chandramouli A.**, Shi J., Feng Y., Holubec H., Shañas R., Bhattacharyya A.K., Zheng W. and Nelson M. (2007). Haploinsufficiency of the cdc2l gene contributes to skin cancer development in mice. *Carcinogenesis*, 28: 2028-2035.

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1. Grover A. Aggarwal P.K., Kapoor A., Katiyar-Agarwal S., Agarwal M., and **Chandramouli A.** (2003). Addressing abiotic stresses in agriculture through transgenic technology. *Current Science* 84: 355-367.

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4. Grover A. and **Chandramouli A.** (2002). Production of abiotic stress tolerant transgenic plants. *Proceedings "Salt Tolerance in Plants: Physiological and Molecular Approaches"*.

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