5-3-2017

The Role of Prostaglandin E2 Signaling in Acquired Oxaliplatin Resistance

Huakang Huang
University of Connecticut Health Center, hkanghuang@gmail.com

The Role of Prostaglandin E2 Signaling in Acquired Oxaliplatin Resistance

Huakang Huang, Ph.D.

University of Connecticut, 2017

The platinum-base chemotherapeutic agent, oxaliplatin, is used to treat metastatic colorectal cancer (CRC). Unfortunately, nearly all patients develop acquired resistance to oxaliplatin after long-term use, limiting its therapeutic efficacy. Recent studies demonstrated synergistic inhibition of colorectal tumor growth by the combination of cyclooxygenase-2 (COX-2) inhibitors with oxaliplatin. The major COX-2 product, prostaglandin E2 (PGE_2), has been implicated in colorectal carcinogenesis; however, it is unknown whether PGE_2 affects colorectal tumor response to oxaliplatin. In this study, we investigated the potential role of PGE_2 in oxaliplatin resistance of human colon cancer cells. Total secreted PGE_2 levels were significantly increased in oxaliplatin-resistant HT29 cells (HT29 OXR) compared to parental cells. This was associated with increased COX-2 (18-fold, 95% confidence interval [CI]=10.71 to 24.35, P=0.008) and reduced 15-PGDH levels (2.18-fold, 95% confidence interval [CI]=0.45 to 0.64, P<0.0001), indicating deregulated metabolic control of PGE_2. Knockdown of microsomal prostaglandin E synthase-1 (mPGES-1) sensitized HT29 OXR cells to oxaliplatin. Selective inhibition of PGE_2 receptor (EP4 receptor) by L-161,982 treatment demonstrated a synergistic effect on oxaliplatin-induced cell apoptosis in OXR cells. L-161,982 also reduced the expression of colonic stem cell markers (CD133 and CD44) expression and tumor sphere formation by OXR cells. Furthermore, we identified that intracellular reactive oxygen species

Huakang Huang, Ph.D.

University of Connecticut, 2017

(ROS) accumulation, a key mechanism of oxaliplatin cytotoxicity, was significantly aggravated by EP4 inhibition. Addition of the antioxidant N-acetyl cysteine (NAC) reversed cellular ROS level in OXR cells and abolished the beneficial effect of EP4 blockade on oxaliplatin efficacy.

Overall, our findings uncover an important role for PGE_2/EP4 signaling in chemoresistance through regulation of oxidative stress and provide the rationale for targeting of EP4 signaling for increased oxaliplatin efficacy in CRC patients.

The Role of Prostaglandin E2 Signaling in Acquired Oxaliplatin Resistance

Huakang Huang

B.M. Peking University Health Science Center, 2012

B.A. Peking University, 2012

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2017

2017

APPROVAL PAGE

Doctor of Philosophy Dissertation

The Role of Prostaglandin E2 Signaling in Acquired Oxaliplatin Resistance

Presented by

Huakang Huang, B.M., B.A.

Major Advisor _____
Daniel W. Rosenberg

Associate Advisor _____
Blanka Rogina

Associate Advisor _____
Kevin Claffey

Associate Advisor _____
Charles Giardina

University of Connecticut

2017

To my parents Yuying Niu and Zhaoyong Huang

ACKNOWLEGEMENTS

I would like to thank Dr. Daniel W. Rosenberg for giving me the opportunity to conduct my thesis work in his laboratory and for giving me the freedom, support and advice to pursue independent research and studies.

I would like to thank my committee members Dr. Kevin Claffey, Dr. Charles Giardina and Dr. Blanka Rogina for all the insightful discussions and suggestions throughout the course of my PhD career.

Many thanks go to all the members of Rosenberg lab, past and present, for their valuable friendship in life and generous help in scientific research.

I also would like to thank my family and friends for their constant support and encouragement through my graduate years.

Lastly, my special thanks go to my husband Mr. Thomas R. Minuto Jr., for his unconditional love and support for my research and career choice.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF COLORECTAL CANCER

1.1.1 Epidemiology of colorectal cancer

Colorectal Cancer (CRC), the cancer that develops in colon and rectum, is the third most common cancer and the third leading cause of cancer death in United States. It is estimated that in 2016, over 134,400 new cases of colorectal cancer will be diagnosed, while about 49,190 Americans will die from colorectal cancer, accounting for 8% of all cancer deaths(1). In United States and worldwide, the incidence and mortality of CRC in women and men is equal; the lifetime probability of developing CRC is 5.56% (1 in 18) for women and 5.88% (1 in 17) for men (2,3) .

The risk of developing colorectal cancer increases with age; about 70% CRC cases occur in those aged 65 or older. This is partially due to the slow progression from precancerous polyp to metastatic CRC, which usually takes 10 to 20 years (4) . Taking advantage of the slow course of CRC development, colonoscopy screening among individuals over the age of 50 helps in early diagnoses and precancerous lesion removal, which results to CRC incidence decline by 3% annually in the past decade(2). However, the high incidence and death number has made CRC a

severe problem for public health; research efforts are needed in understanding CRC pathology and developing effective therapeutic strategies to increase the overall survival rates of CRC patients.

1.1.2 Risk factors

According to the epidemiological studies, the lifetime risk of colorectal cancer for an average US citizen (man or woman) is approximately 5% (5) . Besides age, there are many other factors that are known to increase the risk of developing CRC. These risk factors could largely fall into two categories: non-modifiable factors and modifiable factors. Non-modifiable factors include family history, which is associated with incidence of inherited colorectal cancers, and medical conditions such as Inflammatory Bowel Disease (IBD). Modifiable factors include dietary factors, lifestyle factors (behavior factors) and environmental factors. Evidence from published literatures has suggested that some risk factors are significantly associated with increased colon cancer risk (6) . On the other hand, several factors such as effective health care for medical conditions and healthy lifestyle are found to be inversely associated with CRC risk, indicating possible preventive strategies.

Among all the CRC cases, up to one-third of colon cancers demonstrate increased familial risk, indicating the possible significance of inheritance in risk of developing colon cancer (7) . According to 16 epidemiologic studies based on 8,091 cases of CRC, the risk of CRC for individuals with family history (first-degree relatives) of CRC is significantly higher compared to those with no family history of colon cancer (p=0.001; RR [rate ratio]=1.80, 95%CI

[confidence interval]=1.61-2.02). The pooled analysis of these studies didn't show statistical significant difference base on genders or study design (6) .

Besides the undefined inherited forms of colon cancer, it is known that approximately 5% of CRC cases are associated with well-defined inherited syndromes, including familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC, or Lynch syndrome). FAP is the best-characterized inherited CRC syndrome. FAP syndrome is a autosomal-dominant genetic disorder, its unique feature is the appearance of hundreds even thousands colonic adenomas in affected individuals starting from early adolescence. The risk of colon cancer for classic FAP patient is 100% without treatment. The average age of CRC diagnosis in FAP patients is 39, while 95% of those cases are diagnosed before age 50 (7) . Even for attenuated patients who exhibit a less severe form of FAP, the average lifetime risk of CRC is as high as 69%. Both classic FAP and attenuated FAP are caused by germline mutations in *APC*, which is an important tumor suppressor gene located in chromosome 5q21. The *APC* gene is part of the WNT signaling pathway in GI epithelial cells, and regulates cell growth through inhibiting β-catenin-induced cell proliferation (8) . When the APC gene is silenced/truncated *via* inherited or *de novo* mutations, cells lose the "gatekeeper" and start proliferating rapidly, resulting to increased possibilities of more genetic mutations and malignant transformation, leading to the development of numerous colonic polyps and eventually colorectal cancer.

Another well-known CRC-associated inherited syndrome is HNPCC, also called Lynch syndrome. Lynch syndrome accounts for about 2%-4% of all CRC cases. Although it is rare to observe polyposis in Lynch syndrome patients (distinct from FAP), the affected individuals do

develop colonic adenomas at younger age and bear a significantly higher lifetime risk (50%-80%) of CRC compared to the general population (9) . Lynch syndrome results from germline mutations in a group of genes related to DNA mismatch repair (MMR), including *hMSH2*, *hMLH1*, and *hMSH6*. As the MMR system is crucial for maintaining genomic stability by correcting mismatches during DNA replication in S phase of cell cycle, the defects in MMR genes lead to high-level of microsatellite instability (MSI-H), which induces the malignant transformation of epithelial cells. Besides colon cancer, Lynch syndrome is also highly associated with other types of cancers, including endometrial cancer, gastric cancer and ovarian cancer (10) .

Besides the heritable cancer syndromes, another medical condition called IBD has also been shown to be significantly associated with increased risk of CRC. The two main forms of IBD are Crohn's disease and ulcerative colitis. In a cohort study of 5529 patients with IBD (2857 patients had Crohn's disease and 2672 had ulcerative colitis), the incidence of CRC in IBD patient was significantly higher compared to the non-IBD population (IRR [incident rate ratio]=2.64 for Crohn's disease and IRR=2.75 for ulcerative colitis, respectively) (11) . The risk of CRC has been shown positively related to the duration and anatomic extent of IBD, and the proper health care such as usage of non-steroidal anti-inflammatory drugs (NSAIDs) was associated with decreased risk of developing CRC compared to non-treated IBD patients (12) .

Other than the non-modifiable factors, several lifestyle factors, including diet and behaviors, have also been suggested as risk factors for CRC. For example, population-based studies have shown an overall significant positive correlation between risk of colon cancer and

high consumption of red meats, such beef and pork (13) . In contrast, consumption of fruits and vegetables is found to be inversely correlated with CRC risk (6) . On the other hand, certain behaviors could become CRC risk factors as well. Cigarette smoking has been confirmed as significant causative factor for lung cancer (14) . Studies have shown that long-term heavy smokers of cigarettes, especially those who have used tobacco for more than 30 years, are also at a significant higher risk for developing colorectal cancer, compared to a non-smoking population (15) . Heavy consumption of alcohol was also associated with higher risk of CRC development (RR=1.26 per 20drinks/week), but no statistical significance was found (6) .

Overall, large population-based studies have revealed several significant risk factors for CRC development in an individual's lifetime. The discovery of modifiable factors suggests several primary preventive strategies, such as reducing red meat consumption and maintaining a healthy lifestyle. On the other hand, the studies on non-modifiable factor such as genetic alterations and IBD provide potential targets for secondary prevention and clinical management of colorectal cancer, which will be discussed in the following sections.

1.2 PATHOGENESIS OF COLORECTAL CANCER

1.2.1 Staging of colorectal cancer

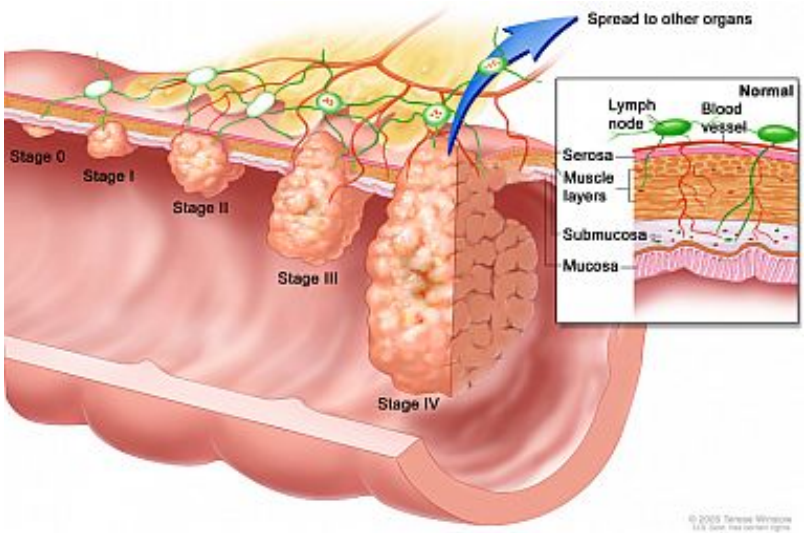
The large intestine is an important part of the digestive (gastrointestinal) system in human body. While the stomach and small intestine are critical for food digestion and most nutrient absorption, colon and rectum are responsible for absorbing water and mineral nutrient, as well as

get rid of the waste. The colon and rectum have complex physiological structures, consisting of four layers from the lumen towards the abdominal cavity: the mucosa, the submucosa, muscularis propria (muscle layer) and serosa. Mucosa is the innermost lining made of epithelial cells and glands (also referred to as crypts), secreting mucus and absorbing nutrients. Its is surrounded by submucosa, where the blood vessels, nerves and connective tissue form a layer to transfer the nutrient and support the mucosa function. The muscularis proporia contains layers of smooth muscle to help food process through the intestine, and the serosa is the outermost layer as a barrier between the large intestine and other vital organs in abdominal organs. The large intestine is also supported by lymphatic system through nearby lymph nodes. Multiple types of immune cells are located through the colonic layers and help maintain the GI homeostasis.

It is known that the almost all colorectal cancers start from the noncancerous abnormal growth (also referred to as "polyp") of epithelial cells within the mucosa layer of colon or rectum. Once the benign to malignant transformation take place, the cancer cells will keep proliferating and acquire invasiveness, leading to continuous growth of tumor through the colon wall, even penetration into the blood or lymphatic system, therefore forming local or distant tumors in organs or lymph nodes (16) . In clinical settings, to assess the prognosis of colorectal cancer and determine the choice of treatment respectively, the American Joint Committee on Cancer (AJCC) has designated the staging of colorectal cancer based on the TNM system (T=primary tumor; N=regional lymph nodes; M=distant metastasis) (17) . According to the pathology and affected areas, five stages are divided and the standard treatment varies between different stages **(Figure 1)**. In stage 0 (Carcinoma in situ), abnormal growth of colonic epithelial cells form precancerous lesions or cancer (polyps) within the mucosa of colon. These polyps can

be removed surgically *via* local excision or resection. In localized stages (stage I & II), the primary tumor spreads across multiple layers but is still restricted within colon wall, so surgical resection of colon segment is the standard treatment. In stage III (regional stage), cancer has penetrated the colon wall and spread to the nearby lymph nodes or tissues. Treatment options include surgical removal (resection) followed by chemotherapy, to avoid tumor recurrence. As for stage IV cancer (distant stage), when cancer has spread to distant lymph nodes or form metastasis in distant organs such as liver, lung or ovary, or recurrent tumors, surgical removal of the affected organs is required. Chemotherapy may be given before or after surgery. Radiation therapy and targeted therapy may be used to improve the survival or relieve symptom of patients.

Besides the staging, another key feature of colorectal cancer is the location of tumors. There are four sections of colon: the ascending colon, which connects with small intestine; the transverse colon which cross the abdomen from left to right; the descending colon; and the sigmoid colon which connects the rectum. Clinically, the ascending and transverse colon are referred to as proximal colon, while the descending and sigmoid colon together are referred to as distal colon. Studies have shown that colorectal tumors located at different locations within the large intestine have very different morphology, histology and epidemiological features (18) . For example, colorectal tumors located with proximal colon tend to have higher prevalence in female and older population and usually exhibit “serrated”, mucinous histological features (19) . These characteristics are significantly associated with different molecular pathways involved in colorectal carcinogenesis, which will be discussed in details in the following section.



Adapted from Advances in colorectal cancer research [Internet].

National Institute of Health, Bethesda, MD.

Available from https://www.nih.gov/research-training/advances-colorectal-cancer-research

Figure 1. Staging of colorectal cancer.

Stage 0 (Carcinoma in situ), abnormal colonic cells form precancerous lesions or cancer in mucosa.

Stage I, cancer is formed in mucosa (innermost layer) and submucosa, but still restricted within the colon wall.

Stage II (localized stage), the primary tumor spreads through the colon wall, might have spread through the serosa (outermost layer) and reach nearby organs.

Stage III (invasive stage), cancer has spread through the colon serosa and reach up to 7 nearby lymph nodes or nearby tissues.

Stage IV (distant stage), primary tumor has spread through colon wall and reached nearby lymph nodes and organs. Cancer cells may also migrate through blood/lymph system and form tumor metastasis in distant lymph nodes or organs, such as liver, lung or ovary.

1.2.2 Genetic and epigenetic alterations in CRC carcinogenesis

It has been shown that during colorectal tumor development, certain gene mutations and epigenetic changes take place in epithelial cancer cells. These alterations lead to dyregulation of several molecular pathways, which are key regulators of cell proliferation and survival, therefore play as driving forces in almost every aspect of colon cancer, including initiation, progression, invasion, metastasis and angiogenesis (20) . In this section, these genetic and epigenetic alterations, their associated molecular pathways in CRC carcinogenesis and their implications for CRC prognosis are discussed.

As mentioned in last section, germline mutations in the *APC* gene are responsible for the FAP syndrome. APC is an important component of the WNT/APC/β-catenin/Tcf complex, dysregulation of which is an early step in the classical adenoma-carcinoma pathway of CRC. The WNT pathway plays an important role in the regulation of both the embryonic development and the adult tissue self-renewal (21) . Under normal conditions, the APC/Axin/GSK-3β complex binds to β-catenin and triggers its phosphorylation, which leads to its further degradation. When Wnt ligands bind to the Frizzled receptor complex, downstream signaling leads to inactivation of the APC/Axin/GSK-3β complex, thereby releasing β-catenin and enabling its translocation to nucleus. This further activates the transcription factor, Tcf, and the Tcf-target genes, regulating multiple biological events, including proliferation and differentiation (22) . However, in colorectal cells from FAP patients or some sporadic CRC patients, the mutations in APC reduce the formation of the APC/Axin/GSK-3β complex, which results in constitutive activation of β-catenin and Tcf, therefore leading to uncontrolled proliferation, differentiation and even migration of cells, initiating colon carcinogenesis. Besides mutations in *APC*, other somatic gene

mutations are also involved in hyper-activation of the WNT pathway, including the mutations in β-catenin itself (present in about 48% colorectal tumors without APC mutations) and its regulators in the Notch pathway (23,24) . Another important oncogene involved in β-catenin regulation is cyclin dependent kinase-8 (*CDK8*), which is activated in approximately 60% of CRC cases (22) . The studies by Firestein and colleagues showed that CDK8 kinase activity is required for β-catenin-driven malignant transformation in colon cancer cells, and the overexpression of CDK8 is significantly correlated with colon cancer mortality (p=0.039; HR [harzard ratio]=1.70; 95%CI=1.03-2.83) (25,26) .

Although the early mutations in the WNT pathway initiate the transformation process of colon epithelial cells, subsequent mutations in other pathways are required for the benign to malignant transformations. Mitogen-activated protein kinases (MAPK) pathway, an important pathway that controls normal cell growth and survival, is confirmed to be involved in colorectal carcinogenesis **(Figure 2)**. The mutations in its key components, the *RAS* and *RAF* oncogenes, are observed in approximately 60% of all colon cancer cases, indicating the importance of the MAPK pathway in CRC (27) . To initiate MAPK signaling, secreted growth factor ligands, such as epidermal growth factor (EGF) bind to the receptor tyrosine kinase (RTK) on the cell surface membrane. Upon the binding, RTKs get phosphorylated and activated, further binding the son of sevenless (SOS) complex. The SOS complex binds to its downstream guanosine diphosphate (GDP)-RAS protein, and facilitates the switch from GDP to guanosine triphosphate (GTP), therefore activating RAS. GTP-RAS recruits and activates the RAF proteins, which in turn activate the downstream MEK and ERK. Activated ERK enters cell nucleus and further activate the transcript factors including Jun and Fos, which bind to AP-1 and activate the transcription of

their target gene, promoting cell proliferation (28) . In normal cells, the activation of MAPK pathway is tightly regulated by RAS-GTPase activating (GAP) protein, which switch GTP to GDP and inactivate RAS. However, during carcinogenesis, mutations in *RAS* proteins, most commonly in *KRAS*, lead to constitutive RAS activation and enable the cells to keep proliferating and escape apoptosis, promoting the adenoma-carcinoma transition (29) . This step is frequently followed by other genetic changes such as *p53* loss of function, to stimulate vigorous proliferation of cancer cells during later stage of colorectal tumorigenesis (30) . On the other hand, *KRAS* mutations are also shown to promote tumor invasion and metastasis in mouse models, whereas suppression of *KRAS* reduces its pro-tumorigenic functions, suggesting that *KRAS* could be a potential therapeutic target for advanced CRC (31) .

In contrast with the significance of RAS mutations in the classic pathway of CRC, another important component of MAPK pathway, BRAF, has been implicated as key player in the development of a distinct subtype of CRC *via* the "serrated" pathway (32) . Oncogenic *BRAF* mutations, most commonly *V600E* mutation, appear in approximately 10% CRC cases and are mutually exclusive with *KRAS* mutations (33) . These *BRAF* mutated colorectal tumors are primarily located in the proximal (right side) colon and exhibit distinct features including mucinous histology, serrated polyps/adenoma and poorer differentiated tumor mass (34,35) . Studies also showed that these tumors are highly methylated compared to the BRAF-wild type ones, thus characterized as CpG island methylator phenotype (CIMP)-high tumors. In contrast, the *KRAS* mutated cancers are usually associated with low level of DNA methylation (CIMP-low). Also, the *BRAF* mutant tumors are significantly correlated with MSI (36) . The studies by Kang group (37) and Thibodeau group (38) showed that the $BRAF^{V600E}$ mutations are strongly

associated with hyper-methylation of hMLH1 promoter rather than hMLH1 germline mutation, while the *KRAS* mutations are associated with hMLH1 un-methylated tumors. Methylation of hMLH1 promoter leads to silencing of hMLH1 and defective DNA mismatch repair. These studies suggest that BRAF-mutated serrated polyps/adenoma might be the precursor of hMLH1-methylated colorectal cancer.

Epidemiological studies showed that compared to BRAF-wild type CRC, *BRAF*-mutated colorectal cancer demonstrate significant prevalence in female (95% *vs* 44%, $p<0.001$) and in patients with advanced age (average age 75 *vs* 66, $p=0.004$) (36) . *BRAF* mutant cancers are also associated with worse prognosis for different stages of CRC, with significantly lower 5-year survival (47.5% *vs* 60.7%, $p<0.01$), regardless of microsatellite stability or mismatch repair proficiency (39,40) . These studies suggest *BRAF* mutation as negative prognostic factor for colorectal cancer. On the other hand, significance of *BRAF* in CRC has been well appreciated, which elicits extensive studies on development of strategies targeting mutated *BRAF* (such as vemurafenib, the first FDA-approved $BRAF^{V600E}$ inhibitor) for CRC therapy.

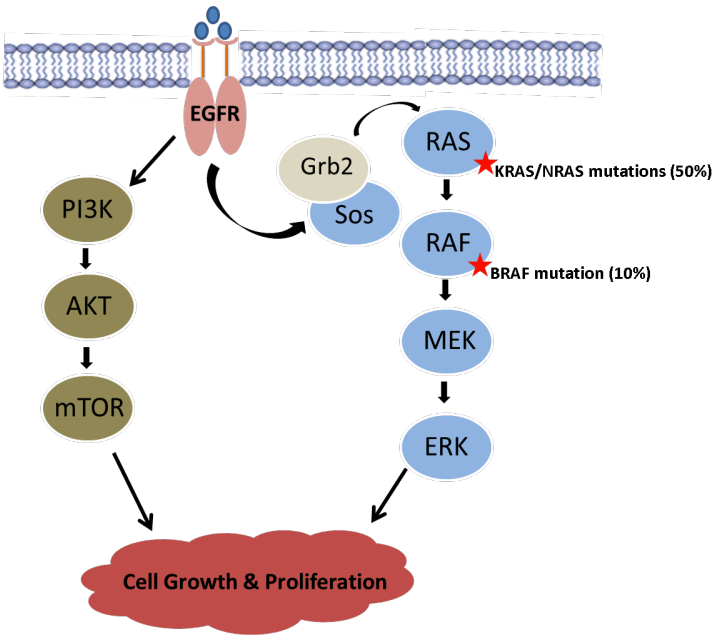
Another important pathway in colorectal carcinogenesis is phosphoinostide-3 kinase (PI3K) pathway **(Figure 2)**. Same as the MAPK pathway, activation of the PI3K pathway starts with growth factors binding to RTKs. Activated RTKs binds to PI3K complex and activate its catalytic subunit, p110. P110 phosphorylates phosphatidylinositol biphosphate (PIP2) to PIP3, and the latter recruits Protein kinase B (PKB, or AKT). Once AKT is phosphorylated and activated by PIP3, it binds to the downstream signaling factors such as Nuclear Factor-κB (NF-κB) or Bcl-2 family proteins, promoting cell metabolism and survival (41) . In normal cells, the

PI3K pathway is tightly regulated by the tumor suppressor gene phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 to inhibit AKT phosphorylation. However, in cancer cells, oncogenic mutations in *PIK3CA* (the gene encodes p110) or nonsense mutations in *PTEN* will lead to constitutive activation of AKT, therefore promote cancer cell proliferation, survival and invasiveness. Mutations in PI3K pathway are observed in 15%-25% of CRC cases and often occur simultaneously with *APC* alterations, resulting to synergistic effects in colorectal carcinogenesis (42) . Interestingly, RAS could activate PI3K pathway by directly binding to normal p110. The mutant RAS could bind *PIK3CA* mutated p110 effectively in cancer cells, suggesting the crosstalk between MAPK and PI3K pathways during colorectal carcinogenesis (43) .

Besides gene mutations and methylations other genetic factors have also been found to play important roles in colorectal carcinogenesis. MicroRNAs (miRNA), originally discovered in *C. elegans*, are small (length of 20-22 nucleotides) non-coding RNA molecules found in most eukaryotes including plants, animals and humans (44) . MiRNAs account for up to 5% human genome and regulate the expression of at least 30% of protein coding genes, particularly genes involved in cell proliferation and differentiation, therefore play important roles in both healthy tissues and cancer (45,46) . For example, studies by James and colleagues demonstrated the reduction of miRNA-143 and miRNA-145 expression in precancerous colonic polyps, compared to normal mucosa, suggesting miRNAs are involved in CRC neoplasia (47) . Altered expression of miRNAs have also been found in colorectal cancer tissues, with distinct expression pattern in accordance with mutations in *KRAS* and *BRAF* (mutually exclusive), suggesting that dyregulated miRNA expression is associated with RAS-RAF signaling in human colon cancer (48) .

Additionally, expression of several miRNAs including miR-18a, miR-21 and miR-203 have been shown correlated with worse prognosis of advanced CRC patients (49,50) , suggesting that the emerging studies on miRNAs could provide potential predictive biomarkers even therapeutic targets to achieve better clinical outcomes.

Overall, a better understanding of the molecular changes and their associated signaling pathways involved in CRC will help to develop novel therapeutic strategies that may improve clinical outcome. The discovery of multiple biomarkers will help develop effective CRC preventive strategies for high-risk populations and give better predictions of prognosis for CRC patients. More importantly, analysis of the genetic and epigenetic profile of a specific patient will help define the most effective targets and aid in the development of individualized treatment approaches, therefore improving the overall survival of CRC patients.



Adapted from Clarke and Kopetz, Journal of Gastrointestinal Oncology, 2015

Figure 2. MAPK pathway and PI3K pathway. Epidermal growth factor (EGF) binds to its EGFR receptor (RTK) and activates downstream RAS-RAF signaling as well as PI3K-AKT signaling, promoting expression of genes involved in cell growth and proliferation. Mutations in RAS, RAF or PI3K leads to constitutive activation of these signaling cascades, resulting in uncontrolled proliferation of affected cells, promoting tumor growth.

1.2.3 Tumor microenvironment and cancer-associated fibroblasts

Besides the malignant transformation in cancer cells, recent studies have also demonstrated the significance of the tumor-stromal interactions during tumorigenesis in different types of cancer, implying that the tumor microenvironment is not just a benign bystander, but actually an important modulator and even key player in tumorigenicity (51) .

In solid tumors, the tumor microenvironment mainly consists of tumor-infiltrating stroma cells, extracellular matrix (ECM) and other non-cellular components. Stromal cells are considered to represent a highly heterogeneous group of different cell type, including fibroblasts, adipocytes, endothelial cells, immune cells and mesenchymal stem cells (MSCs) (52-55) . Although each type of cells maintains distinct properties and functions, the pro-tumorigenic, typically inflamed tumor microenvironment is represented by the coordinated contributions from each cell type *via* extensive intercellular cross talk (56) .

An important group of cells within tumor-associated stroma are referred to as cancer-associated fibroblasts (CAFs). CAFs are comprised of activated fibroblasts originating from a diverse group of cell types, including resident fibroblasts (57) , bone marrow-derived mesenchymal stem cells (58) , adipocytes (59) , endothelial cells (60) and even under certain circumstances the neighboring epithelial cells (61,62) . During the development of primary and metastatic colorectal cancer, upon the interactions between stroma and epithelial-derived cancer cells, normal resident fibroblasts (NRFs) get activated and further acquire a new set of properties; these CAF properties include robust proliferation, enhanced migratory capacity, up-regulation of pro-inflammatory signaling, and increased secretion of cytokines or growth factors

(63) . Although there is no CAF-specific biomarker, CAFs express myofibroblast markers α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP) (64-66) . In several recent clinical studies, a strong association has been demonstrated between elevated levels of CAFs and poor prognosis in patients harboring colorectal tumors (67,68) . High levels of stromal FAP expression have also been considered as an indicator of aggressive tumor behavior, including metastases and recurrence of different malignancies (67,69,70) . These findings suggest that CAFs may be important players during cancer progression, provoking strong interest in understanding their functions during tumor growth and metastasis.

CAFs have been shown to produce various cytokines and growth factors, including IL-6, hepatocyte growth factor (HGF) and insulin-like growth factor (IGF) during colorectal cancer growth (71-74) , to promote cancer cell proliferation and migration. Furthermore, IL-6 can stimulate the expression of vascular endothelial growth factor (VEGF) by CAF in an autocrine manner, promoting the angiogenesis in tumor mass (74) . Interestingly, many growth factors and cytokines are also produced by cancer cells and stimulate the proliferation of CAFs. This crosstalk forms positive feedback loops between tumor and stromal cells, further promoting tumor progression. Recently, several studies have been done to target the crosstalk between cancer cells and CAFs for their anti-cancer therapeutic potential. Cheng and colleagues (75) found that interfering with tumor-stromal interactions could significantly reduced tumor growth in the azoxymethane-dextran sulfate sodium (AOM-DSS) mouse model. A recent study by Li et al. (76) demonstrated that inhibition of CAFs by targeting FAP activity significantly suppressed tumor growth and angiogenesis in a xenograft mouse model. Interestingly, they also discovered that suppression of CAF synergistically enhanced the efficacy of oxaliplatin. Consistent with

these findings, several related studies have also demonstrated that tumor-associated fibroblasts are involved in resistance to chemotherapeutic treatments (e.g. oxaliplatin) through restoring a cancer stem cell phenotype in colorectal cancer (72,77) .

Overall, these findings indicate the significance of tumor-microenvironment, especially cancer-associated fibroblast, in colorectal cancer progression. In addition, it is also suggested that CAFs may be a potent target in CRC prevention and therapy. The current preventive and therapeutic strategies for CRC, and the latest findings will be discussed in the following sections.

1.3 PREVENTION OF COLORECTAL CANCER

1.3.1 Conventional prevention strategies

Given the slow carcinogenesis and multi-stage progression, colorectal cancer is among the malignancies that could benefit from prevention, and effective preventive strategies have been shown to reduce both the incidence and the mortality of CRC (78) . As previously mentioned, almost two-thirds of the total CRC cases are sporadic and are related with modifiable risk factors such as diet and behavior factors (smoking, alcohol and red meat consumption, etc.), suggesting that effective interventions on modifiable factors could possibly reduce the incidence of CRC in average-risk population (free of family CRC history and CRC symptoms). These feasible interventions (also known as health promotion programs) are regarded as primary preventive strategies, which include reducing alcohol consumption and smoking frequency, keeping a diet high in fruits and vegetables while low in red meat, doing regular physical

exercises, etc. It is estimated that approximately 66% of CRC cases are preventable by changing diet and maintaining healthy lifestyle (79) . However, changing diet and lifestyle are effective at long-term CRC prevention and it must be accompanied by preventive strategies with short-term impact, such as CRC screening.

It is well known that the prognosis of CRC is highly associated with the stage at diagnosis (80) , early detection and treatment provide significant advantage in improving the survival rate of CRC patients. Therefore, different from the primary prevention, which provides general protection for healthy individuals from getting CRC, the secondary preventive strategies (CRC screening) actually aim to detect and remove precancerous lesions or even early-stage CRC, and exhibit immediate impact on reducing the incidence and mortality of CRC. There are mainly two types of screening, visualization of large intestine (colonoscopy) and analysis of biological samples (fecal occult blood test, FOBT). Currently, since the risk of CRC increases with age, it is recommended that individuals with average risk (asymptomatic, no risk factors) start population-based screening at age 50. For people with increased risk for CRC, including family/personal history of CRC and personal history of IBD, the screening is suggested to start from age 40 or earlier with short intervals between each screening.

There are several screening methods to visualize the large intestine, including colonoscopy, sigmoidoscopy and CT colonography. Colonoscopy is regarded as the gold standard for CRC diagnosis, and has been shown to significantly reduce the incidence (67% reduction) and mortality (65% reduction) of CRC in several clinical studies (81,82) . Colonoscopy also exhibits better sensitivity and specificity for smaller (6mm~10mm) colonic

lesions than CT colonography (98% vs. 63%) (83) . Moreover, compared to sigmodioscopy, which is only effective on distal colon neoplasm detection, colonoscopy demonstrates better coverage. However, studies have shown that the beneficial effect of colonoscopy on CRC prognosis is strongly associated with the location of CRC (distal colon favorable that proximal) and the specialty of the endoscopist (84) . Colonoscopy also has other disadvantages including cost, lower acceptance and complications due to the invasive nature of the test, which limit the its application.

On the other hand, analyses of biological samples (feces, plasma and urine) have become more accepted population-based screening strategies. These methods include FOBT, fecal/plasma DNA and RNA test, and protein test. Since most cases of CRC tend to bleed during the early stages of tumor development, testing the occult blood in the stool (fecal hemoglobin) has been proved as an effective method for detecting the colonic neoplasia in several clinical studies, with sensitivity ranged approximately from 62% to 80% and specificity ranged from 65% to 98%, depending on whether its guaiac based (gFOBT) or immunological based (FIT) (85) . In fact, due to its low cost and non-invasive nature, FOBT has become the most used population-based screening method for CRC in Europe and worldwide. However, since the tests used in gFOBT are not specific for human hemoglobin, patients are recommended to restrict red meat consumption to avoid false positive and colonoscopy are to be performed on patients with positive FOBT results (86) .

On the other hand, as discussed in previous sections, CRC carcinogenesis is associated with multiple genetic and epigenetic alterations. Besides the genetic mutations in *APC* and MMR

genes, which are the key features for high-risk population, several other genetic and epigenetic markers in biological samples have been developed as diagnostic strategies for detecting colonic neoplasia in average-risk individuals (87) . So far, fecal and plasma marker panels include gene mutations in *APC*, *KRAS*, *p53* and genes involved in EMTs; methylations in such gene promoters and RNA expression levels of genes such as *COX-2* have also been tested for CRC diagnosis (88,89) . A recent study by Link and colleagues also suggested that abnormal levels of fecal miRNAs such as miR-21 and miR-106a could be used as biomarkers for colonic neoplasia screening with specificity of approximately 75% in average-risk population (90) . Although the DNA/RNA tests have higher cost and need improvement in specificities, analyses of genetic and epigenetic biomarkers have exhibited remarkable accuracy and better coverage for diagnosis of early lesions than conventional methods (91) . More studies are required for development of biomarker panels to achieve better specificity and lower cost for CRC early diagnosis.

1.3.2 Aberrant Crypt Foci as surrogate biomarkers for colon cancer

Colorectal carcinogenesis is known to arise from pre-neoplastic lesions comprised of abnormal epithelial cells. Although the conventional CRC pathway is regarded as "polyp-adenoma-carcinoma" pathway, recent studies suggested that aberrant crypt foci (ACF), described as the cluster of colonic crypts morphologically different from normal surrounding mucosa, is in fact the earliest neoplastic lesion in CRC progression and might be a surrogate biomarker for clinical CRC prevention.

ACF was first discovered in AOM mouse model by Bird in 1987 using methylene blue staining (92) , and has intrigued many related pre-clinical and clinical studies ever since. Similar

as methods used in animal studies, human ACF could be detected in high-magnification chromoscopic colonoscopy (HMCC) through staining. Compared to regular endoscopic technologies, HMCC has higher magnification and sensitivity for flat or depressed lesions, and is widely used in clinical settings to reduce the false-negative rate of CRC screening (93) . In HMCC, ACFs stand out from the background as elevated, usually deeper stained cluster of larger crypts with abnormal shape, and can be removed by endoscopic biopsy for further histological and molecular analysis (94) .

Based on histological features, almost all ACFs can be classified into two main subtypes: hyperplastic ACF and dysplastic ACF. Hyperplastic ACFs are characterized as larger and longer crypts (compared to normal ones) with abnormal, sometimes serrated luminal openings. Similar as hyperplastic polyps, the hyperplastic ACFs exhibit hyper-proliferation of epithelial cells, represented by upward expansion of positive staining for proliferating cell nuclear antigen (PCNA) and Ki-67 protein in abnormal crypts (95) . On the other hand, dysplastic ACFs may not be larger than normal crypts or having serrated features, but demonstrate histologic features of dysplasia, including nuclei elongation, stratification, or polymorphism in epithelial cells, and positive staining of PCNA and Ki-67 in upper crypts (96) . Similar as adenomas or colorectal cancers with distinct histological characters, different ACFs result from correlated molecular features and have been implied as precursors for different CRC progression pathways.

Multiple studies have shown that ACFs share similar molecular features, including genetic and epigenetic alterations with CRC, providing strong support for their role as pre-neoplastic lesions for CRC. For example, alterations in WNT pathway such as *APC* mutations

and β–catenin nuclear expression, have been found in both ACFs and CRC. Interestingly, in sporadic *APC* mutations and β–catenin nuclear translocation are more likely to be found in dysplastic ACFs, while *KRAS* mutations occur more frequently in hyperplastic ACFs (97) . In FAP patients who carry germline mutations in *APC,* the prevalence of dysplastic ACFs is significantly higher than hyperplastic ACFs (98) . On the other hand, the serrated hyperplastic ACFs often carry oncogenic mutations of *KRAS* or *BRAF* (mutually exclusive), correlated with DNA hyper-methylation (CIMP), suggesting that hyperplastic ACFs may be the precursor lesions of the serrated CRC pathway (99) . Moreover, the level of microsatellite instability (MSI) has been shown gradually increasing in ACFs to adenoma and carcinoma, compared to the normal colonic mucosa (100) , suggesting ACFs could be early precursors in Lynch syndrome associated colorectal cancers. Moreover, a recent study by our lab deciphered the cellular interplay between ACF and adjacent normal-appearing stroma in CRC patients, highlighting the activation in NF-κB pathway and stromal fibroblasts, suggesting that ACF, as the earliest pre-neoplastic lesion in colorectal carcinogenesis, is associated with inflammation and altered stromal microenvironment (101) .

Overall, clinical and pre-clinical studies have indicated ACFs as the pre-neoplastic lesions of CRC and potential biomarker for CRC prevention. Further studies such as more complete characterization of genomic profile of ACFs, better understanding of molecular pathways involved in CRC carcinogenesis, especially the crosstalk between neoplastic lesions and stromal microenvironment are required for promoting the clinical application of ACF as surrogate marker for CRC.

1.3.3 Prostaglandin E2 (PGE_2) and colorectal cancer prevention

Besides the population-based CRC screening through colonoscopy, developing effective chemo-preventive strategies to reduce CRC incidence in high-risk population is another promising field. Since the 1980s, studies have shown that regular use of non-sterodial anti-inflammatory drugs (NSAIDs) such as aspirin, sulindac, and ibuprofen is associated with significantly reduced risk of CRC (102-104) . Recent clinical trials indicated the long-term (5 years or longer) low-dose (75mg or less daily) use of aspirin significantly reduce colorectal cancer incidence (~30%) and mortality (~40%) (105) . The effect of NSAIDs on CRC prevention is believed to be largely attributed to their suppression of pro-inflammatory prostaglandin synthesis by cyclooxygenase (COX) enzyme inhibition (102) . COX inhibition leads to less synthesis of down-stream prostanoids, including prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) **(Figure 3)**. These lipids are critical in various physiological processes (i.e. inflammation, platelet aggregation, wound healing) and PGE_2 is known as the most important bioactive lipid in the human body, particularly with respect to its effects on inflammation and tumorigenesis (106) .

PGE_2 has been shown to regulate various physiological and pathological events. Within the digestive system, PGE_2 helps to maintain mucosal integrity and maintain GI track homeostasis (107) . During acute or chronic inflammation, PGE_2 promotes early inflammatory response by facilitating immune cells infiltration and also resolute inflammation by regulating cytokines and chemokine expression (i.e. IL-2, CCL19) (108) . Normally PGE_2 synthesis is tightly regulated through modulation of the expression or activity of its synthases (COX enzymes and PGE_2 terminal synthases). However, during colorectal tumorigenesis or chronic

inflammation the levels of PGE_2 are significantly elevated within the colonic mucosa (109) , due in part to the coordinated up-regulation of COX-2 and mPGES-1, an effect that is caused by growth factors and/or inflammatory stimuli such as LPS (106) .

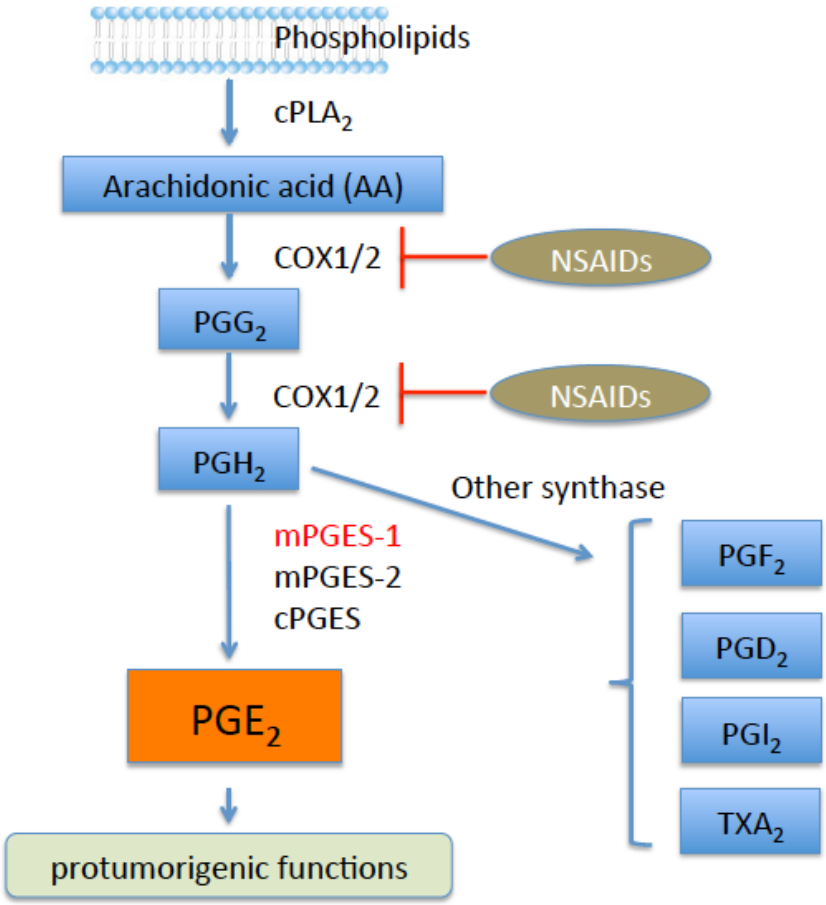


Figure 3. PGE_2 synthesis pathway. Arachdonic acid is released from membrane phospholipids by calcium-dependent phospholipase A2 (cPLA2), and gets rapidly oxidized to unstable PGG_2, then reduced to PGH_2. Both steps are catalyzed by COX enzymes (COX-1 and COX-2). PGH_2 is subsequently converted to PGE_2 or other prostanoids, including prostaglandin (PG) D_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). The three specific terminal synthases for PGE_2 generation are microsomal PGE synthase 1(mPGES-1), mPGES-2, and cytosolic PGE synthase (cPGES). NSAIDs inhibit the activities of COX enzymes and suppress the synthesis of prostaglandins.

Besides the up-regulation of PGE_2 levels in CRC patients, other studies have shown that PGE_2 may promote colorectal tumorigenesis directly *in vitro* and *in vivo*. In multiple CRC mouse models, it is shown that administration of exogenous PGE_2 or a PGE_2 analogue increases the incidence and multiplicity of intestinal tumors (110,111) , while PGE_2 suppression through genetic deletion of COX-2 or COX-2 inhibitor treatment leads to decreased small intestinal and colorectal tumorigenesis (112,113) . Moreover, the terminal synthase of PGE_2, mPGES-1, has been shown to be functionally linked with COX-2 overexpression and PGE_2 level elevation during colon cancer development (114) . Studies in our lab have shown that genetic deletion of mPGES-1 selectively blocks inducible PGE_2 synthesis within the colonic mucosa, and significantly suppresses genetic or carcinogen-induced intestinal cancer in mouse models (115,116) , indicating mPGES-1 as a potential target for selective PGE_2 suppression for CRC prevention.

In addition, PGE_2 may also play an important role within the tumor microenvironment by facilitating tumor-associated angiogenesis and metastasis during CRC progression. Angiogenesis, the process that new blood vessels generate from existing system, is required for providing blood perfusion and supplying oxygen/nutrient to local proliferating cells, therefore is critical for both normal organogenesis (i.e. embryogenesis and adult tissue regeneration) and cancer progression (117) . Angiogenesis is a dynamic process regulated by pro-angiogenic factors, such as hypoxia-inducible factor-1α (HIF-1α), VEGF or CXCL1, and anti-angiogenic factors, such as anti-angiogenic peptides (i.e. prolactin) and interferon-α (IFN-α). During tumor progression, pro-angiogenic mechanism, mainly driven by HIF-1α upregulation and VEGF overexpression, overrides anti-angiogenic effects, known as *angiogenic switch*, to promote

neovascularization in tumor tissue and facilitate tumor growth. Pro-angiogenic factor overexpression can be induced by hypoxia, pro-inflammatory cytokines or growth factors (118) . Recent studies have shown that PGE_2 signaling induces pro-angiogenic factor expression and promotes angiogenesis in several malignancies, including breast, lung, ovarian, colon and prostate cancers (119-122) . In breast cancer mouse model, mammary epithelial cancer cell secreted PGE_2 activates PKA pathway and induces VEGF expression in tumor stroma, increasing micro-vessel density and promotes tumor progression (120) . Importantly, many studies have shown that in CRC animal models, lack of PGE_2 signaling by COX-2 knockout or EP receptor antagonist treatment leads to significant decreased tumor vessel density and slower tumor progression (123,124) . NSAIDs or selective COX-2 inhibitors treatment also block PGE_2-induced angiogenic factor production and suppress tumor-associated angiogenesis, suggesting that PGE_2 may play an important role in CRC angiogenesis (125) . Another aspect of PGE_2 pro-tumorigenic effects is associated with immunosuppression during tumor metastasis. Studies showed that PGE_2 could suppress cytotoxic (CD8+) T cell anti-tumor effect by directly down-regulating dendritic cell-mediated antigen presentation both *in vivo* and *in vitro* (126,127) . PGE_2 also reduces the level of anti-tumor cytokines (i.e. TNF-α, IFN-γ and IL-2) secreted by CD4+ T cells (128) . Moreover, PGE_2 has been shown to promote tumor immunosuppression by inducing differentiation of Gr1+ CD11b+ myeloid-derived suppressor cells (MDSCs) and M2-like macrophages in multiple malignancies including lung, breast and cervical carcinoma, to suppress anti-tumor immunity and promote tumor metastasis (129,130) .

On the other hand, PGE_2 has been shown important for the pro-tumorigenic functions of the aforementioned CAFs. Gene expression analysis of colonic fibroblasts demonstrated a

significant increase in the levels of COX-2 in CAFs compared to normal fibroblasts during colorectal cancer initiation and growth (66,131-133) . The work by Konstantinopoulos and colleagues (134) suggested that the up-regulation of COX-2 in colorectal cancer-associated fibroblasts is associated with activation of AP-1 and NF-kB transcription factors; Studies by the Lance group (131,135-138) and Zhu group (73) showed that COX-2 expression in CAFs can be induced by pro-inflammatory factors including IL-1β, TNF-α, deoxycholic acid and HGF through PKC-mediated mechanisms. Increased COX-2 expression and PGE2 secretion by CAF promotes the proliferation and invasiveness of epithelial colon cancer cells in a paracrine manner, partially by activation of EP4 receptor signaling in cancer cells (139) . Pre-treatment of cancer cells with COX-2 inhibitors may abolish the pro-tumorigenic function of CAFs and suppress colon cancer cell proliferation and invasion (73,139) . Overexpression of COX-2 has also been observed in invasive adenocarcinomas and liver metastases in advanced colon cancer patients (132,140) , suggesting that COX-2 may also be an important modulator the in metastasis-promoting effects of CAFs (64) .

Overall, these studies suggest that PGE_2 plays an important role in CRC tumorigenesis and may be a potent target for CRC prevention. However, clinical studies demonstrate that long-term intake of NSAIDs could inhibit either COX-1 activity and suppresses platelets TXA_2 production, or block COX-2 mediated PGI_2 generation, thereby adversely affecting cardiovascular homeostasis and resulting in severe side-effects, such as increased risk of stroke, heart attack or GI bleeding (141,142) . Therefore, recently several selective mPGES-1 inhibitors (i.e. MF63, PF-9184) have been developed and their efficacy as selective PGE_2 suppressor for CRC prevention while circumventing the side effects is under investigation.

1.4 TREATMENT OF COLORECTAL CANCER

1.4.1 Clinical therapeutics

Despite the advances in preventive strategies, once an individual is diagnosed with CRC, effective clinical treatments are critical for disease outcome and patient survival. As mentioned in section 1.2.1, the clinical treatments for CRC vary based on the stage of diagnosis, but in most cases, the therapeutic strategies are comprised of surgery and adjuvant radiotherapy or chemotherapy. Recently, with a better understanding of the molecular mechanisms involved in development of CRC, several targeted therapies have also been used as a complementary treatment in clinical trials or first-line treatments for metastatic CRC patients (78) .

Surgery is the standard treatment for CRC diagnosed at all stages. For carcinoma *in situ* and certain cases of stage I cancers (tumors that have not exceeded the upper third of the submucosa within the colon wall), cancerous lesions can be removed by the endoscopic microsurgery (143) . For more advanced primary tumors in colon or rectum, colectomy or total mesorectal excision (TME), meaning the total removal of the affected section of colon or rectum, usually accompanied by resection of nearby lymph nodes, are performed to reduce the risk of tumor recurrence (144) . For patients diagnosed with metastatic CRC, the mostly common metastatic site is the liver (approximately 20% of CRC cases have liver metastases). Liver metastases resections are performed based on the evaluation of a number of prognostic factors, including the numbers of metastases, extra-hepatic disease and sufficient liver reserves. With effective treatments, there can be a 5-year disease free survival (DFS) in some patients (145) .

Surgical treatment for stage II or more advanced CRC cases are usually accompanied by adjuvant radiotherapy or chemotherapies. Due to the anatomic complexity in pelvis and absence of serosa in rectum, preoperative or postoperative radiotherapy (radiotherapy fraction of 50.4Gy in 28 fractions) are required to reduce the regional recurrence (146) . Concomitantly, chemotherapies such as 5-fluorouracil (5-FU) and oxaliplatin, are used for both advanced rectal and colon cancer, to prevent both local recurrence (rectal cancer) and distant metastasis (colon cancer). Oxaliplatin is a platinum derivative and forms crosslinks with DNA strand, while 5-FU is a thymidylate synthase (TS) inhibitor and blocks DNA synthesis. These conventional chemotherapeutic agents attack rapidly-proliferating cells by inhibiting DNA/RNA synthesis or microtubule function, resulting to cell death and tumor growth suppression. Due to the adverse effects on normal proliferating cells, in clinical settings, these chemotherapeutic agents are usually given in combination, such as FOLFOX (5-FU, oxaliplatin, folinic acid), or CapeOx (capecitabine with oxaliplatin) (147) . Administration of chemotherapy of 6 months is known to improve patient survival by approximately 10% to 15% (148) . However, due to their high toxicity, the use of certain agents (i.e. oxaliplatin) in patients with lower tolerance (aged 70 or older) is highly limited (16) . Another clinical problem is treatment failure, as tumor develops resistance to chemotherapy (chemo-resistance), which leads to lack of response or tumor recurrence. Chemo-resistance is more common among advanced CRC patients; most of the metastatic colorectal patients develop resistance against chemotherapy within 8 months (149) . The chemo-resistance of CRC and possible mechanisms will be discussed in the following sections in detail.

Besides surgery and conventional adjuvant therapies, recent advances in molecular mechanisms of colorectal carcinogenesis and progression has inspired the development of targeted therapies, the therapeutic strategies aiming at specific targets critical for CRC development. For example, monoclonal antibodies against EGFR (i.e. Cetuximab, Panitumumab) or VEGF (Bevacizumab) have been developed to suppress EGF signaling and angiogenesis in CRC and other malignancies (i.e. melanoma) (16) . Some clinical trials showed that the combination of targeted therapies with conventional chemotherapy increases patient survival with less toxicity, but their efficacy in CRC is controversial (103,150) . In addition, resistance to targeted therapy also develops in advanced CRC patients, and more studies are required to optimize these targeted therapies for clinical use (151) .

1.4.2 Chemo-resistance of colorectal cancer

Despite the impressive advances in cancer research and development of clinical therapeutics in the past several decades, treatment failure due to resistance against chemotherapy, known as chemo-resistance, remains one of the biggest challenges in the fight against cancer (152) . In clinical settings, despite the standard application and modest initial response to chemotherapy in advanced CRC patients, most metastatic CRC patients develop resistance against current chemotherapeutic agents (i.e. oxaliplatin, 5-FU) and die from tumor metastasis within 2 years (149,153) . Understanding the underlying mechanisms and developing novel therapeutic strategies to overcome chemo-resistance, is a key question in the field of cancer therapy (154) .

There are two forms of cancer cell resistance to chemotherapy: primary or intrinsic resistance which leads to non-response to initial drug treatment, and secondary or acquired resistance which develops after initial response to drug treatment. Both types of resistance are believed to result from genetic or epigenetic alterations in cancer cells. However, intrinsic resistance is likely to be caused by genetic alterations existing prior to drug treatment, while in the case of acquired resistance, changes in gene expression or epigenetic deregulation might be induced by the initial drug treatment.

Multiple mechanisms have been shown to be involved in chemo-resistance, and the most common forms are due to de-regulations of critical cell proliferation or survival signaling pathways. Genetic mutations or altered DNA methylation result in either activation of oncogenes or inactivation ("loss-of-function") of tumor suppressor genes. Therefore, not only does these genetic changes promote cell transformation and proliferation, but also provide cell survival signals to render resistance against chemotherapy-induced cell death. For example, activating mutations in *PI3KCA* leads to constitutive activation of the PI3K/Akt/mTOR pathway, promoting cell survival upon stress (i.e. chemotherapy, radiation) (41) . On the other hand, in some cancer cells, an important tumor suppressor PTEN, which regulates Akt pathway, is inactivated *via* promoter methylation, leading to both intrinsic and acquired chemoresistance (155) . Similarly, alterations in DNA repair pathways or their downstream apoptotic pathways, such as gene mutations for either MMR proteins or B-cell lymphoma 2 (Bcl-2) protein family, could also affect the cellular response to drugs and promote drug resistance in cancer cells (156) .

It is worth mentioning that when cancer cells exhibit reduced sensitivity to chemotherapy after initial response, they are likely to develop resistance simultaneously to multiple structurally/functionally unrelated drugs. This phenomenon is known as multidrug resistance (MDR), which is mainly mediated by multidrug resistance proteins from the ATP-binding cassette (ABC) transporter family, such as P-glycoprotein (MDR-1/P-gp or ABCB1) and multidrug resistance protein 1 (MRP1 or ABCC1) (157) . ABC transporters work as membrane-embedded efflux pumps for various drugs, and their expression can be induced by chronic exposure of cancer cells to drugs (158) . Overexpression of these proteins will increase drug efflux and lower the intracellular concentration of anti-neoplastic drugs, therefore reduce their efficacy. MDR is one of the major mechanisms involved in CRC chemoresistance (159) .

Besides the commonly known mechanisms, recent studies have indicated that cancer stem cells (CSCs) are not only important for tumor initiation, but also play key roles in tumor recurrence after chemotherapy (160) . CSCs are defined as tumorigenic cancer cells with stem cell like properties including slow proliferation, self-renew and differentiation abilities. CSCs are found in a wide variety of malignancies including GI cancers and exhibit capability to drive primary tumor initiation in xenograft mouse models (161) . As current chemotherapies mainly target rapidly proliferating cells, CSCs are more resistant to cytotoxic drugs and able to mediate tumor recurrence (expansion) after chemotherapy (162) . Other mechanisms such as MDR and anti-apoptosis signaling have also been associated with CSC chemoresistance in colon cancer (163) . Developing a better understanding of CSC-mediated chemoresistance mechanisms is critical for enhancing chemotherapeutic efficacy and improving advanced CRC patient survival.

The involvement of CSCs in colon cancer resistance against oxaliplatin will be discussed in greater detail later in this dissertation.

1.4.3 Targeting PGE_2 for treatment of colorectal cancer

As discussed previously (section 1.3.3), the multi-functional bioactive lipid PGE_2 has been well known for its various pro-tumorigenic effects in colorectal cancer initiation and progression, and has provoked many studies as a potent target for CRC prevention (104) . However, recent studies haves shown that PGE_2 signaling may be involved in tumor response or resistance to current anti-cancer therapies, suggesting that PGE_2 or its selective synthase may serve as potential targets for adjuvant therapy in cancer treatment (164) . For example, several preclinical studies have shown that PGE_2 suppression by NSAIDs or COX2 inhibitors, including celecoxib and sulindac, could effectively enhance chemotherapy efficacy or even abrogate chemo-resistance in various cancer types (165,166) . In recent neo-adjuvant clinical trials, the combination of COX-2 inhibitors with standard breast cancer therapy aromatase inhibitors (AI) have shown promising efficacy and safety for treatment of metastatic breast cancer (167) .

In colorectal cancer, the beneficial effect of PGE_2 suppression has been confirmed in multiple studies in which COX-2 inhibitors are combined with current chemotherapeutic agents, including 5-FU and oxaliplatin. For example, Zhang and colleagues discovered that combination of celecoxib and 5-FU significantly inhibited colon tumor growth *via* activation of cytochrome C mediated apoptotic pathway in subcutaneous xenograft mouse model (168) . Lin and colleagues demonstrated that combination of celecoxib and oxaliplatin could significantly reduce expression

of survivin protein expression and increase cell death compared to oxaliplatin alone (169) . Furthermore, studies by the Zhao group showed that addition of celecoxib not only increases cell apoptosis and facilitates tumor shrinking, but also significantly reduces angiogenesis (VEGF mRNA expression and microvessel density) in a mouse xenograft model of colon cancer (170) . These combination therapy studies suggest that COX-2 inhibition could improve chemotherapeutic efficacy in CRC. However, the side effects, including increased cardiovascular risk, have become great hurdle for clinical application of COX-2 inhibitors (142) .

Fortunately, the beneficial effect of COX-2 inhibition in combined treatment of CRC is possibly due to blockade of PGE_2 signaling. Studies have shown that PGE_2 promotes colon cancer cell growth and inhibits cell apoptosis through PI3K pathway activation (171,172) . Interestingly, besides chemotherapy-induced cell death, Tessner and colleagues found that PGE_2 could also reduce radiation-induced epithelial apoptosis in mouse small intestine and human colon cancer cell line, possibly through AKT mediated anti-apoptotic pathway (173) . These studies suggest that PGE_2 signaling could promote cancer cell survival in different conditions (spontaneous or under stress), implying that the aforementioned COX-2 inhibition may enhance oxalipatin efficacy possibly by suppressing PGE_2 mediated anti-apoptotic mechanisms.

Besides cell survival signaling, the COX-2/PGE_2 pathway has also been associated with CSC-mediated cancer chemoresistance (164,165) . Several studies showed that a combination treatment of NSAIDs or COX-2 specific inhibitors could enhance the efficacy of chemotherapies on colon cancer cells, while long-term use of aspirin or celecoxib has been shown to improve the overall survival of advanced CRC patients in several clinical trials (174-176) , indicating the

potential clinical benefits of COX-2 inhibition in colon cancer therapy. These findings suggest that PGE_2 may play an important role in colorectal tumor response to chemotherapy, and targeting PGE_2 could provide a novel strategy to enhance treatment efficacy and combat chemo-resistance. In my thesis study, the significance of PGE_2 signaling in colorectal cancer cell survival will be examined in depth, and the potential of targeting PGE_2 as adjuvant therapeutic strategy to enhance the efficacy of oxaliplatin or circumventing oxaliplatin resistance in CRC will be evaluated.

1.5 Genetically engineered mouse models of colorectal cancer

1.5.1 Germline genetically mutant models

Although cell culture system is widely used for mechanism studies and early phase/high-throughput drug screening, preclinical animal models are critical *in vivo* platforms for biomarker identification and drug development in colorectal cancer research, given their great advantages in both time and cost. There are mainly two types of mouse CRC models, xenograft models and genetically engineered mouse models (GEMMs). Xenograft models are built by subcutaneously or orthotopically transplanting *in vitro* passaged CRC cells or patient-derived colorectal tumors into immunodeficient mice. Although these models are simple to use and relatively cheaper than GEMMs, their drawbacks such as host immune deficiency and tumor-stromal mismatch have limited their value in testing and predicating the efficacy of novel anti-cancer drugs. On the contrary, GEMMs recapitulate spontaneous colorectal tumorigenesis in immunocompetent mice

by genetically modifying critical genes in human CRC development (i.e. *APC, KRAS*), thus circumvent the limitations of xenograft models.

The very first GEMM for CRC was developed in 1990s. Moser and colleagues discovered a mouse lineage that exhibits inherited predisposition to spontaneous intestinal tumorigenesis, and named it Min (multiple intestinal neoplasia) (177) . Min mice were then confirmed to carry a nonsense mutation in *APC* genes, which is analogous to the *APC* mutations found in FAP patients and some sporadic CRC patients (178) . Ever since, more CRC mice models have been established with various *APC* germline mutations (179) . Because homozygous *APC* mutations are proved embryonically lethal, all these mice models are heterogeneous (180) . Interestingly, despite the variations in sizes and numbers, most polyps or adenomas developed in these *APC* mutated mice are located in small intestine rather in colon.

Although *APC* gene mutations are critical for initiating human colorectal tumorigenesis, subsequent genetic/epigenetic alterations in other key genes (i.e. *KRAS, TP53, PTEN*) are required for carcinoma development. Recently, different GEMMs have been established by crossing *APC* mutant mice with other mutant mice, providing great models for studying the role of different factors in multistep colorectal carcinogenesis. For example, while $APC^{Min+/-}$ mice mostly develop benign intestinal adenomas, invasive carcinomas are developed in $APC^{Min+/-}$ $PTEN^{+/-}$ mice, suggesting that *PTEN* loss-of-function is critical for malignant transformation in colorectal carcinogenesis (181) . On the other hand, genetic deletion of COX-2 or mPGES-1 reduces intestinal polyp formation in Min mice, indicating the significance of PGE_2 synthesis in intestinal tumorigenesis (113,116) . In addition to mechanism studies, germline *APC* mutant

mice have also been used widely in development of preventive and therapeutic strategies for human CRC (182) . However, due to the inherent heterogeneity in tumor growth, it is difficult to determine if the tested drug is preventing tumor formation or regress established tumors in these germline mutant mice, therefore creating hurdles for implementation of preclinical results into clinical applications.

1.5.2 Cre recombinase-based genetic models

To circumvent the embryonic lethality and other limitations of germline mutant mice models, another type of GEMMs, the inducible genetic mice models, were created using *Cre*-loxP system. This system is established by employing a loxP-flanked transcriptional/translational stop cassette (neostop) located within the first intron of a target gene (with desirable mutations). In the absence of *Cre*, expression of the mutant gene is suppressed by neostop. When *Cre* recombinase gets activated and removes the neostop, the mutated gene will be expressed, providing conditional gene modifications (183) . The first conditional *APC* knockout mice were generated by Shibata and colleagues through injection of recombinant adenovirus expression *Cre* recombinase into mice colons (184) . *Cre*-mediated conditional knockout of *APC* resulted in colonic adenoma formation in 4 weeks and invasive adenocarcinoma development after 1 year. The adenovirus expressing *Cre* method was further modified by Hung and colleagues to achieve more reproducible distal colonic adenoma formation to test drugs for sporadic CRC treatment (185) .

In addition, promoter-driven *Cre* recombinase expression has also been used to generate tissue-specific gene modification in mice. In 2004, Robine group established an intestinal epithelium-specific *Cre* expressing model by inserting *Cre* under the control of *Villin* promoter

(186) . This Villin-Cre system has been widely used for studying the functions of different genes in colorectal carcinogenesis, including APC, KRAS, TGFβ (187,188) . Besides *Villin* promoter, other promote has also been used combined with *Cre* to achieve more specific genetic modifications in CRC GEMMs (189,190) . Moreover, the applications of tamoxifen-regulated *Cre* expression allowed the generation of inducible GEMMs and further strengthened their specificity as preclinical platforms for mechanism studies and drug development.

1.6 EXPERIMENTAL DESIGN

Specific Aim 1. ***To assess the significance of PGE_2 signaling in oxaliplatin resistance in human CRC cells.***

The efficacy of oxaliplatin, the first-line platinum-derivative for CRC treatment, has been strongly limited by acquired resistance developed in advanced CRC patients after long-term exposure, while the direct mechanism for oxaliplatin resistance remains unclear. Recent studies showed that the co-treatment of COX-2 inhibitors could improve the efficacy of oxaliplatin on suppressing CRC cell proliferation and colorectal tumor growth, *in vitro* and *in vivo*, respectively. In this aim, we want to determine the significance of PGE_2, the main product of COX-2 pathway, in CRC oxaliplatin resistance using oxaliplatin resistant (OXR) human colon cancer cells. The metabolism of PGE_2 in resistant cells and the effect of PGE_2 suppression on oxaliplatin cytotoxicity will be determined. The significance of each EP receptor signaling in oxaliplatin resistance will also be tested. This work will reveal the direct involvement of PGE_2 in

oxaliplatin resistance and evaluate the potential of PGE_2 signaling as target for CRC adjuvant therapy.

Specific Aim 2. ***To investigate the association between PGE_2 signaling and potential chemo-resistance mechanisms in human CRC in vitro.***

Given the clinical obstacles associated with oxaliplatin resistance in advanced CRC patient treatment, developing a better understanding of its underlying molecular mechanisms of resistance is critical for novel target discovery and the further development of adjuvant therapeutic strategies. Besides the conventional mechanisms (i.e. MDR, apoptotic regulation), recent studies have suggested that cancer stem cells (CSCs) may be involved in oxaliplatin resistance. In this aim, we will assess the stem-like properties of oxaliplatin resistant (OXR) colon cancer cells, and determine the significance of PGE_2 and its receptor signaling in CSC subpopulation of the heterogeneous OXR cancer cells. Another mechanism associated with chemoresistance is through modulation of oxidative stress. In this aim, we also will investigate the modulation of reactive oxygen species (ROS) in OXR cells and evaluate the effect of PGE_2 signaling suppression on oxaliplatin-induced oxidative stress and cytotoxicity in OXR colon cancer cells.

Specific Aim 3. ***To determine the histological and molecular alterations of pre-neoplastic lesions in the inducible $BRAF^{V600E}$ mutation mouse model.***

The colonic pre-neoplastic lesions, such as aberrant crypt foci (ACF), are regarded as precursors to CRC. Constitutive BRAF activation has been shown to be associated with the "alternative" serrated pathway of CRC, and oncogenic *BRAF* mutations are found in hyperplastic

ACFs, supporting the hypothesis of a "hyperplastic ACF-hyperplastic polyp-serrated adenoma/carcinoma" pathway. To evaluate the significance of *BRAF* mutation for early neoplasia of serrated CRC, in this aim, we generated a tamoxifen-induced $BRAF^{V600E}$ mutation specifically in intestinal Lgr5+ cells in B6 mice, and characterized the histological and molecular alterations of pre-neoplastic lesions. This study will demonstrate the significance of the *BRAF* mutation in colorectal serrated neoplasia and help establish the *BRAF* mutation-driven ACF model for development of CRC preventive strategies.

CHAPTER 2

TARGETING PGE_2 SIGNALING IN OXALIPLATIN RESISTANT HUMAN COLON CANCER CELLS

2.1 INTRODUCTION

Prostaglandin E2 (PGE_2), one of the most abundant products of prostaglandins pathways in human body, has been shown tightly involved in human colorectal caricinogenesis (125) . PGE_2 level is elevated in CRC patients, resulting from the hyperactivation of its synthesis pathway. Two of the key enzymes for PGE_2 generation, cyclooxygenase-2 (COX2) and microsomal prostaglandin E synthase-1 (mPGES1) have been shown overexpressed in both colorectal cancer cells and preclinical CRC models (191) . Since PGE_2 exerts multiple pro-tumorigenic functions, including promoting cancer cell proliferation and survival, assisting tumor angiogenesis and metastasis, suppressing anti-tumor immunity, it has been regarded as a potent target for CRC prevention (106) . Large scale randomized clinical trials have demonstrated that long-term intake of nonsteriodal anti-inflammatory drugs (NSAIDs) or COX-2 inhibitors could reduce CRC incidence by 30% among high-risk population (105) .

Recently, studies suggested that PGE_2 might also play important role in tumor response to chemotherapy. Preclinical studies have suggested that PGE2 suppression by COX2 inhibitors

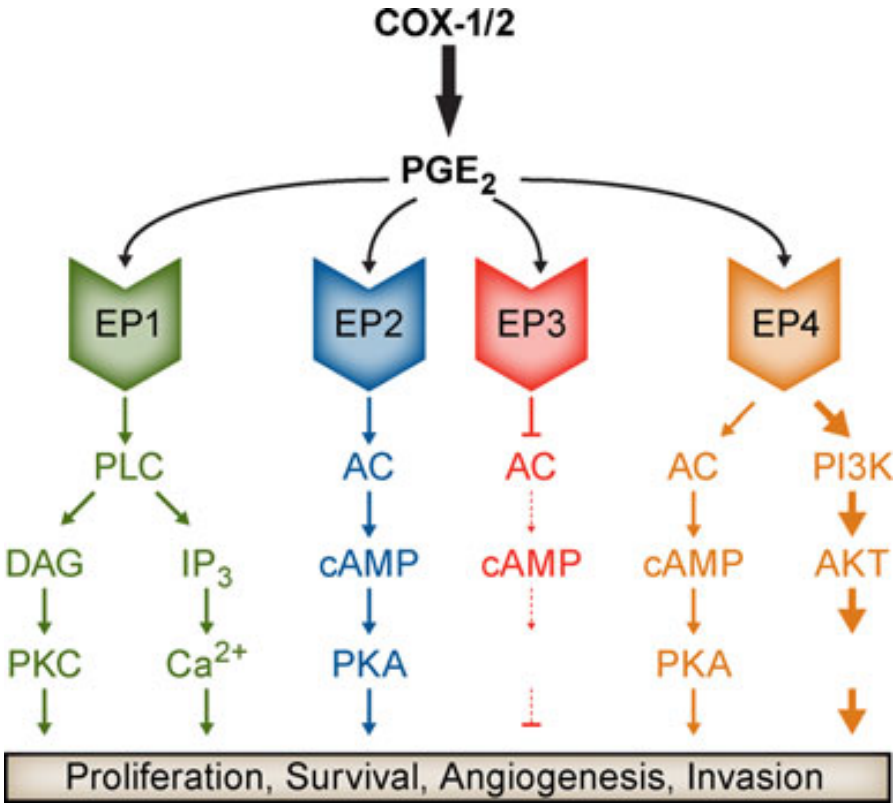
such as celecoxib and sulindac could effectively enhance chemotherapy efficacy, even abrogate chemo-resistance in preclinical models for breast cancer and bladder cancer (165,167) . In addition, treatment of COX-2 inhibitors showed synergistic effect on tumor growth inhibition when combined with chemotherapeutic drugs (i.e. oxaliplatin or 5-FU) in human cancer cell studies or CRC mice models, suggesting that the COX-2/PGE_2 pathway may be a potential target to enhance chemotherapy efficacy and combat chemo-resistance (168-170) . However, the adverse effects of COX-2 inhibitors, such as increased cardiovascular risk and GI bleeding, has resulted to strong limitation for its clinical use (141) . Therefore, further studies are needed to discover more specific targets for circumventing the adverse effects but retaining the anticancer benefits of PGE2 suppression.

To solve this problem, many studies have focused on identifying the specific receptors of PGE_2 and signaling pathways that mediate the biological functions of PGE_2. There are four pharmacologically distinct, plasma membrane G-protein coupled receptors (GPCR) that binds to PGE2 on cell membrane, known as EP receptors (EP1, EP2, EP3, and EP4). Each EP receptor activates different downstream signaling pathways, resulting to different functions in both normal and malignant cells (192,193) **(Figure 4)**. Recent studies suggested that each EP receptor plays different role during colorectal tumor development. For example, by crossing selective homozygous EP gene knockout mice with *APC* mutant mice, Taketo and colleagues found out that EP2 accelerates intestinal polyposis in *APC* mutant mice, while EP1 and EP3 receptor signaling don't affect intestinal polyp formation (194) . In contrast, Wakabayashi group revealed that down-regulation of EP3 receptor expression resulted to higher incidence of carcinogen-induced mouse colon tumor generation (195) . Moreover, studies have shown that

EP4 receptor, although originally identified as similar to EP2 receptor, could couple with Giα, activate phosphatidylinositol 3-kinase (PI3K) and β–catenin, thus plays unique roles in many physiologic and pathophysiologic events (196) . Specifically, EP4 receptor signaling has been extensively studied for its pro-tumorigenic functions, including promoting cell proliferation and survival, tumor metastasis, and suppressing antitumor immunity (197,198) . These findings suggest that EP receptors play important roles in CRC progression, but their functions may be situation dependent, so it is important to identify the significance of each EP receptor and their downstream signal pathways in different CRC models.

Given the results of studies on COX-2 inhibitors and PGE_2 in CRC chemotherapeutics, we *hypothesize* that PGE_2 promotes human colorectal cancer cell survival against oxaliplatin treatment *via* its downstream receptors (EP1-4) signaling; blocking PGE2-EP signaling pathways could enhance oxaliplatin efficacy on resistant human CRC cells. To test our hypothesis, in the following study, we evaluated the significance of PGE_2 in oxaliplatin resistance using an established oxaliplatin resistant cell culture model. Oxaliplatin resistant (OXR) human CRC cells were generated from chemo-naïve human CRC cell line HT29, by chronic exposure of increasing concentrations of oxaliplatin. We compared both PGE_2 metabolism pathways in both resistant and parental cell lines to build the associated between PGE_2 metabolism and oxaliplatin resistance. The causation was confirmed by measuring oxaliplatin efficacy on OXR cells after PGE_2 suppression *via* blocking its terminal synthase mPGES-1 expression. We also examined the roles of each EP receptor in oxaliplatin resistance using highly selective EP receptor antagonists and evaluate EP4 receptor downstream signaling in both OXR and chemo-naïve cell lines. By identifying the specific EP receptor signaling involved in oxaliplatin resistance, further

studies may discover potential targets for adjuvant therapy to maximize the benefit for overcoming oxaliplatin resistance while avoiding side effects of PGE2 suppression in CRC treatment.



Adapted from Rundhaug, Simpler, Surh and Fischer, Cancer Metastasis Rev, 2011

Figure 4. Signaling pathways activated by the EP receptors. EP receptors are G-protein couple receptors (GPCRs) located on the cell membrane. When binding to the ligand PGE_2, EP1 activates phospholipase C (PLC), which catalyze the generation of 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+}. DAG activates the protein kinase C (PKC) pathway and promotes cell proliferation. EP2 and EP4 both activate adenylate cyclase (AC), which increase cAMP generation and PKA pathway activation. However, EP3 inactivates AC and suppress PKA signaling. EP4 also activates PI3K/AKT pathway, which increases cell proliferation and survival.

2.2 MATERIALS AND METHODS

Materials. Human colon cancer cell lines HT29 and RKO were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The HT29 Oxaliplatin-resistant (OXR) cell line was generated as previously described (199) . Briefly, chemo-naïve HT29 cells were exposed to increasing concentrations of oxaliplatin over a three-month time-frame, with the final concentration maintained at 2μM. Cell culture media and serum were purchased from Life Technologies (Carlsbad, CA). Oxaliplatin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). PGE2, EP receptor selective antagonists and EP4 receptor agonist were purchased from Cayman Chemicals (Ann Arbor, MI).

Cell Culture Conditions. Human cancer cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 in MEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, L-Glutamine, MEM vitamin solution, sodium pyruvate and MEM non-essential amino acids. Oxaliplatin resistant cells were maintained in 2μM oxaliplatin, but were cultured in oxaliplatin-free media at least 24 hours prior to experimentation. Cells were confirmed to be free of Mycoplasma using Mycoplasma Detection Test (200) . All experiments were performed at 70% cell confluence with no more than 20 cell passages. Results from all studies were confirmed in at least three independent experiments.

Cell Viability Assay. Cell sensitivity to drugs was assessed using cholorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.

Briefly, cells were seeded in 48-well plates overnight and treated with or without drugs. After 72 hours, 100µl MTT solution (Sigma) was added to each well and incubated for 1h at 37°C. Medium was then aspired and 300µl DMSO was added. Colorimetric analysis was performed at a wavelength of 570nm using a standard microplate reader. IC50 curves were generated with GraphPad Prism (software version 5.0c) using variable slope model.

Gene Knockdown Using Small Interfering RNA (siRNA). Cells were seeded in 6-well plates or 48-well plates overnight. Cell layers were rinsed twice with sterile PBS followed by the addition of OPTI-MEM (Life Technologies) containing K2 transfection reagent (1µl/100µl, Biontex) and siRNA against mPGES-1 (5nM siGENOME SMARTpool siRNA targeting human PTGES, Target Sequence: GCA CGC UGC UGG UCA UCA A, GGG CUU CGU CUA CUC CUU U, GGA UGC ACU UCC UGG UCU U, UGG CAC ACA CCG UGG CCU A) or control siRNA (5nM siGENOME Non-Targeting siRNA) (Dharmacon). After overnight incubation, the media was changed to MEM supplemented with 10% FBS. After 48-72h, culture medium was removed and stored at -80°C for subsequent PGE2 level determination, and the cells were harvest for RNA isolation or protein extraction.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using the RNeasy Mini kit (Qiagen Inc.). cDNA was synthesized using Superscript III according to the manufacturer's protocol (Invitrogen) mRNA expression levels of genes of interest were examined with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on the ABI-7500 platform (Applied Biosystems). The levels of RNA expression were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The

primers used for PCR amplification were: 5'-GAA GAA GGC CTT TGC CAA C-3' and 5'-GGG TTA GGA CCC AGA AAG GA-3' for mPGES-1; 5'-TCA TGC TCA ACG AGA AGG AG-3' and 5'-CTC GCG GAC AAT GTA GTC AA-3' for mPGES-2; 5'-AAG TCG ACT CCC TAG CAG CA-3' and 5'-TCC CTT CGA TCG TAC CAC TT-3' for cPGES; 5'-CAC AAC ACT TCA CCC ACC AG-3' and 3'-CGG GTA CAT TTC TCC ATC CA-5' for COX-1; 5'-TGA AAC CCA CTC CAA ACA CA -3' and 5'-GAG AAG GCT TCC CAG CTT TT-3' for COX-2; 5'-GTA AAG CTG CCC TGG ATG AG-3' and 5'-TGT CCA GTC TTC CAA AGT GGT-3' for 15-PGDH; 5'-ACA ACT TTG GTA TCG TGG AAG G -3' and 5'-CAG TGA GCT TCC CGT TCA G-3' for GAPDH. For each experiment, PCR amplifications with no cDNA were performed as negative controls. The levels of RNA expression were normalized to GAPDH. PCR products were analyzed on 2% agarose gel with ethidium bromide (E-gel, Invitrogen), together with 1 kb plus DNA ladder (Invitrogen).

Protein Extraction and Western Blotting. To isolated protein, cultured cell monolayers were washed twice with ice-cold PBS and treated with lysis buffer (1xRIPA buffer, 1:50 protease inhibitor and 1:50 phosphatase inhibitor, Sigma) for 5 minutes on ice. The cell lysates were ultrasonicated (Sonic Dismembrator Model 100, Fisher Scientific, MA) and centrifuged. The protein concentration was determined using the Protein Assay solution (Bio-Rad Laboratories, Inc., CA). 30μg of protein was loaded for electrophoresis (Bio-Rad) and transferred to PVDF membrane (Immobilon-P membrane, EMD Millipore, MA). The membranes were blocked in 5% non-fact dry milk in TBST (1x TBS, 0.1% Tween 20) for 1 hour. Blots were incubated with primary antibodies overnight at 4°C and HRP-conjugated secondary antibodies for 1 hour at room temperature. HRP was visualized with enhanced luminal reagent (Immobilon Western,

EMD Millipore, MA). Antibodies used for Western blotting analyses were as follows: rabbit anti-mPGES-1 (Abnova, Taiwan), rabbit anti-COX-2, rabbit anti-EP1, rabbit anti-EP2, rabbit anti-EP3, rabbit anti-EP4 (Cayman Chemicals, MI), rabbit anti-cleaved PARP, rabbit anti-phospho-Akt, rabbit anti-Bcl2, rabbit anti-Bax (Cell Signaling Technologies, MA), mouse anti-β actin (Sigma, MO).

PGE_2 ELISA Essay. To measure total secreted PGE_2 level, cell culture supernatants were collected and PGE_2 concentrations were measured by commercial ELISA kit (Cayman Chemical, MI) according to manufacturer's protocol.

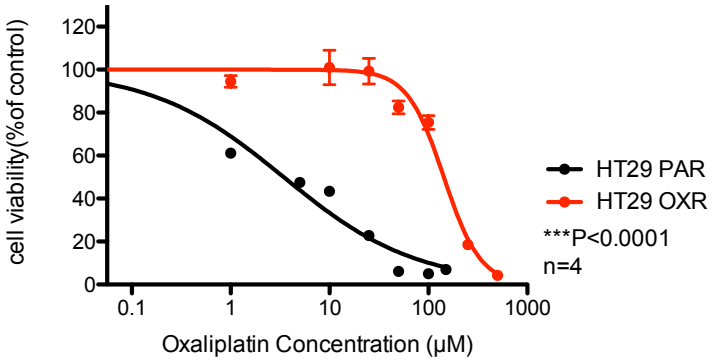
Flow Cytometry and Cell Cycle Analysis. Drug-treated cells or control cells were collected and fixed in cold methanol, and stained with propidium iodide (PI) or 7-AAD (Sigma, MO). After staining, Cells were collected and analyzed for DNA content using LSR-II Flow Cytometer (BD Biosciences, CA). All Analyses were performed in triplicate and 50,000 gated events/sample were counted using FlowJo 10.3 software (FlowJo LLC). Cell Cycle stages and apoptosis rate were analyzed using ModFit LT 3.3.11 software (Verity Software House).

Statistical Analysis. Data from all experiments was analyzed using the Student's t test or two-way analysis of variance (ANOVA) when appropriate for analysis by GraphPad Prism (software version 5.0c). For MTT assay, 50% inhibitory concentrations of oxaliplatin were calculated and compared using extra sum-of-squares F test. Results were considered as statistically significant at a P value of less than 0.05. All statistical tests were two-sided.

2.3 RESULTS

HT29 oxaliplatin-resistant (HT29 OXR) cells exhibited lower sensitivity to both oxaliplatin and 5-FU. HT29 oxaliplatin-resistant (HT29 OXR) cells were generated by chronic exposure (up to 3 months) of parental HT29 cells to increasing concentrations of oxaliplatin, with a final concentration maintained at 2μM (199) . Long-term treatment with low concentration of oxaliplatin induces a significant increase in colon cancer cell survival in the presence of oxaliplatin (IC50 value: 134.1μM compared to 3.4μM; $P<0.0001$), compared to the chemo-naïve parental cell line (Fig. 5A). The sensitivity of both HT29 OXR cells and HT29 parental cells to another first-line CRC chemotherapy, 5-FU were also tested. The resistant cells also showed significantly less sensitivity to 5-FU cytotoxicity compared to the parental cells (IC50 value: 67.35μM compared to 12.66μM; $P<0.0001$)(Fig. 5B). These results suggest that certain molecular mechanism in OXR cells may be involved in both oxaliplatin resistance and 5-FU resistance.

A.



B.

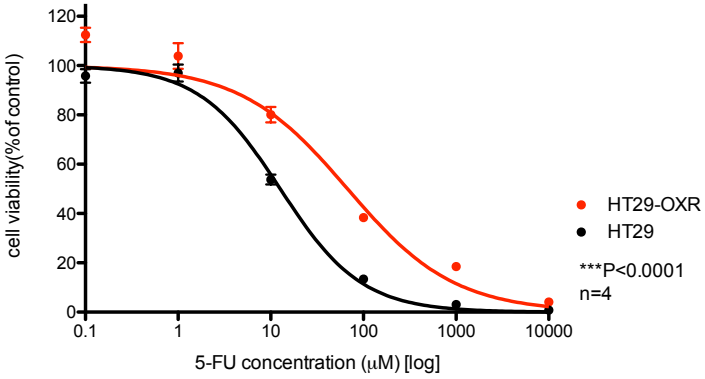
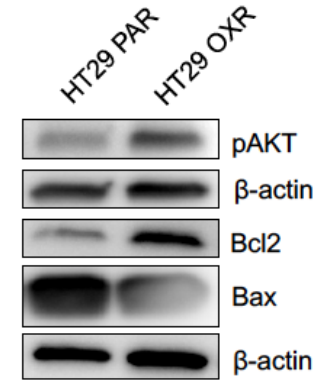


Figure 5. HT29 oxaliplatin-resistant (HT29 OXR) cells exhibited higher cell survival rate upon treatment of both oxaliplatin and 5-FU. (A) HT29 PAR and OXR cells were treated with increasing concentrations of oxaliplatin for 72 hours. (B) HT29 PAR and OXR cells were treated with increasing concentrations of 5-FU for 72 hours. Cell viability was assessed using the MTT assay.

Anti-apoptotic pathway activation is upregulated in oxaliplatin resistant cells compared to parental cell line. To understand the direct mechanisms involved in chemoresistance in OXR cells, we tested anti-apoptotic pathway activation status in both OXR cells and parental cells without presence of oxaliplatin. Compared to parental cells, the OXR cells showed significant up-regulation of AKT phosphorylation level, and concomitant higher Bcl-2/Bax ratio at basal level (Fig. 6A), suggesting an activation of anti-apoptotic pathway in OXR cells. We also tested the protein level of MDR-1 (P-glycoprotein), the most common multidrug resistance protein from ABC transporter family. Surprisingly, there was no difference in the protein levels of MDR-1 in both cell lines (Fig. 6B), suggesting that MDR mechanism may not be playing an important role in chemoresistance of the OXR cells.

A.



B.

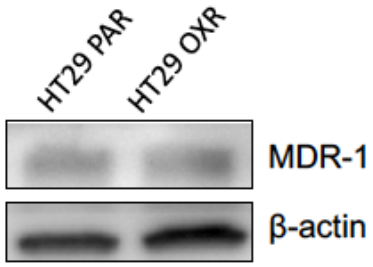
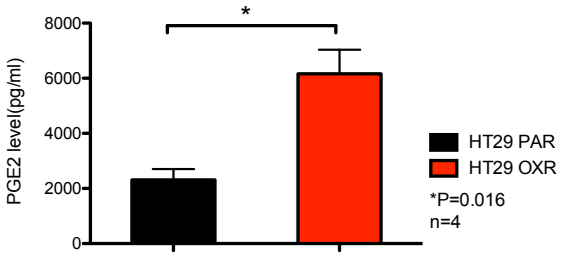


Figure 6. Oxaliplatin resistant cells demonstrated activation of anti-apoptotic pathway but not multi-drug resistance mechanism. Both HT29 OXR cells and HT29 PAR cells were cultured in oxaliplatin free condition for 72 hours before protein extraction. (A) Western Blot analysis for the phosphorylated AKT, Bcl-2 and Bax protein. (B) Western Blot analysis for MDR-1 protein in both cell lines. β-actin was used for standard normalization.

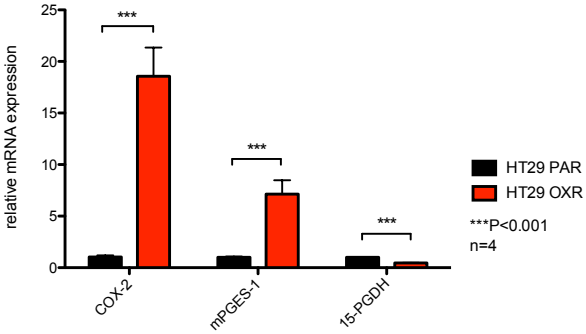
Deregulation of PGE_2 metabolism in oxaliplatin resistant colon cancer cells. Many studies have suggested that PGE_2 signaling promotes cell anti-apoptotic pathway activation in CRC. Recently, metabolic deregulation of PGE_2 has been associated with chemotherapy efficacy and chemoresistance in cancer. To determine the PGE_2 metabolism during the development of chemoresistance, we measured the secreted PGE2 levels in both parental (chemo-naïve) HT29 cells and the oxaliplatin-resistant derivative (HT29 OXR) cells. We found that HT29 OXR cells maintained higher level of secreted PGE_2 (~3 fold increase; $P<0.05$) compared to the parental cell line (Fig. 7A), suggesting significant deregulation in PGE_2 metabolism in HT29 OXR cells.

To understand the mechanism of PGE_2 up-regulation in OXR cells, we measured mRNA expression and protein levels of several key enzymes involved in PGE_2 metabolism. Compared to the parental cells, HT29 OXR cells exhibited increased expression of COX-2 (18-fold; $P<0.001$) and the terminal PGE_2 synthase, microsomal prostaglandin E synthase-1 (mPGES-1) (7-fold increase; $P<0.001$), respectively, suggesting activation of the PGE_2 synthesis pathway in these cells. Meanwhile, a significant reduction of both mRNA (50% reduction; $P<0.0001$) and protein levels of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the key PGE_2 catabolic enzyme, were detected in HT29 OXR cells compared to the parental cell line, indicating down-regulation of PGE_2 catabolism (Fig. 7B-C). However, there is no difference in the mRNA expression level of COX-1, mPGES-2 and cPGES (Fig. 7D). Taken together, our results demonstrate a marked loss of metabolic control over PGE_2 in oxaliplatin resistant colon cancer cells, suggesting an associated between PGE_2 deregulation and oxaliplatin resistantance.

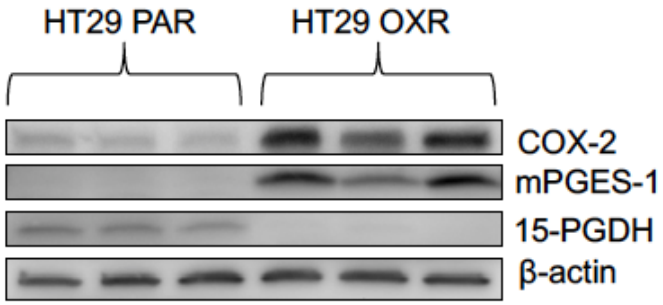
A.


*
8000
6000
4000
2000
0
PGE2 level(pg/ml)
HT29 PAR
HT29 OXR
*P=0.016
n=4

B.


25
20
15
10
5
0
relative mRNA expression
***
***
***
COX-2
mPGES-1
15-PGDH
HT29 PAR
HT29 OXR
***P<0.001
n=4

C.


HT29 PAR
HT29 OXR
COX-2
mPGES-1
15-PGDH
β-actin

D.

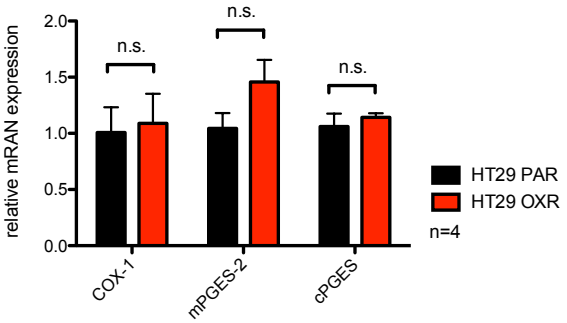
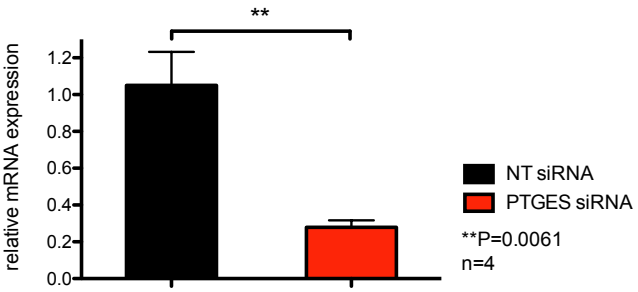

2.0
1.5
1.0
0.5
0.0
relative mRAN expression
n.s.
n.s.
n.s.
COX-1
mPGES-2
cPGES
HT29 PAR
HT29 OXR
n=4

Figure 7. Deregulation of PGE2 metabolism in HT29 OXR cells. Both HT29 OXR cells and HT29 PAR cells were cultured in oxaliplatin free condition for 72 hours before supernatant collection and RNA/protein extraction. (A)Total secreted PGE2 levels in both cell lines measured using ELISA. (B) mRNA expression and (C) cellular protein level of COX-2, mPGES-1 and 15-PGDH in both cell lines were determined using RT-PCR and Western Blotting analysis, respectively. (D) mRNA expression of COX-1, mPGES-2 and cPGES were measured using RT-PCR analysis.

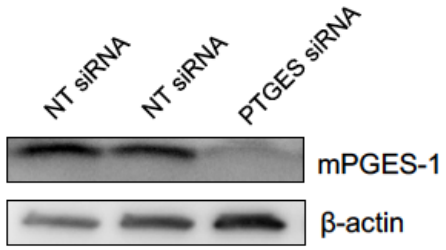
PTGES knockdown sensitizes HT29 OXR cells to oxaliplatin. In order to determine whether PGE2 suppression affects oxaliplatin resistance in human CRC, we further inhibited PGE2 synthesis by OXR cells via siRNA-mediated knockdown of PTGES, the gene encoding mPGES-1. Significant reductions in both mRNA expression (~70% reduction; $P<0.01$) and protein levels of mPGES-1 were found 48 hours after siRNA treatment (Fig. 8A-B). Concomitantly, gene silencing of mPGES-1 in HT29 OXR cells reduced PGE2 synthesis by ~85% ($P<0.001$) (Fig. 8C).

PGE_2 suppression through PTGES knockdown increased cell sensitivity to oxaliplatin treatment (IC50) by 33%, as measured by the MTT cell viability, compared to the non-targeting (NT) siRNA treated HT29 OXR cells (Fig. 9A). In addition, PTGES knockdown also reduced phosphorylation of AKT in HT29 OXR cells, suggesting that PGE_2 suppression may affect the activation of a key survival pathway in oxaliplatin resistance of human CRC (Fig. 9B).

A.



B.



C.

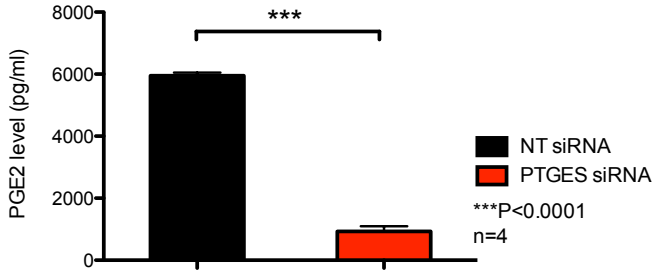
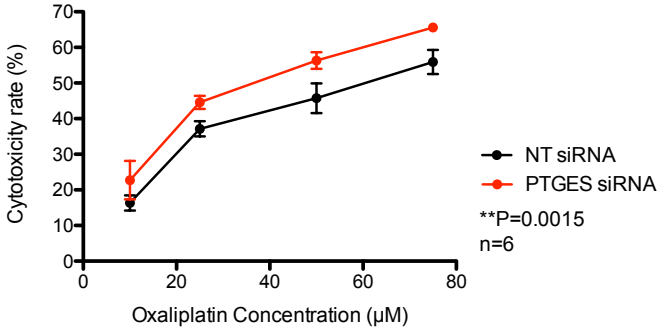


Figure 8. SiRNA silencing of mPGES-1 suppressed PGE_2 synthesis in HT29-OXR cells. HT29 OXR cells were treated with PTGES or Non-targeting (NT) siRNA (0.1μg siRNA per 2.5x104 cells) for 48 hours following by RNA/protein extraction and supernatant collection. (A) mRNA expression and (B) protein level of mPGES-1 were measured by RT-PCR analysis and Western Blotting analysis, respectively. (C) Total secreted PGE2 level was measured by ELISA.

A.



HT29 OXR	NT siRNA	PTGES siRNA
OX IC50(μM)	55.57	37.43**

B.

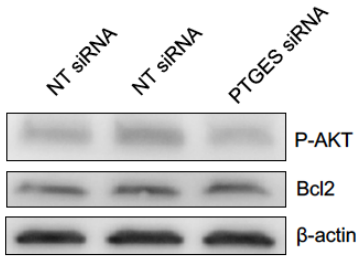


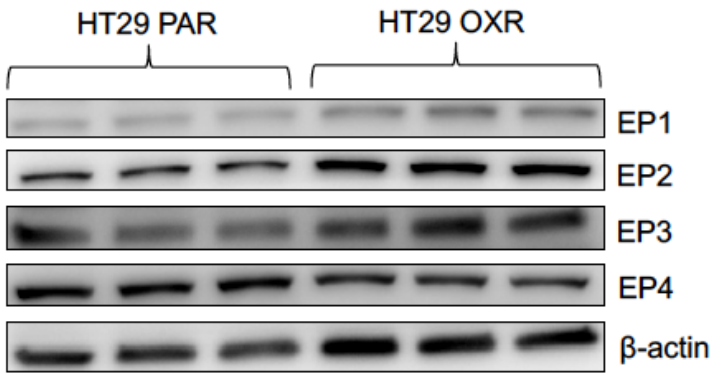
Figure 9. PGE_2 suppression sensitizes HT29 OXR cells to oxaliplatin cytotoxicity. (A)HT29 OXR cells were treated with increasing concentrations of oxaliplatin for 72h after PTGES siRNA or non-targeting(NT) siRNA treatment. Cell viability was assessed using the MTT assay. Cytotoxicity rate was defined as the percentage of dead cells in oxaliplatin treated cells compared to untreated cells. (B) Western Blot analysis for the indicated protein extracted from HT29 OXR cells treated with PTGES siRNA or non-targeting (NT) siRNA.

A selective EP4 receptor antagonist sensitizes resistant cells to oxaliplatin. The biological function of PGE2 is controlled in part by its direct binding to a set of G-protein-coupled receptors (GPCRs): EP1, EP2, EP3 and EP4 receptors (192) ; these EP receptor subtypes signal through distinct downstream pathways to afford different cellular functions. To understand the mechanism of PGE_2 suppression in oxaliplatin resistance, we ought to determine the significance of each EP receptor in OXR cells. First we measured the protein levels of the EP receptors between chemo-naïve HT29 parental cells and OXR cells, but did not see significant difference (Fig. 10A). To determine the effects of specific EP receptor inhibition in OXR cells, we blocked EP receptor activity using a set of selective EP receptor antagonists and assessed the cytotoxicity of oxaliplatin via cell viability assay. The addition of the EP4 antagonist L-161,982 (10μM) significantly increased oxaliplatin induced cytotoxicity (~1.7-fold increase; $P<0.05$) compared with oxaliplatin alone in HT29 OXR cells. In contrast, addition of L-161,982 had no significant effect on oxaliplatin efficacy on HT29 parental cells (Fig. 10B; Table 1). Selective blockade of the other EP receptors (1-3) failed to demonstrate a synergistic effect on oxaliplatin cytotoxicity (Fig. 10C).

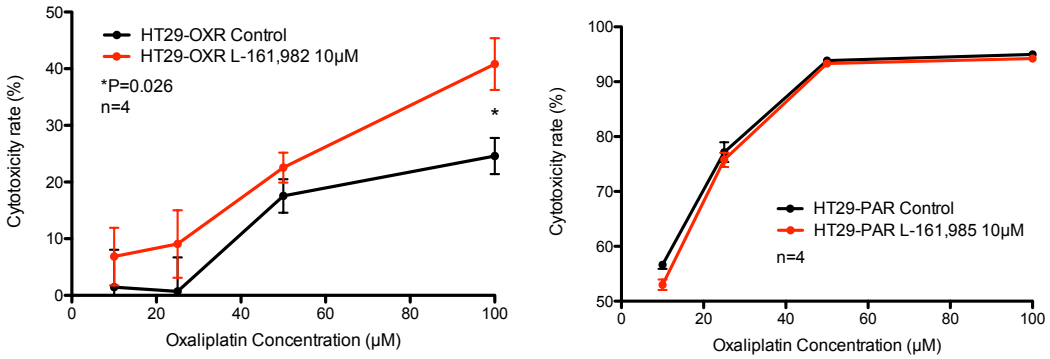
Oxaliplatin exerts its cytotoxicity by inducing extensive DNA damage (201) . This in turn causes cell cycle arrest and intrinsic cell apoptosis (202) . To gain further insight into potential mechanisms by which EP 4 receptor blockade may synergize with oxaliplatin-induce cell death, we measured the effects of the EP4 antagonist L-161,982 on oxaliplatin-induced cell cycle arrest and apoptosis using FACS analysis. The combination treatment of L-161,982 (10μM) and oxaliplatin for 48 hours significantly increased the apoptotic cell population of OXR cells compared to oxaliplatin alone (Fig. 11A; Table 2). To evaluate the level of cell apoptosis in

treated HT29 OXR cells, we measured the protein markers involved in cell apoptosis and survival pathway. The levels of PARP cleavage induced by oxaliplatin were increased by L-161,982 treatment, indicating increased apoptosis of HT29 OXR cells. In contrast, the phosphorylation of AKT and the Bcl2/Bax ratio were reduced, suggesting reduction of cellular survival pathway activation in response to oxaliplatin (Fig. 11B). Taken together, our results show that selective inhibition of EP4 receptor activity by L-161,982 suppresses cell survival and synergistically enhances oxaliplatin cytotoxicity in oxaliplatin resistant cells.

A.


HT29 PAR
HT29 OXR
EP1
EP2
EP3
EP4
β-actin

B.


HT29-OXR Control
HT29-OXR L-161,982 10μM
*P=0.026
n=4
Cytotoxicity rate (%)
Oxaliplatin Concentration (μM)
HT29-PAR Control
HT29-PAR L-161,985 10μM
n=4
Cytotoxicity rate (%)
Oxaliplatin Concentration (μM)

C.

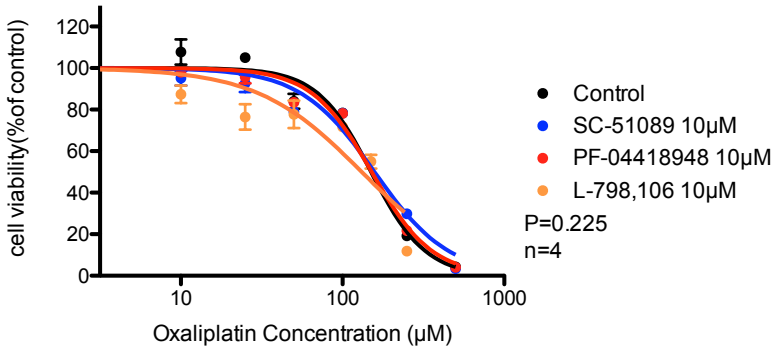

cell viability(%of control)
Oxaliplatin Concentration (μM)
Control
SC-51089 10μM
PF-04418948 10μM
L-798,106 10μM
P=0.225
n=4

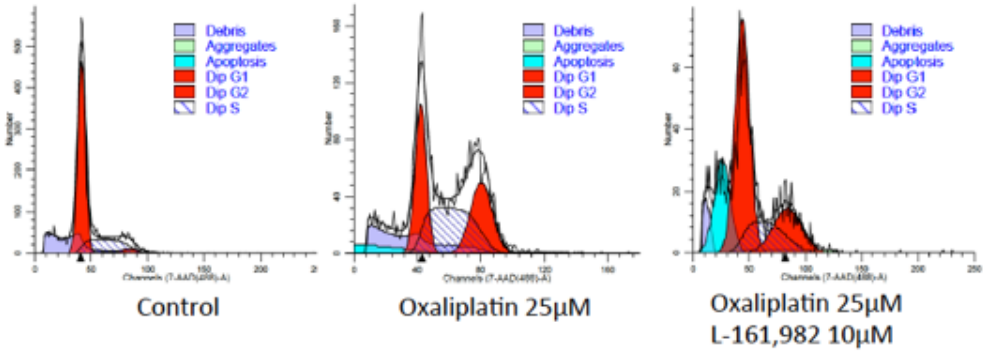
Figure 10. Selective EP4 blockade synergistically enhanced oxaliplatin efficacy in HT29 OXR cells. (A) Both HT29 OXR cells and HT29 PAR cells were cultured in oxaliplatin free condition for 72 hours before protein extraction. The protein levels of all EP receptors (1-4) were measured using western blotting analysis. (B) HT29-OXR cells or HT29 PAR cells were either treated with different concentrations of oxaliplatin alone (control) or co-treated with 10μM L-161,982 for 72h. Cell viability was assessed using the MTT assay. Cytotoxicity rate was defined as the percentage of dead cells In oxaliplatin treated cells compared to untreated cells. (C) HT29 OXR cells were either treated with increasing concentrations of oxaliplatin alone (control) or co-treated with 10μM SC-51089 (EP1 selective antagonist)/ 10μM PF-04418948 (EP2 selective antagonist)/ 10μM L-798,106 (EP3 selective antagonist) for 72h. Cell viability was assessed using the MTT assay.

Table 1. 50% inhibitory concentrations of oxaliplatin in both cell lines were measured using MTT assay.

Cells	Control	L-161,982 10μM
HT29 PAR	3.435	5.34
HT29 OXR	143.1	104.2***

(***P<0.0001;n=4)

A.



B.

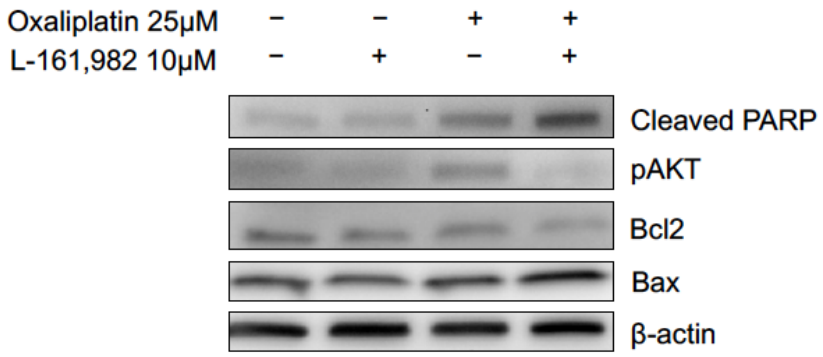


Figure 11. Selective EP4 blockade increased oxaliplatin induced cell apoptosis in HT29 OXR cells. HT29 OXR cells were treated with vehicle control or 25μM oxaliplatin alone or 25μM oxaliplatin+10μM L-161,982 for 48h followed by PI staining or protein extraction. (A) Cell cycle analysis was done using flow cytometry. (B) Levels of cleaved PARP, phosphorylated AKT and Bcl2/Bax protein were detected using western blotting analysis.

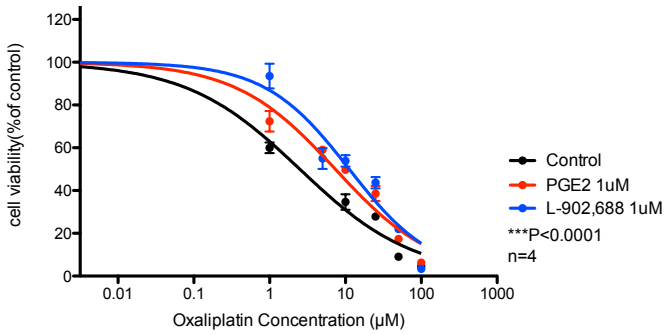
Table 2. The percentages of cycle stages and apoptosis rate in each group were calculated by computer modeling using ModFit LT.

	G1(%)	G2(%)	S(%)	Apoptosis(%)
Control	72.45	3.21	24.33	0.00
OX25μM	31.31	28.39	40.30	6.75
OX25μM +L-161,982 10μM	58.38	21.35	20.27	19.79

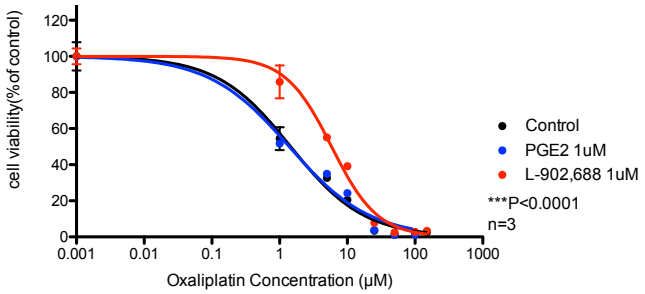
(n=3)

Selective EP4 receptor agonist increased cell survival against oxaliplatin of chemo-naïve cancer cells. EP4 receptor signaling has been shown important for chemo-naïve cancer cell proliferation and survival upon stress (radiation, chemotherapy) (172,196) . To test the effects of EP4 receptor signaling on oxaliplatin cytotoxicity to chemo-naïve cancer cells, we treated two parental human adenocarcinoma cell lines, HT29 and RKO, with combinations of oxaliplatin and the EP4 receptor selective agonist, L-902,688 for 72 hours. The addition of L-902,688 significantly increased cell survival in both HT29 cells (IC50 value: 2.50μM compared to 14.14μM; $P<0.001$) and RKO cells (IC50 value: 1.47μM compared to 5.86μM; $P<0.001$) compared to oxaliplatin alone, measured by cell viability assay (Fig. 12A-B and Table 3). In addition, co-treatment of L-902,688 and oxaliplatin reduced protein levels of cleaved PARP cleavage in both cells lines, compared to oxaliplatin alone, indicating reduced apoptosis (Fig. 12C-D). These results further establish the significance of EP4 receptor signaling on oxaliplatin sensitivity of chemo-naïve colon cancer cells, suggesting that EP4 could also be a potential target to enhance oxaliplatin efficacy in initial CRC chemotherapy.

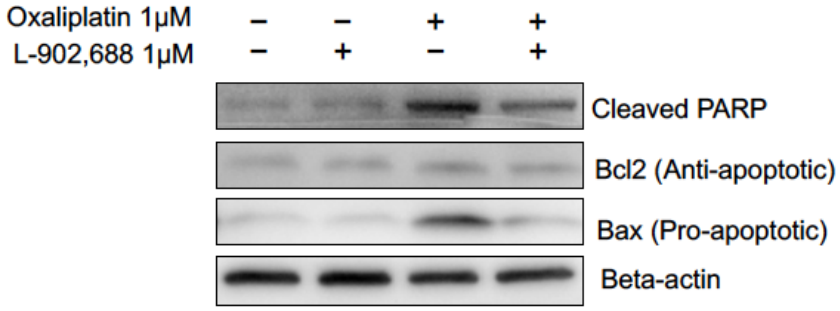
A.


cell viability(%of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Oxaliplatin Concentration (μM)
Control
PGE2 1uM
L-902,688 1uM
***P<0.0001
n=4

B.


cell viability(%of control)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
1000
Oxaliplatin Concentration (μM)
Control
PGE2 1uM
L-902,688 1uM
***P<0.0001
n=3

C.


Oxaliplatin 1μM
L-902,688 1μM
Cleaved PARP
Bcl2 (Anti-apoptotic)
Bax (Pro-apoptotic)
Beta-actin

D.

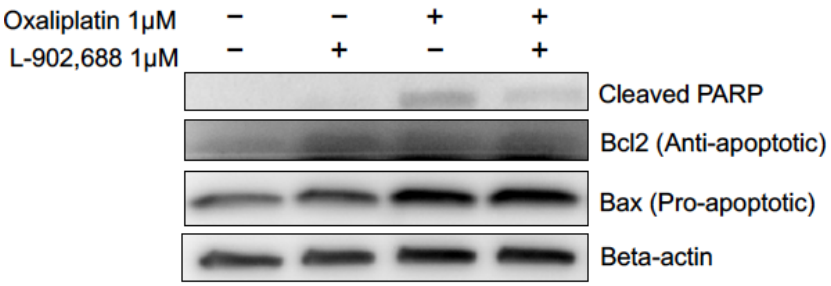

Oxaliplatin 1μM
L-902,688 1μM
Cleaved PARP
Bcl2 (Anti-apoptotic)
Bax (Pro-apoptotic)
Beta-actin

Figure 12. Selective EP4 agonist reduced oxaliplatin sensitivity in HT29 parental cells. Parental human colon cancer cell lines HT29 and RKO were either treated with different concentrations of oxaliplatin alone (control) or co-treated with 1μM PGE_2 or 1μM L-902,688 for 72h before cell staining or protein extraction. The levels of cell viability (A) HT29 and (B) RKO were assessed using the MTT assay. Cytotoxicity rate was defined as the percentage of dead cells In oxaliplatin treated cells compared to untreated cells. Levels of cleaved PARP and Bcl2/Bax protein were detected in both (C) HT29 and (D) RKO cells using western blotting analysis.

Table 3. 50% inhibitory concentrations of oxaliplatin in both cell lines were measured using MTT assay.

Cell line	Control	PGE_2 1μM	L-902,6881μM
HT29	2.51	7.36***	11.21***
RKO	1.46	1.39	5.860***

(n=4)

2.4 DISCUSSION

The role of PGE_2 in colorectal cancer development has been studied extensively for decades. Several clinical trials have confirmed the chemo-preventive benefits of long-term treatment with NSAIDs, such as aspirin or celecoxib (105) . Recent studies have shown synergistic effects of COX-2 inhibition on improving the efficacy of chemotherapeutic agents (5-FU and oxaliplatin) in both cell culture and preclinical colon cancer models (166,168,170) . For example, short-term (up to 72 hours) treatment of COX-2 inhibitors (sulindac sulfide, indomethacin, NS-398) has been shown to enhance efficacy of 5-FU and oxaliplatin on human CRC cells (203,204) . The synergistic effect on oxaliplatin cytotoxicity is associated with significantly reduced PGE_2 level and increase expression of pro-apoptotic proteins (i.e. Cytochrome C, caspase-3, caspase-9). Moreover, COX-2 may facilitate CRC chemo-resistance by up-regulating the expression of ABC transporters and MDR1/P-gp, which mediates multidrug resistance and enhance the drug removal from colon cancer cells (205,206) . These studies introduced new aspects of NSAIDs application in CRC treatment. However, the use of COX-2 inhibitors have been associated with increased risk of cardiovascular events, such as myocardial infarction and strokes, due to the suppression of cardio-protective PGI_2 (prostacyclin) (207,208) . Therefore, it is important to develop strategies that retain the anticancer benefits of COX-2 inhibition while avoiding the cardiovascular side effects. Our findings suggest that inhibition of PGE_2/EP4 receptor signaling may be a good adjuvant therapeutic strategy to enhance oxaliplatin efficacy and circumvent oxaliplatin resistance, ultimately increase the survival of CRC patients.

In consistent with its significant pro-tumorigenic functions, elevated level and deregulated metabolism of PGE_2 has been observed in colon cancer cell lines and patients, compared to healthy controls (106,125,209) . In addition, previous studies have shown that chronic oxaliplatin exposure induce multiple alterations in morphology and gene expression patterns of human colorectal cancer cell, which may lead to COX-2 overexpression and activation of eicosanoid signaling, resulting to aggravated tumor metastasis signaling, suggesting COX-2 and PGE_2 signaling may be involved in CRC oxaliplain resistance (199,210) . In the present study, to mimic the development of oxaliplatin resistance of human CRC *in vitro*, we used the well-established drug resistant cancer cell model, in which acquired resistance against oxaliplatin was induced through long-term low dose oxaliplatin treatment to human colon cancer cells. First we found that chronic treatment of oxaliplatin to human colon cancer cells resulted to significantly reduced sensitivity to not only oxaliplatin, but also 5-FU, another first-line chemotherapy, in spite of different mechanism of act by these drugs, indicating that there are multiple mechanisms involved in the drug resistance in OXR cells. Further test on cell apoptotic pathway and multidrug resistance mechanism revealed significant increases in both AKT phosphorylation and Bcl-2/Bax ratio, but no difference in MDR1 expression, suggesting deregulation of cell apoptotic pathway but not MDR in these cells.

Next, to examine the association between PGE_2 metabolism and oxaliplatin resistance, we measured secreted PGE_2 level and key enzymes involved in PGE_2 metabolism pathways. We found a significant increase in the concentrations of PGE_2 in the medium of HT29 OXR cells compared to HT29 parental cells, which is likely due to both its increased synthesis and decreased catabolism. The elevated PGE_2 level is concomitant with up-regulation in AKT

phosphorylation and Bcl-2/Bax ratio. Using siRNA-mediated PTGES knockdown, we found that PGE_2 suppression significantly reduces both AKT phosphorylation and cell survival against oxaliplatin cytotoxicity. A possible explanation for the effect of PGE_2 suppression on OXR cell survival, is that the down-regulation of PGE_2 synthesis reduced its signaling through EP2 or EP4 receptor, which is known to activate AKT pathway and exert anti-apoptotic and pro-survival functions in colon cancer cells upon extracellular stress as PGE_2 signaling has been shown in association with cell survival pathway activation in colonic epithelial cells (173,211) . As recent studies also associated PI3K/AKT pathway with chemoresistance in human colon cancer cells (212,213) , suggesting that elevated PGE_2 signaling may promote oxaliplatin resistance through AKT-mediated mechanisms in OXR cells.

Although PGE_2 suppression did somewhat sensitize OXR cells to oxaliplatin cytotoxicity, this effect likely results from blocking the downstream signaling of all the EP receptors, therefore lack specificity. EP receptors are known to each activate distinct downstream signaling pathways and mediate different functions in cancer progression and treatment (214) . Studies using mouse models with genetic ablation of selective EP receptor subtypes have demonstrated the very different even conflicting roles played by EP receptors in colorectal cancer pathogenesis (192) . For example, Sonoshita and colleagues determined that abrogation of the EP2 decreases intestinal polyposis in ApcΔ716 mice, while genetic inactivation of EP1 and EP3 receptor signaling has no effect on polyp formation (194) . However, in azoxymethane (AOM)-induced colon cancer models, EP1 knockout mice developed fewer colonic neoplastic leisons than wild-type mice, while EP3 knockout mice showed enhanced colon carcinogenesis (195,215) . On the other hand, preclinical studies have shown that EP4 receptor promotes colon

cell proliferation and survival, tumor metastasis, and suppressing antitumor immunity (197,198,216) . Interestingly, EP4 receptor signaling has also been shown to transactivate EGF receptor, thus establishing a crucial crosstalk between PGE2 and EGF signaling pathways that promotes colorectal polyps growth (110) . These studies revealed the sophisticated cellular functions of EP receptors, highlighting the importance of specifying the role of each EP receptor in different models. To determine which EP receptor signaling mediates the pro-resistant function of PGE_2 in the OXR cells, we treated cells with selective antagonists against each EP receptor. We found that blockade of EP4 receptor signaling by L-161,982 provided comparable synergistic effects on oxaliplatin efficacy as PGE_2 suppression, while inhibition of the other EP receptors did not affect oxaliplatin resistance in OXR cells. Concomitant with less cell viability, we also found increased cell death (higher sub-G1 proportion in cell cycle test), decreased AKT phosphorylation and increased PARP cleavage in OXR cells co-treated with L-161,982 and oxaliplatin, compared to treatment of oxaliplatin alone. These findings suggest that PGE_2 promotes cellular survival specifically through EP4 signaling, which could be a mechanism of oxaliplatin resistance in OXR cells.

In summary, we have demonstrated a critical role of PGE_2/EP4 receptor signaling in promoting oxaliplatin resistance in human colorectal cancer cells, possible *via* mechanisms that involve cellular anti-apoptotic pathways. Combining with other studies on EP4 signaling in CRC progression (196) , EP4 receptor and its signaling may serve as potent target for adjuvant therapeutic strategy in human CRC treatment. Further studies are needed to explore the potential of inhibiting EP4 or its downstream signaling using pharmaceutical molecules to increase

oxaliplatin efficacy, circumvent oxaliplatin resistance, and ultimately increase the survival of advanced CRC patients.

CHAPTER 3

SIGNIFICANCE OF PGE_2 SIGNALING IN MOLECULAR MECHANISMS INVOLVED IN HUMAN CRC OXALIPLATIN RESISTANCE

3.1 INTRODUCTION

Oxaliplatin (1R, 2R-diaminocyclohexane oxalatoplatinum (II)) is a third generation platinum compound and the only FDA-approved platinum-based first-line treatment for advanced CRC patients. Different from other platinum-derivatives (i.e. cisplatin), oxaliplatin has shown promising activity in CRC, with patient response rates of 12% to 24% as a single agent. When combined with 5-FU as first-line therapy, the response rates increase to 60% or higher in patients with previously untreated advanced colorectal cancer (217) .

The major mechanism of action by platinum drugs is that they bind to DNA covalently to form platinum-DNA crosslinks, which cause DNA distortions, DNA damage response and apoptosis pathway activation, therefore leading to cell death. However, cancer cells develop various mechanisms, including loss of DNA mismatch repair, apoptosis pathway inactivation, survival signaling enhancement, and increase of drug export mediated by MDR transporter, to escape platinum-induced cell death and acquire drug resistance (147) . Due to its different mechanisms of act, oxaliplatin is able to circumvent the intrinsic resistance mechanism and show

efficacy during the initial treatment for colorectal tumors. However, almost all advanced CRC patients develop acquired resistance after exposure to oxaliplatin for 6 months and eventually get tumor metastases (218) . The molecular aspects of oxaliplatin resistance have been shown different from drug resistance against other platinum-based compounds and remain unclear yet (219) . To enhance oxaliplatin efficacy and overcome the acquired resistance against oxaliplatin, it is very important to gain better understanding on the molecular mechanisms of CRC oxaliplatin resistance, for novel target discovery or adjuvant therapy development.

It has been shown that besides the conventional chemo-resistance mechanisms, cancer cells may take advantage of other strategies to escape the cytotoxic effect by chemotherapeutic agents and acquire resistance (152,220) . Recently, cancer stem cells (CSC) have gained intense interests for not only being crucial tumor initialing cells (TICs) in tumor progression, but also key players in chemo-resistance of different malignancies (160,165) . CSCs are known as a subpopulation of cancer cells in tumor mass, which maintain stem cell like properties. Besides the intrinsic properties like slow proliferation, which already makes CSCs less sensitive to most chemotherapy that targets fast-proliferating cells, CSCs haven been shown to pick up several other mechanisms including MDR (through overexpression of ABC transporters) and hyper-activation of anti-apoptotic pathways (160,221) . After the initial response (tumor regression) to chemotherapy, these resistant CSCs are responsible for driving tumor growth and tumor replase, eventually lead to cancer related deaths. By generating chronic oxaliplatin resistant colon cancer cells *in vitro*, Lee Ellis group have shown that long-term exposure to low concentration of oxaliplatin could induce acquired drug resistance in human CRC cells, concomitant with enrichment of CSC like subpopulation and phenotypic changes consistent with EMT, suggesting

CSC may be involved in oxaliplatin resistance of human CRC (199,220) . Interestingly, in a very recent study, Kurtova and colleagues associated PGE_2 with chemoresistance through regulation of CSC subpopulation for the first time (165) . They found that through blockade of PGE_2 signaling using neutralizing antibody or celecoxib, they were able to abolish the CSC repopulation and attenuate tumor recurrence in xenograft model of chemo-resistant bladder cancer. These intriguing findings reveal a novel chemo-resistant mechanism mediated by PGE_2 possibly through CSC regulation.

On the other hand, modulation of oxidative stress has been shown as the key mechanism involved in cytotoxicity. Many conventional chemotherapeutic drugs attack DNA in both nucleus and mitochondria, where the most reactive oxygen species (ROS) is generated. The damage in mitochondria DNA causes deregulation of mitotic enzyme expression and generates high level of ROS, triggering the intrinsic cell apoptotic pathway, leading to cell death (222) . Because proliferating cancer cells usually contains multiple genetic mutations and high oxidative stress, they are more susceptible to DNA damage an intrinsic apoptosis induced by pharmacologically generated ROS (223) . However, after long-term exposure to chemotherapy, cancer cells develop strategies such as up-regulation of antioxidant capacity to get adapted to intrinsic oxidative stress, therefore confer drug resistance (223,224) .

Recently, oxaliplatin has been shown to induce colon cancer cell death through generating high level of ROS; regulation of ROS-related mechanisms could enhance oxaliplatin sensitivity in human colorectal cancer cells, suggesting it as a potential target to circumventing oxaliplatin resistance in human CRC (225,226) . A recent study by Gallick group showed that

treatment of oxaliplatin acutely activates Src, through intracellular ROS-dependent mechanism. Src is the nonreceptor protein tyrosine kinase that correlates with high disease stage and poorer patient survival. They also found that in oxaliplatin resistant cells, Src is constitutively activated. Inhibiting Src by tyrosine kinase inhibitor dasatinib enhances oxaliplatin efficacy *in vitro* and *in vivo*, suggesting a potential oxaliplatin resistance mechanism in human colorectal cancer mediated by ROS (227) . Recently, Mo and colleagues found that inhibition of PGE_2/EP4 signaling using selective EP4 receptor antagonist L-161982 could significantly increase cellular ROS level and inhibit myoblast proliferation in vitro, providing an association between EP4 receptor signaling with regulation of intracellular ROS metabolism, suggesting that PGE2/EP4 signaling may affect colorectal cancer cell sensitivity to oxaliplatin through modulation of intracellular oxidative stress (228) .

Taken together, studies suggest that PGE_2 signaling may be involved in both CSC and ROS mediated drug resistance mechanism and promote oxaliplatin resistance in human CRC. Here, we *hypothesize* that PGE_2/EP4 signaling promotes human colon cancer cell survival against oxaliplatin treatment *via* regulating CSC and ROS related mechanisms; selective blockade of PGE_2/EP4 signaling could modulate stem cell phenotype and oxidative stress in human colon cancer cells and circumvent oxaliplatin resistance in human CRC. To address our hypothesis, we conducted the following study to evaluate both CSC and ROS-related mechanisms in human CRC using an established oxaliplatin resistant cell culture model as described before. We assessed the phenotype of CSC subpopulations in both resistant and parental cell lines, and determined the effect of PGE_2 suppression or PGE_2 signaling blockade on the CSC tumorigenic properties using selective EP receptor antagonist. We also examined the

intracellular ROS level in both cell lines and evaluated the effect of EP4 inhibition on OXR cell survival and intracellular ROS metabolism, either as single treatment or combined with oxaliplatin. The impact of antioxidants on cell survival was also tested. By identifying the connection between PGE_2/ EP4 signaling and the potential mechanisms involved in oxaliplatin resistance, we provide strong evidence for EP4 as a potent target to tackle the problem of oxaliplatin resistance in CRC treatment.

3.2 MATERIALS AND METHODS

Materials. Human colon cancer cell line HT29 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The HT29 Oxaliplatin-resistant (OXR) cell line was generated as previously described (199) . Briefly, chemo-naïve HT29 cells were exposed to increasing concentrations of oxaliplatin over a three-month time-frame, with the final concentration maintained at 2μM. Cell culture media and serum were purchased from Life Technologies (Carlsbad, CA). Oxaliplatin, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). PGE2, EP receptor antagonist L-161,982 was purchased from Cayman Chemicals (Ann Arbor, MI).

Cell Culture Conditions. Human cancer cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 in MEM, supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, L-Glutamine, MEM vitamin solution, sodium pyruvate and MEM non-essential amino acids. Oxaliplatin resistant cells were maintained in 2μM oxaliplatin, but were cultured in oxaliplatin-free media at least 24 hours prior to experimentation. Cells were confirmed to be free of Mycoplasma using Mycoplasma Detection Test (200) . All experiments were performed at 70% cell confluence with no more than 20 cell passages. Results from all studies were confirmed in at least three independent experiments.

Cell Viability Assay. Cell sensitivity to drugs was assessed using cholorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.

Briefly, cells were seeded in 48-well plates overnight and treated with or without drugs. After 72 hours, 100µl MTT solution (Sigma) was added to each well and incubated for 1h at 37°C. Medium was then aspired and 300µl DMSO was added. Colorimetric analysis was performed at a wavelength of 570nm using a standard microplate reader. IC50 curves were generated with GraphPad Prism (software version 5.0c) using variable slope model.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using the RNeasy Mini kit (Qiagen Inc.). cDNA was synthesized using Superscript III according to the manufacturer's protocol (Invitrogen) mRNA expression levels of genes of interest were examined with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on the ABI-7500 platform (Applied Biosystems). The levels of RNA expression were normalized to GAPDH. The primers used for PCR amplification were: 5'-CAA GTT CAA GCA GCT CTA CCG-3' and 5'-GCT CCT GCA ACT CCT CAA AG -3' for HOMX1; 5'- AAG AAA GGA TGG GAG GTG GT-3' and 5'-CAG AAC AGA CTC GGC AGG AT-3' for NQO1; 5'-TTG CAA CCA ATT TGG ACA TC-3' and 5'- GTT CTG CCC ATT CAC CTC AC-3' for GPX; 5'-CTG GGA GCT CTT CTG ACT GG-3' and 3'-TGG TGC CTC AGG TTG TTA AA-5' for DUOX2; 5'-AGG CTG TAC CAG TGC AGG TC-3' and 5'-CAA TAG ACA CAT CGG CCA CA-3' for SOD1; 5'-CGT CAC CGA GGA GAA GTA CC-3' and 5'-TAG GGC TGA GGT TTG TCC AG-3' for SOD2; 5'-AAA CCA GTG GAT CTG CCA AC-3' and 5'-ACG TAG CCG AAG AAA CCT CA-3' for NFE2L2; 5'-ACA ACT TTG GTA TCG TGG AAG G -3' and 5'-CAG TGA GCT TCC CGT TCA G-3' for GAPDH. For each experiment, PCR amplifications with no cDNA were performed as negative controls. The levels

of RNA expression were normalized to GAPDH. PCR products were analyzed on 2% agarose gel with ethidium bromide (E-gel, Invitrogen), together with 1 kb plus DNA ladder (Invitrogen).

Sphere Formation Assay. Tumor sphere formation was evaluated as previously described with modifications (229) . Briefly, 100 cells were cultured in a 96-well ultra-low attachment surface plate (Corning Life Sciences) with serum-free DMEM/F12 medium containing B27 supplement, 20 ng/mL EGF and 20 ng/mL FGF (Invitrogen) for 7 days. The sphere numbers in each well were quantified. After 5 days, cells were supplemented with fresh SCM for another 100µl/well. The formation of spheres was evaluated day 1, 3, 5 post seeding by light microscopy and the number of spheres was counted at day 7 as indicator of cell sphere forming ability.

Immunofluorescence. Drug treated or control tumor spheres were grown on 8 chamber glass slide (BD Falcon) for 1 week and fixed in 4% paraformaldehyde for 15mins, followed by permeabilization and blocking by 5% goat serum/0.3% Triton X-100 in 1xPBS for 1 hour at room temperature followed by incubation with primary antibodies overnight at 4°C and secondary antibodies for 1h at room temperature in the dark. Nuclei were counterstained with 4',6-diamidino-2 phenylindole (DAPI). Tumor spheres were mounted on to glass slides and visualized using an Olympus fluorescence microscope (Olympus Corp.).

ROS Detection. Cellular ROS levels were detected with H2DCFDA (Life Technologies) staining as previously described with small changes (230) . Briefly, cells were treated with drugs or vehicle control. After 48 hours, cells were washed twice with serum free media then incubated with 2 µM H2DCFDA in 2% FBS media at 37°C for 30mins. Following incubation, cells were

washed twice with serum free media twice, trypsinized and collected for flow cytometry analysis. None staining group as negative control and cells treated 2 hours treatment of 0.03% H_2O_2 served as positive control. The levels of ROS in tested groups are measured by the percentage of H2DCFA positive stained cells in total cell population.

Glutathione (GSH) Assay. To measure GSH level, cells were treated with drugs or vehicle control for an 8-hour time-frame. Cells were collected using rubber policeman and cell numbers were counted. Cell were then sonicated and centrifuged. Supernatants were deproteinated and stored at -20°C until GSH measurement. Total GSH levels were then measured using a commercial glutathione assay kit (Cayman Chemical, MI) according to manufacturer's protocol.

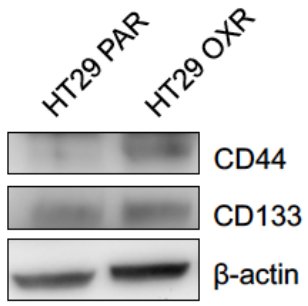
Statistical Analysis. Date from all experiments was analyzed using the Student's t test or two-way analysis of variance (ANOVA) when appropriate for analysis by GraphPad Prism (software version 5.0c). For MTT assay, 50% inhibitory concentrations of oxaliplatin were calculated and compared using Extra sum-of-squares F test. Results were considered as statistically significant at a P value of less than 0.05. All statistical tests were two-sided.

3.3 RESULTS

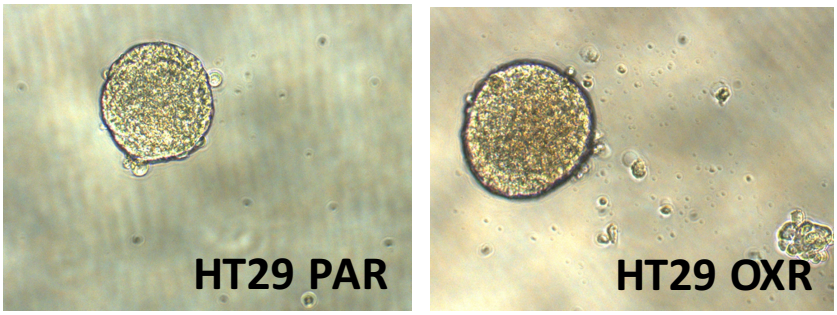
PGE_2 level is associated with Cancer Stem Cell Enrichment in Oxaliplatin Resistant Cells. One important mechanism of cancer chemoresistance is the enrichment of cancer stem cells (CSC) in tumors (160,165) . Previous studies associated overexpression of stem cell markers and expansion of CSC subpopulations with oxaliplatin resistance in human CRC (199,220) . Consistent with these findings, in the present study, HT29 OXR cells showed increased protein levels of colonic stem cell markers (CD133 and CD44) compared to parental cells (Fig. 13A). Moreover, in tumor sphere formation assays, both HT29 PAR and OXR cells formed viable tumor spheres with similar morphology. However, HT29 OXR cells demonstrated a 2.5-fold increase ($P<0.001$) in tumor sphere formation over a 7-day time period, indicating enhanced CSC capacity compared to HT29 parental cells (Fig. 13B).

As our previous work showed deregulated PGE_2 metabolism in HT29 OXR cells, we'd like to determine if PGE_2 signaling is involved in the enhancement of tumor sphere-forming ability and CSC subpopulation of OXR cells. PGE_2 suppression via siRNA knockdown of PTGES for 48 hours led to a significant reduction in the number of tumor spheres formed by the resistant cells (~65% reduction; $P<0.01$), suggesting that PGE_2 may directly contribute to the tumorigenic behavior of OXR cells (Fig. 14B).

A.


HT29 PAR
HT29 OXR
CD44
CD133
β-actin

B.


HT29 PAR
HT29 OXR

C.

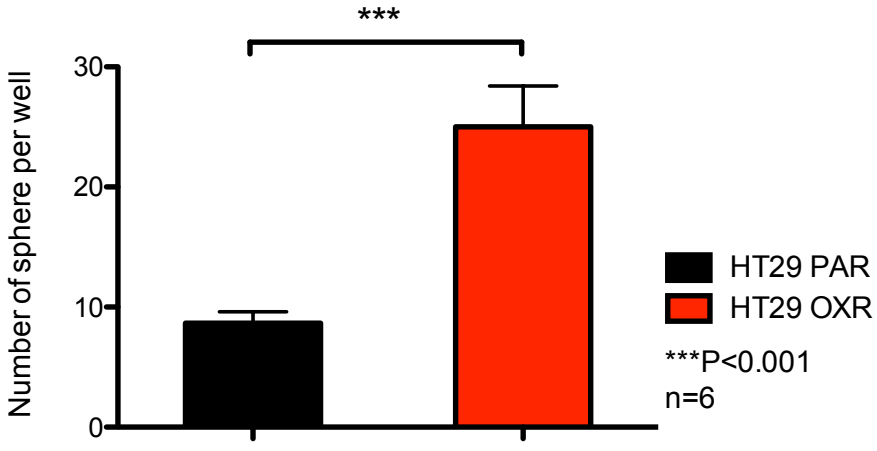
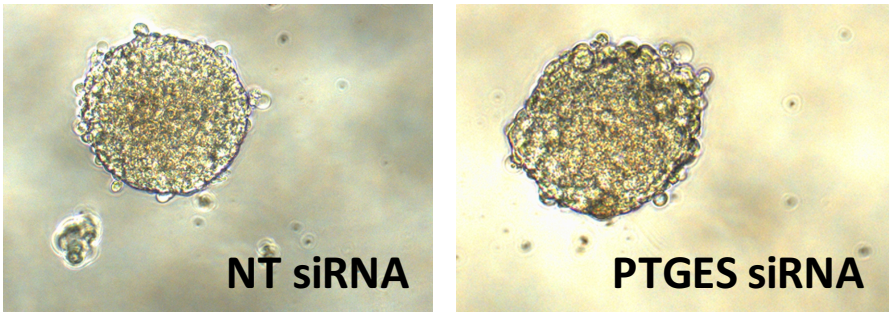

***
Number of sphere per well
30
20
10
0
HT29 PAR
HT29 OXR
***P<0.001
n=6

Figure 13. Oxaliplatin resistant cells demonstrated enrichment of tumor initiating cell subpopulaion. (A) HT29 PAR and OXR cells were cultured in drug-free medium for 72 hours followed by protein extraction.Protein levels of stem cell marker CD44 and CD133 were detected in both HT29 PAR and HT29 OXR cells using western blotting analysis. β-actin was used for standard normalization. (B) HT29 PAR and OXR cells were plated in ultra-low attachment 96 well plates (100 cells per well) for 1 week. The morphology of tumor spheres formed by both cells were observed under microscope and (C) the numbers of viable tumor sphere per well in each group were counted.

A.



B.

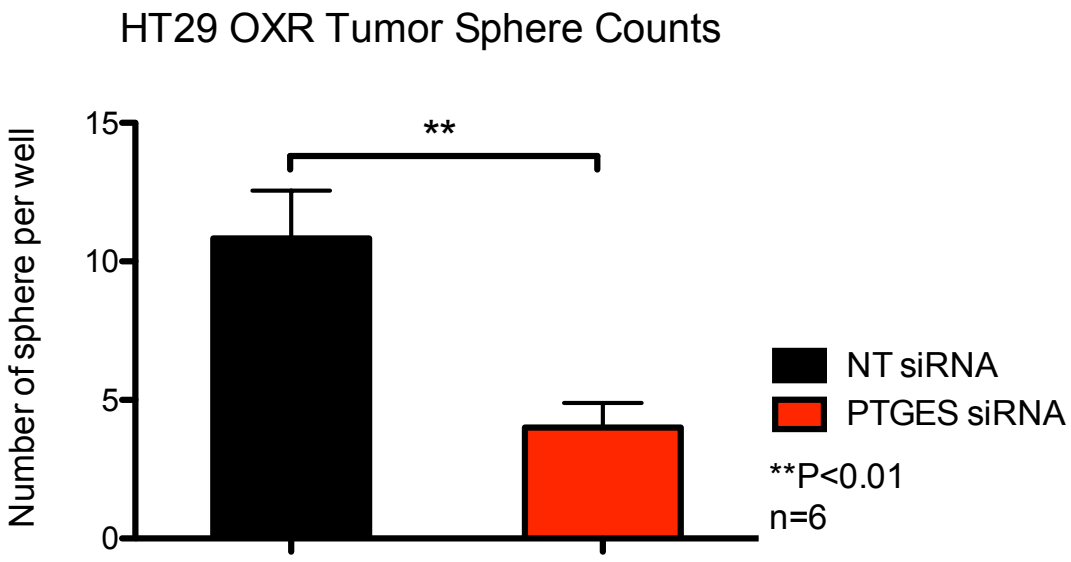
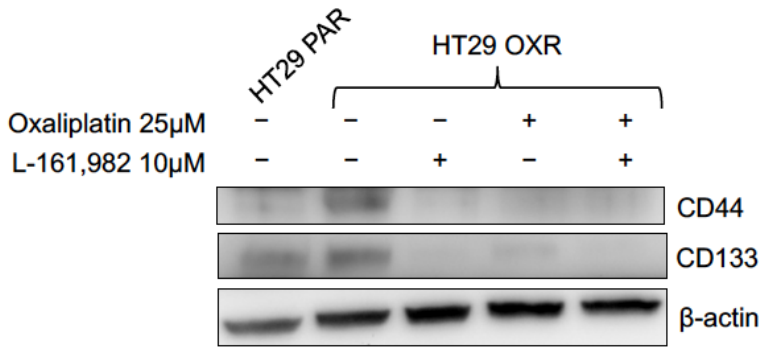


Figure 14. PTGES knockdown significantly reduced *in vitro* tumor sphere formation by HT29-OXR cells. HT29 OXR cells were treated with PTGES siRNA or non-targeting (NT) siRNA for 48 hours followed by tumor sphere formation assay. After plated for 1week in ultra-low attachment 96-well plates (100 cell per well), (A) the morphology of tumor spheres formed by both cells were observed under microscope and (B) the numbers of viable tumor sphere per well were counted.

Blocking PGE_2/EP4 receptor Signaling impaired Tumorsphere Forming Capacity of Oxaliplatin Resistant Cells. To examine the significance of PGE_2/EP4 signaling in CSC subpopulations of OXR cells, the protein levels of both CD133 and CD44 were measured at 48 hours after EP4 inhibition. We found that treatment with L-161,982 reduced the protein levels of both CD44 and CD133 in OXR cells (Fig. 15A). In tumor sphere assay, treatment with L-161,982 significantly reduced the number of tumor spheres formed by HT29 OXR cells, regardless of oxaliplatin treatment (Fig. 15B). Interestingly, treatment of oxaliplatin alone did not affect the number of tumor spheres, but significantly reduced the size of tumor spheres formed by OXR cells with or without the presence of L-161,982, suggesting that oxaliplatin treatment could suppress the proliferation of CSC cells, but not the tumor initiation (Fig. 16). In addition, blockade of EP4 signaling in HT29 OXR cells significantly reduced the expression of stem cell markers (CD44 and CD133) within the tumor spheres (Fig. 17). These results suggest that PGE_2/EP4 signaling is critical for growth and abilities of CSC subpopulations in OXR cells, which mediate a key mechanism for cell survival and repopulation against oxaliplatin cytotoxicity.

A.



B.

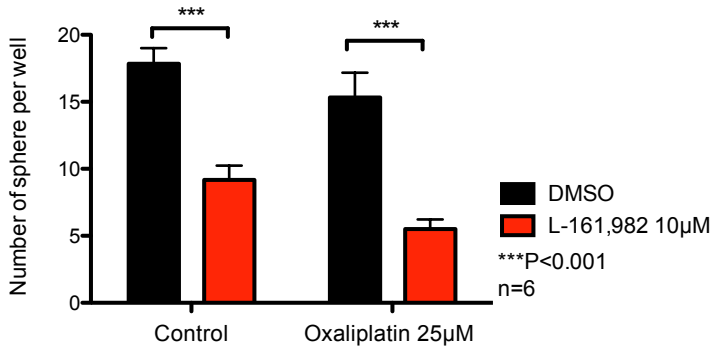


Figure 15. Selective EP4 blockade significantly reduced *in vitro* tumor sphere formation of HT29-OXR cells. (A) HT29 PAR and OXR cells were cultured in oxaliplatin free medium for 24 hours, then treated with indicated combination of oxaliplatin and L-161,982 for 48 hours followed by protein extraction. The protein level of stem cell markers CD44 and CD133 were detected using Western Blot analysis. β-actin was used for standard normalization. (B) HT29 OXR cells were seeded in ultra-low attachment plate (100 cells per well) and treated with indicated combination of oxaliplatin and L-161,982 for 1week. At day 7, numbers of viable tumor sphere per well were counted.

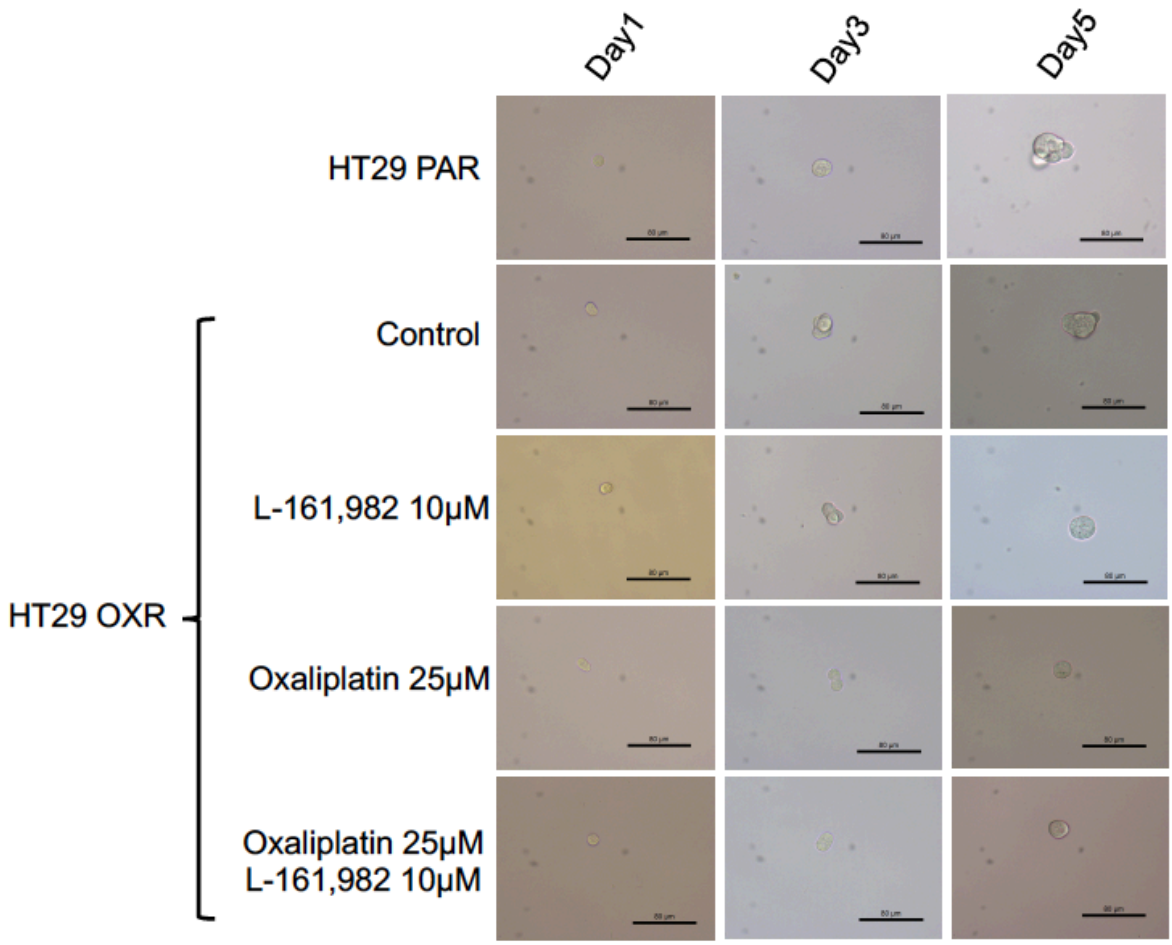


Figure 16. Morphology of tumor spheres formed by HT29 PAR and OXR cells with combined treatment of oxaliplatin and L-161,982. HT29 PAR cells and OXR cells were seeded in ultra-low attachment plate (100 cells per well) for 1week with indicated combination of oxaliplatin and L-161,982. At day 1, 3, 5 post seeding, the morphology of tumor sphere formed was observed under microscope.

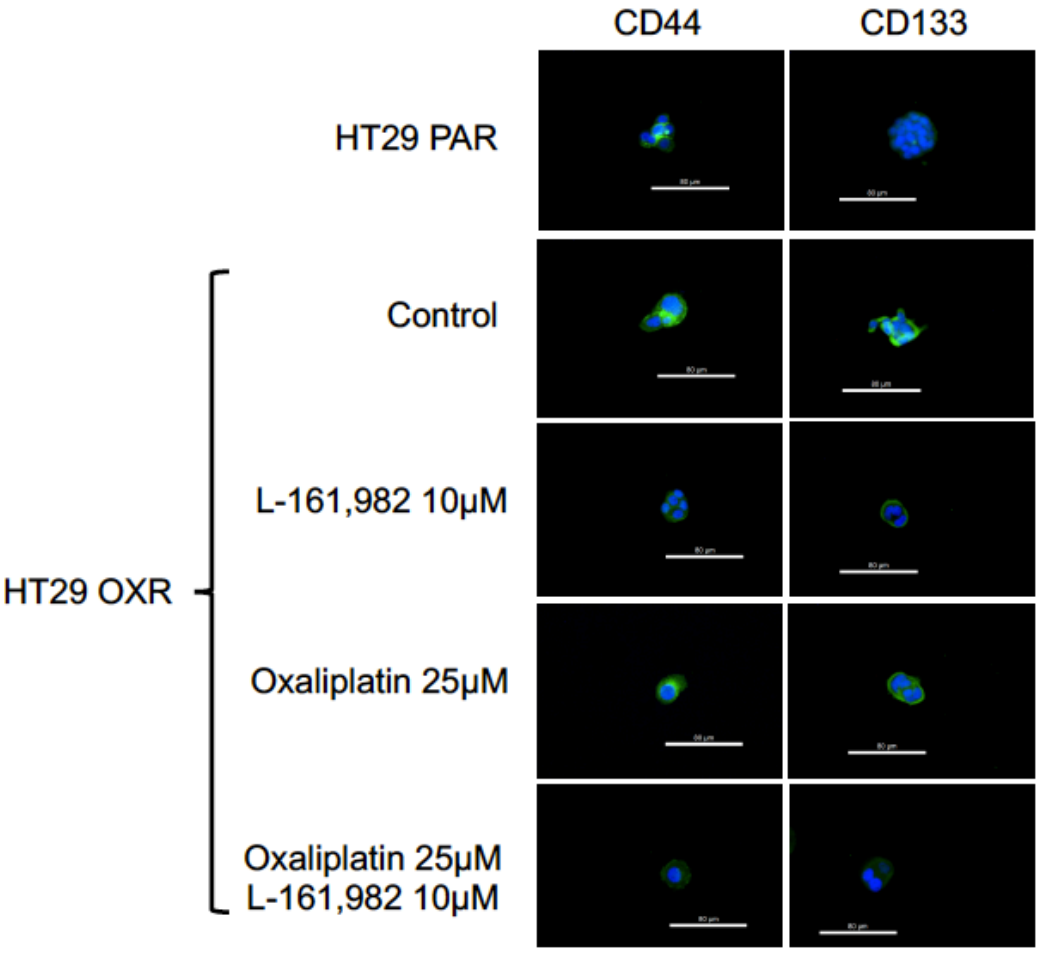
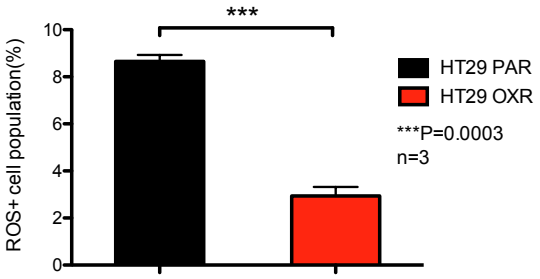


Figure 17. Selective EP4 blockade significantly reduced stem cell marker expression in tumor sphere formed by HT29 PAR and OXR cells. HT29 PAR cells and HT29 OXR cells were seeded in ultra-low attachment plate and treated as indicated for 1week. At day 7, the expression levels of CD44 and CD133 in tumor sphere were detected using Immunofluorescence Analysis.

PGE_2/EP4 receptor signaling is associated with deregulation of cellular oxidative stress in oxaliplatin resistant cells. Recent studies have suggested that oxaliplatin activates apoptotic pathways in colon cancer cells by inducing the accumulation of cellular reactive oxygen species (ROS), while cancer cells may adapt oxidative stress modulation mechanism to evade ROS-mediated cell death (230) . To determine whether a ROS-related mechanism is involved in oxaliplatin resistance in OXR cells, we measured cellular ROS levels in both parental HT29 and HT29 OXR cells using H2DCFDA staining followed by flow cytometry. Compared to HT29 parental cells, oxaliplatin resistant cells maintained significantly lower (~60%; $P<0.001$) basal levels of ROS, suggesting deregulation of ROS metabolism in OXR cells (Fig. 18A). In addition, 48 hours of treatment with oxaliplatin (50μM) significantly increased ROS in OXR cells. Interestingly, although treatment with L-161,982 (10μM) alone did not affect ROS level, the addition of L-161,982 caused a further elevation in oxaliplatin-induced ROS accumulation (~2.2-fold; $P<0.0001$) in HT29 OXR cells, indicating a strong association between EP4 signaling and ROS metabolism in cancer cell oxaliplatin resistance (Fig. 18B).

A.



B.

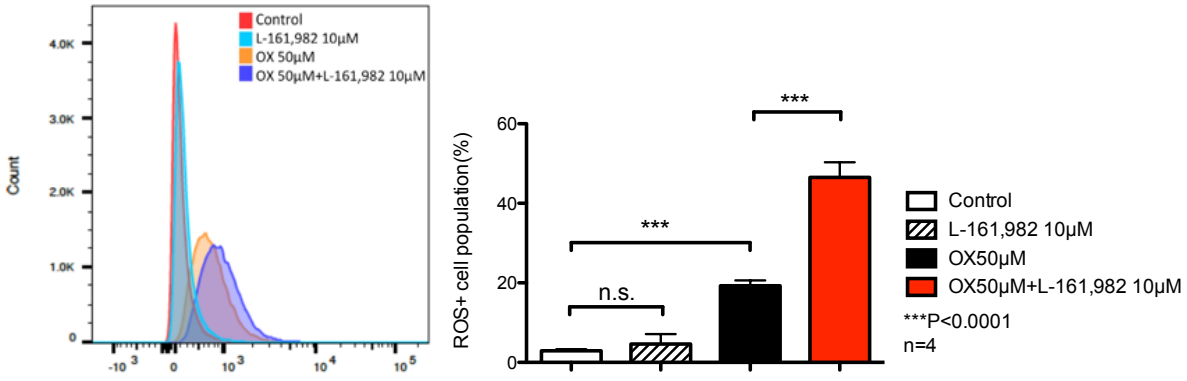
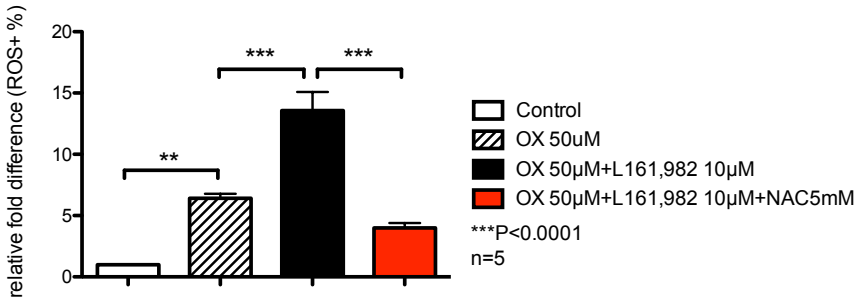


Figure 18. Blocking PGE_2/EP4 signaling reduced cellular reactive oxygen species (ROS) level in HT29 OXR cells. (A) HT29 PAR and OXR cells were cultured in oxaliplatin-free medium for 72 hours before cell collection. The basal level of ROS in HT29 PAR and OXR cells were detected with H2DCFDA staining and measured by flow cytometry analysis. (B) HT29 OXR cells were treated with vehicle control or combination of oxaliplatin(50μM) and L-161,982(10μM) for 48 hours before cell collection. The cellular level of ROS were detected with H2DCFDA staining and measured by flow cytometry analysis. Results are presented as the percentage of fluorescence positive population.

Blockade of EP4 signaling using L-161,982 sensitize HT29 OXR cells to oxaliplatin cytotoxicity *via* reactive oxygen species (ROS) mediated mechanism. To determine the significance of ROS mediated mechanism in the effect of EP4 selective inhibition on oxaliplatin resistance, we treated HT29 OXR cells with combination of oxaliplatin, L-161,982 and an antioxidant GSH precursor, N-acetyl cysteine (NAC). We found that the effects of L-161,982 on maintaining higher ROS levels upon oxaliplatin treatment was reversed by addition of NAC (Fig. 19A). Consistent with the results on ROS levels, the addition of NAC to oxaliplatin caused a synergistic effect with L-161,982 on oxaliplatin cytotoxicity as well, leading to increased cell survival and oxaliplatin resistance in HT29 OXR cells (Fig. 19B).

To understand the underlying mechanism of ROS regulation by EP4 selective inhibition, we measured the mRNA expression of enzymes involved in ROS metabolism and the cellular level of the non-enzymatic ROS scavenger, Glutathione (GSH). We found that treatment of oxaliplatin resulted in significant changes of GSH level within 48-hours post treatment, while L-161,982 treatment reduced the levels of GSH (Fig. 20A). We also tested the expression of genes encoding enzymes associated with GSH synthesis and utilization. Blockade of EP4 receptor for 48 hours significantly reduced the mRNA expression of the ROS-detoxifying enzyme, glutathione peroxidase (GPX), and the cysteine provider, γ-glutamyltraspeptidase (GGT) (~50% reduction; $P<0.00001$ and ~70% reduction; $P<0.0001$, respectively) (Fig. 20 B-C). However, the mRNA expression levels of other enzymes in ROS clearance were not affected by treatment of L-161,982 (Fig. 21). Taken together, these results suggest that selective inhibition of EP4 signaling enables tumor cells to maintain higher levels of cytotoxic ROS through suppression of GSH dependent ROS detoxification mechanisms.

A.



B.

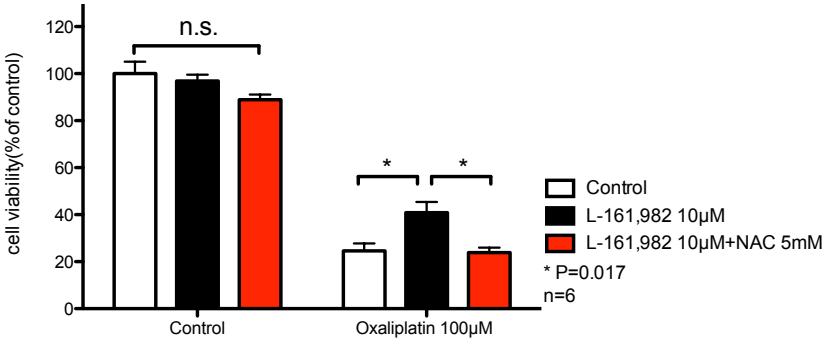
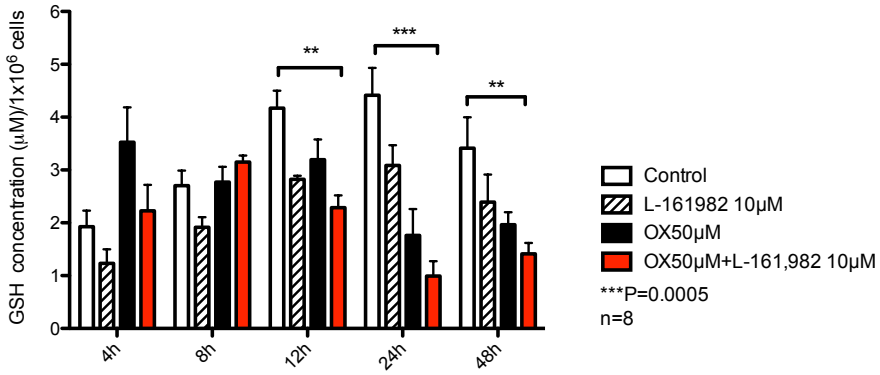
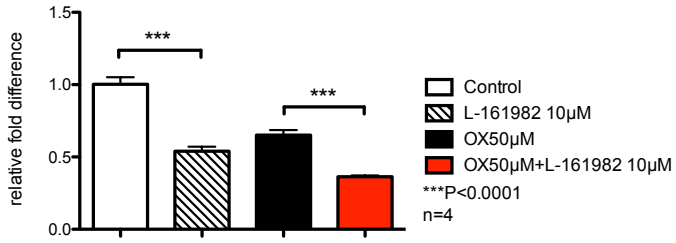


Figure 19. Synergistic effects of L-161,982 on oxaliplatin efficacy in HT29 OXR cells were cellular reactive oxygen species (ROS) levels dependent. (A) HT29 OXR cells were treated with indicated combination of oxaliplatin (50μM) and L-161,982 (10μM) and NAC (5mM) for 48 hours, followed with H2DCFDA staining. The cellular ROS level was measure by flow cytometry analysis. (B) HT29 OXR cells were treated with indicated combination of oxaliplatin and L-161,982 and NAC for 72 hours, followed by MTT assay. Cell viability rate was defined as the percentage of viable cells in each group compared to untreated cells.

A.



B.



C.

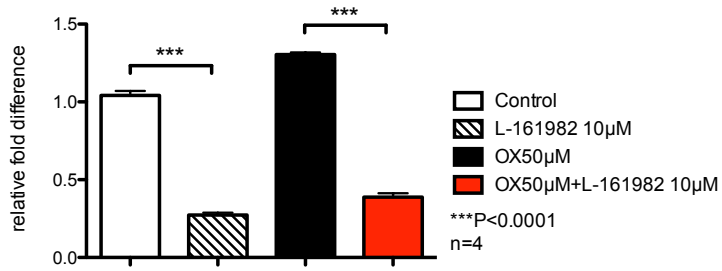
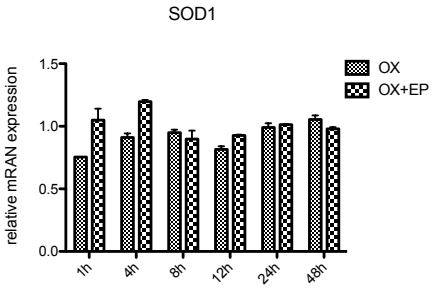
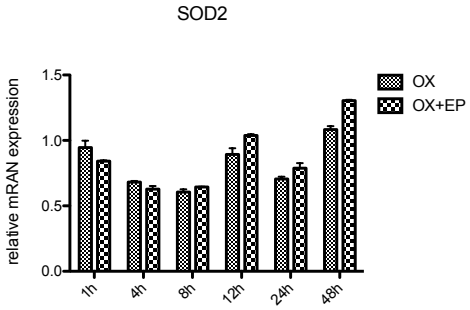


Figure 20. Inhibition of EP4 signaling suppressed Glutathione (GSH) level and utilization in HT29 OXR cells. HT29 OXR cells were treated with indicated combination of oxaliplatin (50μM) and L-161,982 (10μM) for 48 hours, followed by supernatant collection and RNA extraction. (A) The levels of cellular GSH at different time points were measured using commercial GSH assay kit. (B-C) mRNA expression levels of GPX2 and GGT at 48 hours were measured by RT-PCR analysis.

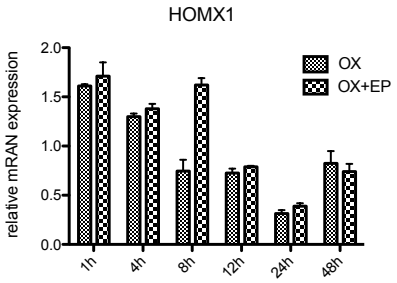
A.


SOD1
relative mRAN expression
1.5
1.0
0.5
0.0
1h
4h
8h
12h
24h
48h
OX
OX+EP

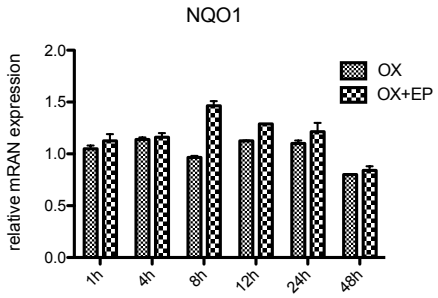
B.


SOD2
relative mRAN expression
1.5
1.0
0.5
0.0
1h
4h
8h
12h
24h
48h
OX
OX+EP

C.


HOMX1
relative mRAN expression
2.0
1.5
1.0
0.5
0.0
1h
4h
8h
12h
24h
48h
OX
OX+EP

D.


NQO1
relative mRAN expression
2.0
1.5
1.0
0.5
0.0
1h
4h
8h
12h
24h
48h
OX
OX+EP

E.

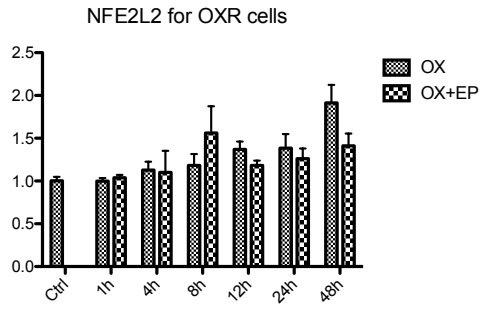
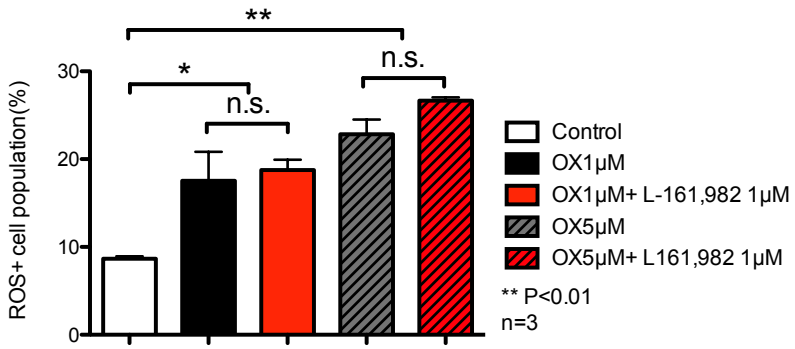


Figure 21. Inhibition of EP4 signaling did not affect mRNA expression of GSH-independent enzymes in HT29 OXR cells. HT29 OXR cells were treated with indicated combination of oxaliplatin (50μM) and L-161,982 (10μM) for 48 hours, followed by total RNA extraction. The mRNA expression levels of SOD1, SOD2, HOMX1, NQO1 and NFE2L2 were measured by RT-PCR analysis.

Blockade of EP4 signaling using L-161,982 did not affect cellular ROS level in HT29 parental cells. Our previous results showed that EP4 inhibition did not affect oxaliplatin cytotoxicity of parental cells (Fig. 10B). To determine whether EP4 inhibition affect cellular ROS metabolism in HT29 parental cells, we treated HT29 cells with combination of oxaliplatin and L-161,982. Addition of L-161,982 did not further increase ROS levels induced by oxaliplatin alone in HT29 parental cells (Fig. 22A). However, combined treatment with oxaliplatin and the EP4 receptor agonist, L-902,688, suppressed oxaliplatin-induced ROS up-regulation (Fig. 22B). This data is in consistent with the results from cell viability experiments, in which treatment of L-902,688 significantly increased cell survival in HT29 parental cells (IC50 value: 2.50μM compared to 14.14μM; $P<0.001$) (Fig. 12A). Taken together, these results suggest that EP receptor signaling is an important factor in oxidative stress regulation during cancer cell response upon oxaliplatin treatment.

A.



B.

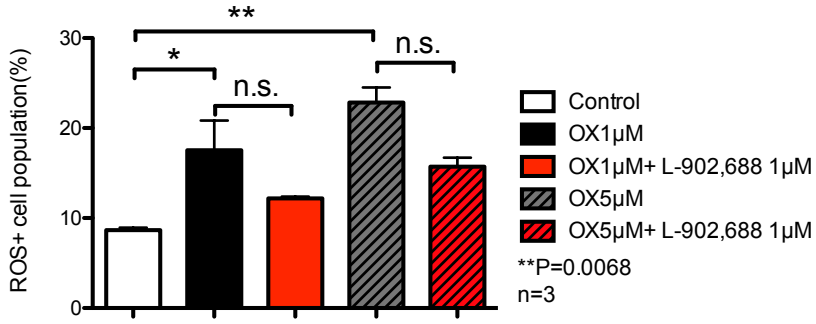


Figure 22. Regulation of EP4 signaling affects oxidative stress level in HT29 cells. (A) HT29 cells were treated with indicated combination of oxaliplatin and L-161,982 for 48 hours followed with H2DCFDA staining. The cellular ROS level was measure by flow cytometry analysis. (B) HT29 cells were treated with indicated combination of oxaliplatin and L-902,688 for 48 hours followed with H2DCFDA staining. The cellular ROS level was measure by flow cytometry analysis.

3.4 DISCUSSION

It has been shown that long term exposure of chemotherapeutic agents induce acquired resistance in colon cancer cells, leading to tumor recurrence and metastases after initial response. The common mechanisms involved in colon cancer chemoresistance include hyper-activation of cell survival/anti-apoptotic pathways, deregulation of DNA repair pathways and multidrug resistance (MDR) mechanisms (218,219) . Recently studies suggested that mechanism associated with tumor initiation and metastases, such as enrichment of cancer stem cells and epithelial-to-mesenchymal transition (EMT) of cancer cells in tumor mass, could also mediate the drug resistance in human CRC (199) . On the other hand, the recent work by Wang and colleagues suggested that PGE_2/EP4 signaling could promote the expansion of CSC in colorectal cancer and mediate tumor metastases, indicating that PGE_2/EP4 signaling may also mediate drug resistance through regulation CSC subpopulation of colon cancer cells (231) .

Previous work by Ellis lab demonstrated a significant enrichment of tumorigenic CSC subpopulation in oxaliplatin resistant colon cancer cells, implying an association of CSC and oxaliplatin resistance in HT29 OXR cells (220) . In the present study, we explored the potential association between deregulated PGE_2 synthesis and the capacity of cancer stem cells in resistant cells. HT29 OXR cells demonstrated stronger tumorigenic ability by forming more tumor spheres than parental cells, while PGE_2 suppression by siRNA-mediated PTGES knockdown significantly reduced the number of tumor spheres formed by OXR cells. These results suggest that PGE_2 (and possibly its downstream signaling) plays an important role in maintaining the expansion of CSC subpopulation in HT29 OXR cells.

To further determine the specific downstream signaling of PGE_2 that is responsible for its CSC promoting effect, we blocked EP4 recepotor signaling using selective antagonist L-161,982. Treatment of L-161,982 led to a marked reduction in both tumor sphere formation and the expression of the CSC markers, CD44 and CD133 in OXR cells. These findings may be due in part to the down-regulation of AKT pathway, since studies have shown that EP4 signal through PI3K-AKT pathway, and AKT signaling is critical for CSC proliferation or survival (232,233) .

Besides enrichment of cancer stem cells, another mechanism that cancer cells may utilize to acquire chemoresistance is the upregulation of antioxidant capacity (223) . Oxidative stress is a key mechanism involved in chemotherapy-induced cancer cell death. Due to vigorous metabolism and multiple genetic mutations, cancer cells usually maintain higher level of ROS generation of ROS compared to normal cells, making them more susceptible to DNA damage and intrinsic apoptosis induced by pharmacologically generated ROS, providing an important anticancer strategy (222,224) . However, consistent exposure to chemotherapy-induced oxidative stress may exert selective pressure and induce adaption to intrinsic oxidative stress in the survivor cells, therefore confer drug resistance (223) . Recently, oxaliplatin was shown to induce colon cancer cell death through ROS generation (225) , suggesting that regulation of oxidative stress may affect the sensitivity of human colon cancer cells to oxaliplatin cytotoxicity (226) . Mo and colleagues found that inhibition of EP4 receptor signaling using L-161,982 significantly increased cellular ROS level and inhibit cell proliferation in myoblast (228) . In the present study, the HT29 OXR cells demonstrate significantly reduced basal level of ROS compared to the parental cells, suggesting a possible adaption to oxidative stress induced by long-term exposure of oxaliplatin. Although the treatment with L-161,982 alone did not affect the ROS

level in cancer cells, blockade of EP4 receptor signaling by L-161,982 further boosted the overproduction of ROS by oxaliplatin in OXR cells, but not parental cells, suggesting that the synergistic effect of EP4 inhibition on oxaliplatin efficacy is possibly mediated through modulation of oxidative stress. Furthermore, treatment of HT29 OXR cells with antioxidant NAC reversed the effects of L-161,982 on both ROS level regulation and oxaliplatin cytotoxicity. These results present suggest that PGE_2/EP4 signaling plays an important role in regulation of oxaliplatin induced oxidative stress in HT29 OXR cells, explaining the beneficial effect of EP4 inhibition on oxaliplatin efficacy.

In cancer cells, ROS level is balanced by both ROS generation and scavenging (222) . Our findings suggest that treatment of L-161,982 alone does not affect cellular ROS level; However, EP4 inhibition could increase cellular ROS in the presence of oxaliplatin. These findings not only confirmed that oxaliplatin could induced ROS generation as key mechanisms of act, but also revealed an important role of PGE_2/EP4 signaling in ROS scavenging, instead of ROS generation. To further determine the ROS clearing mechanism regulated by EP4 signaling, we tested the mRNA expression of enzymes involved in ROS clearance and the cellular level of GSH, the critical non-enzymatic ROS scavenger in cancer cells (234) . Although we didn't not found significant difference in many ROS scavenging enzymes (including SOD enzymes, HOMX1, NQO1, NFE2L2) upon the blockade of EP4 signaling, we did find that treatment of OXR cells with L-161,982 suppressed the cellular level of GSH and reduced its utilization by inhibiting the mRNA expression of GSH peroxidase (GPX), therefore reducing ROS elimination. Taken together, these results suggest that treatment of oxaliplatin increase cellular level of ROS in cancer cells, while in HT29 OXR cells, deregulated PGE_2/EP4 signaling promotes the

clearance of ROS through GSH mediated mechanisms. Inhibition of PGE_2/EP4 signaling could suppress GSH mediated ROS scavenging, therefore help maintain higher cellular level of ROS and induce cell death.

In summary, we have demonstrated two important mechanisms involved in oxaliplatin resistance of human colon cancer cells: CSC enrichment and modulation of oxidative stress. More importantly, we discovered the direct association of PGE_2/EP4 receptor signaling in both mechanisms. Our results demonstrate direct evidence supporting the critical role of PGE_2/EP4 signaling in maintaining enriched CSC subpopulation and regulating ROS-dependent mechanism for oxaliplatin cytotoxicity in HT29 OXR cells, suggesting a critical role of PGE_2/EP4 signaling in promoting oxaliplatin resistance in human colorectal cancer cells. Combining with other studies on EP4 signaling in CRC progression (196) , our findings provide the logical basis of targeting PGE_2/EP4 signaling for increasing oxaliplatin efficacy, circumventing oxaliplatin resistance, and ultimately increase the survival of advanced CRC patients.

CHAPTER 4

HISTOLOGICAL AND MOLECULAR ALTERATIONS OF ABERRANT CRYPT FOCI IN THE INDUCIBLE BRAF^{V600E} MUTATION CRC MOUSE MODEL

4.1 INTRODUCTION

Colorectal Cancer (CRC) is the third most common cancer and the fourth leading cause of cancer death that accounts for approximately 609,000 deaths worldwide. In order to develop effective preventive and therapeutic strategies and improve clinical outcomes for CRC patients, numerous studies have been focusing on the molecular genetics of CRC tumorigenesis over the past three decades (31) . In the "classic" model of colorectal tumorigenesis proposed by Fearon and Vogelstein, the development of colorectal cancer follows the "adenoma-carcinoma" sequence, driven by a relative limited number of genetic alterations including oncogene activation and silencing of tumor-suppressor genes (235) . In this genetic model, the inactivation of *APC* gene leads to the appearance of adenomatous polyps, the neoplastic precursor lesions in colonic mucosa, therefore is regarded as the initiating step of human CRC. This step is followed by further mutations in other genes such as *TP53* and *KRAS*, leading to the development of large adenoma and carcinoma. This stepwise pathway is regarded as the classic chromosomal instability (CIN) pathway and account for approximately 70% of human CRC cases and have been extensively studied for preventive (screening) and treatment purposes.

Besides the CIN pathway, recent studies have indicated that approximately 30% of colorectal carcinomas develop via an alternative pathway, the "serrated" pathway, named by the histological feature of saw-toothed (serrated) crypts in the precursor colonic polyps (236) . This concept of alternative pathways derived from the study on precancerous polyps. Previously, all hyperplastic polyps are considered as benign lesions without malignant potential. In 1996, Torlakovic and Snover described the histological difference between serrated polyps in serrated polyposis syndrome (SPS) patients and the sporadic hyperplastic polyps, and observed serrated adenomas and cancers developed in SPS patients to establish a strong association between serrated polyps and serrated CRC (adenocarcinoma) for the first time (237) . Their studies suggested that the hyperplastic polyps (now named as serrated polyps) are in fact a heterogeneous group and some of the hyperplastic polyps are precancerous. The following studies confirmed their findings and established the serrated pathway of CRC in which the serrated polyps, instead of the traditional adenoma, represent the precursors of colon cancer. According to the latest World Health Organization (WHO) classification, the serrated polyps are categorized into three groups: the hyperplastic polyps (HPs), the sessile serrated adenoma (SSA) and the traditional serrated adenoma (TSA) (238) . Although the malignant potential of these polyps may vary, all three types of polyps share the same histologic feature of saw-toothed crypts.

Distinct from the traditional colorectal tumors that harbor *APC* and *TP53* mutations, the serrated CRCs have been associated a distinct set of molecular features, such as MAPK pathway activation, primarily *via* either *BRAF* or *KRAS* mutations, CpG island methylator phenotype (CIMP) and DNA microsatellite instability (MSI) (236) . Based on these genetic characteristics,

in 2007, Jass and colleagues (239) proposed the following molecular profiles to classify three subtypes of serrated adenomas: (1) *BRAF* mutant, CIMP-high, MSI-high; (2) *BRAF* mutant, CIMP-low, MSI-low and (3) *KRAS* mutant, CIMP-low, MSS/MSI-low (239) . The first two groups share *BRAF* mutations and are more strongly linked to the serrated neoplasia pathway, in which the SSAs arise.

BRAF is a serine/threonine protein kinase that plays critical role in EGFR mediated MAPK pathway signaling in cancer cells. Activated by the upstream RTKs, the small GTPase RAS, BRAF (and other isoforms including ARAF and CRAF) further activate MAPK signaling to promote cell proliferation, growth and differentiation. BRAF is also found to mediate cell migration, pro-survival and anti-apoptosis in cancer cells, therefore plays an important role in tumor development of multiple malignancies (240) . In 2002, Rajagopalan and colleagues discribed *BRAF* mutations in colon cancer patients for the first time, and also found that the BRAF and KRAS mutations are mutually exclusive in human CRC (241) . Until now, oncogenic mutations in the *BRAF* gene are found in approximately 10% CRC patients and have been extensively studied for its clinical relevance. Compared to the *APC* or *KRAS* mutations, *BRAF* mutations are often found enrich in proximal (right colon), serrated colorectal tumors and usually associated with higher age, female gender and overall poor prognosis (28) . *BRAF* mutated colon tumors have also displayed intrinsic resistance to BRAF inhibitors (i.e. vemurafenib) or anti-EGFR treatments (242) . A better understanding of the biology associated with *BRAF* mutations is imperative to develop effective strategies for the prevention and treatment of serrated pathway CRC.

Among over 30 single-site missense mutations that are mostly found within the kinase domain of *BRAF* gene, a T1799A transversion, which causes Glu for Val substitution that encodes constitutive active $BRAF^{V600E}$ accounts for 90% of *BRAF* mutations in human cancers including melanoma and CRC (28,243) . $BRAF^{V600E}$ mutations have been found in over 80% serrated carcinomas and 62% of micro vesicular hyperplastic polyps (MVHPs), leading to the hypothesis that $BRAF^{V600E}$ mutation is a driver of serrated pathway and *BRAF* mutated HPs may be the precursors of serrated CRC (242) . In 2013, Rad and colleagues (244) demonstrated that $BRAF^{V600E}$ mutation could initiate serrated pathway of intestinal tumorigenesis using a conditional $BRAF^{V637E}$ (the murine counterpart to human $BRAF^{V600E}$) knock-in mice (*Vil-Cre;* $Braf^{LSL\text{-}V637E}$ mice). In this model, the *Cre*-induced expression of mutated BRAF in epithelia of small and large intestine induced the development of generalized MSI-high serrated hyperplasia in both SI and colon, with progressed to dysplasia at age of 10 months. Inactivation of p16 further promotes the development of advanced carcinoma in *Vil-Cre;* $Braf^{LSL\text{-}V637E}$ mice. These results suggest that serrated pathway could be initiated by *BRAF* mutation, yet requires tumor suppressor inactivation for advanced CRC progression.

The study by Rad and colleagues (244) established an initiating role for oncogenic BRAF in serrated adenomas, but whether $BRAF^{V600E}$ mutation drives the development of pre-neoplastic lesions such as aberrant crypt foci (ACF) in the serrated pathway of CRC is still unknown. In 2007, the Hans Clevers group (189) discovered the exclusive expression of Lgr5, a Wnt target gene, in the intestinal stem cells (ISCs) at the base of intestinal crypts. To study the potential role of the $BRAF^{V600E}$ mutation in colonic pre-neoplastic lesions and provide a valuable tool for developing preventive and therapeutic strategies for sporadic serrated CRC, in the

following study we generated a conditional *BRAF*^{V600E} knock-in mouse target specifically to ISCs in C57/B6 mice. We further assessed the impact of tissue-specific, conditional *BRAF*^{V600E} mutations on the histological and molecular features of the colonic mucosa. By characterizing the pre-neoplastic lesions induced by mutated BRAF, we reveal the link between hyperplastic ACF and neoplasia in the *BRAF*-mutant serrated pathway of CRC. Further studies may discover potential biomarkers for evaluating malignant potentials of hyperplastic ACFs and preventing serrated polyps malignant transformations.

4.2 MATERIALS AND METHODS

Generation of *LSL-Braf ^{V600E};Lgr5-EGFP-IRES-creERT2* compound mutant mice (Braf-Lgr5 mice). Both *LSL-Braf ^{V600E}* mice and *Lgr5-EGFP-IRES-creERT2* mice on the C57BL/6 background were purchased from The Jackson Laboratory and the generation of the mouse lines has been previously described (189,245) . To generate the compound mutant mice, male *LSL-Braf ^{V600E}* mice were crossed with female *Lgr5-EGFP-IRES-creERT2* mice. Genotyping was done by tail biopsy. Mice were maintained in a temperature-controlled, light-cycles room and allowed free access to drinking water and standard lab mouse chow in the animal facility of University of Connecticut Health Center (UCHC). Animals were weighed monthly and checked twice a week for signs of weight loss or abnormal behaviors. Animal experiments were conducted with approval from the Institutional Animal Care and Use Committee (IACUC), UCH.

Tamoxifen treatment. Sixty three-week-old male and female mice were separated and Braf-Lgr5 mice were randomly grouped for the following experiments. There are five male mice and five female mice in each group. At 6 week of age, mice received two intraperitoneal (i.p.) injections of either tamoxifen (Sigma-Aldrich; 200mg/kg body weight) or corn oil (0.2ml) within a timeframe of 72 hours. Body weight was recorded monthly. Mice were sacrificed at 4 weeks, 8 weeks and 12 weeks after the last injection, respectively. Colons were harvested, flushed immediately with ice-cold phosphate-buffered saline (PBS) and excised longitudinally. Specimens were fixed-flat in 10% neutral formalin solution overnight and stored in 70% ethanol for further analysis.

Quantification of colonic lesions. Whole-mount fixed colon specimens were stained with 0.2% methylene blue and the number of ACFs per field (20x) were counted under a dissecting microscope. The criteria for ACF examination were described in previous studies (246,247) . The extent of hyperplasia in the colon was evaluated by H&E staining.

Immunohistochemistry. Small intestine and colon tissues were Swiss-rolled, paraffin-embedded and sectioned at 7-μm thickness. Tissue sections were de-paraffinized and incubated with 1 to 3 % hydrogen peroxide for 20 min and blocked with 10% normal goat serum in TBST for 1 hour at room temperature. Sections were then incubated overnight at 4°C with anti-Ki-67 (1:600; Cell Signaling) or anti-green fluorescence protein (GFP; 1:200; Cell Signaling) followed by incubation with the anti-rabbit SignalStain Boost IHC Detection Reagent (HRP, Cell Signaling) for 30 min at room temperature. Signal was detected using diaminobenzidine solution (Vector Laboratories). Tissues were counterstainedwith hematoxylin. Images were captured using QCapture PRO software (QImaging, Surrey, BC, Canada).

Statistical Analyses. Data from all experiments was analyzed using the Student's t-test or two-way analysis of variance (ANOVA) when appropriate for analysis by GraphPad Prism (software version 5.0c). Results were considered as statistically significant at a P-value of less than 0.05. All statistical tests were two-sided.

4.3 RESULTS

Oncogenic *BRAF^{V600E}* mutation activates MAPK signal transduction in colonic crypts. To determine the impact of an oncogenic *BRAF^{V600E}* mutation alone on normal colonic mucosa, we generated the Braf-Lgr5 mice, in which the mutant *BRAF* gene expression is inducibly expressed only in cre-expressing Lgr5+ stem cells at the bottom of the intestinal crypts. At six weeks of age, Cre expression was induced in mice *via* two i.p. injections of tamoxifen (200mg/kg body weight in corn oil) within a timeframe of 72 hours. For control group, mice were injected with corn oil. Mice were sacrificed at age of 10 weeks, 14 weeks or 18 weeks. The body weight was measured at the time of sacrifice and colons were collected for further analysis **(Fig. 23)**. Four weeks after last injection, mice that received tamoxifen (TAM group) injections showed elevated protein levels of phosphor-Erk in colonic epithelium compared to mice in control group, indicating activation of MAPK signaling through oncogenic *BRAF^{V600E}* expression **(Fig. 24A)**. However, no significant difference in body weight was found between TAM groups and control groups **(Fig. 24B)**.

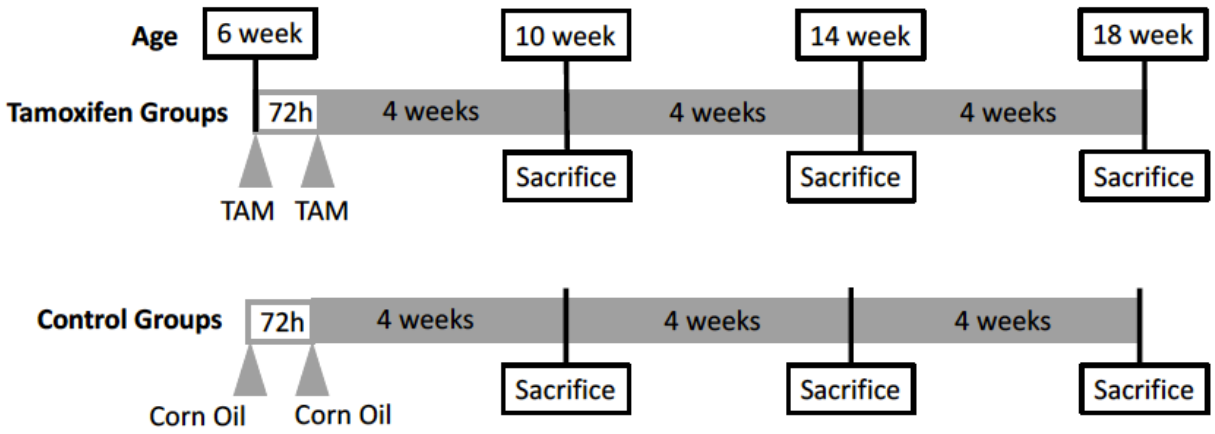
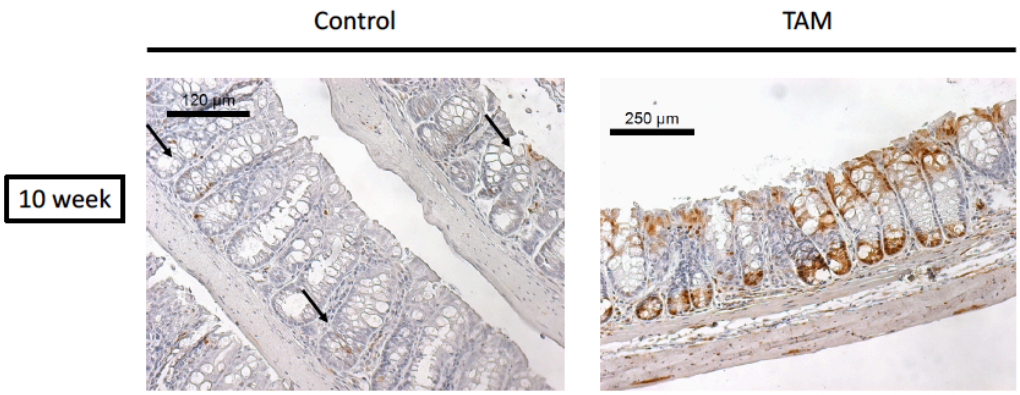


Figure 23. Study Design. At 6 weeks of age, a total of 60 LSL-Braf ^{V600E};Lgr5-EGFP-IRES-creERT2 mice (30 male and 30 female)were randomized and placed into 6 groups. In Tamoxifen groups (n=10 each group), mice received injection of tamoxifen (200mg/kg in 20mg/ml of corn oil, i.p.) twice in 72 hours, and sacrificed at 4weeks, 8 weeks and 12 weeks post injection. In Control groups (n=10 each group), mice received injection of corn oil (0.2ml, i.p.) twice in 72 hours, and sacrificed at 4weeks, 8 weeks and 12 weeks post injection.

A.


Control
TAM
10 week
120 µm
250 µm

B.

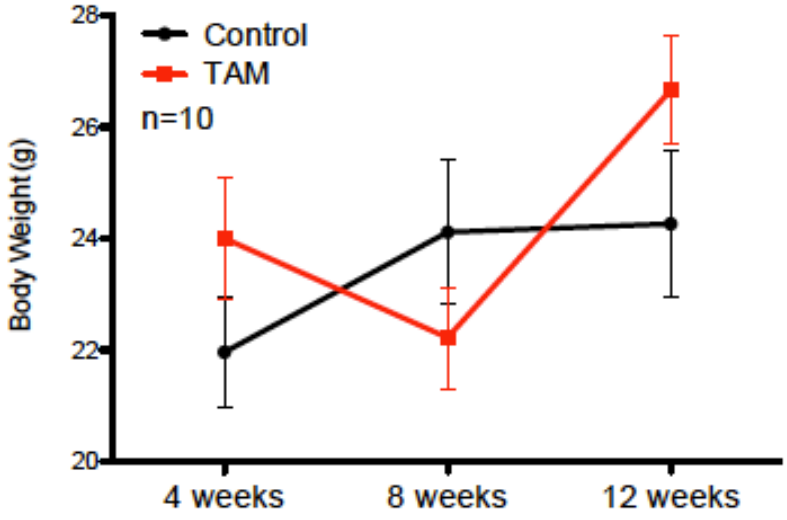
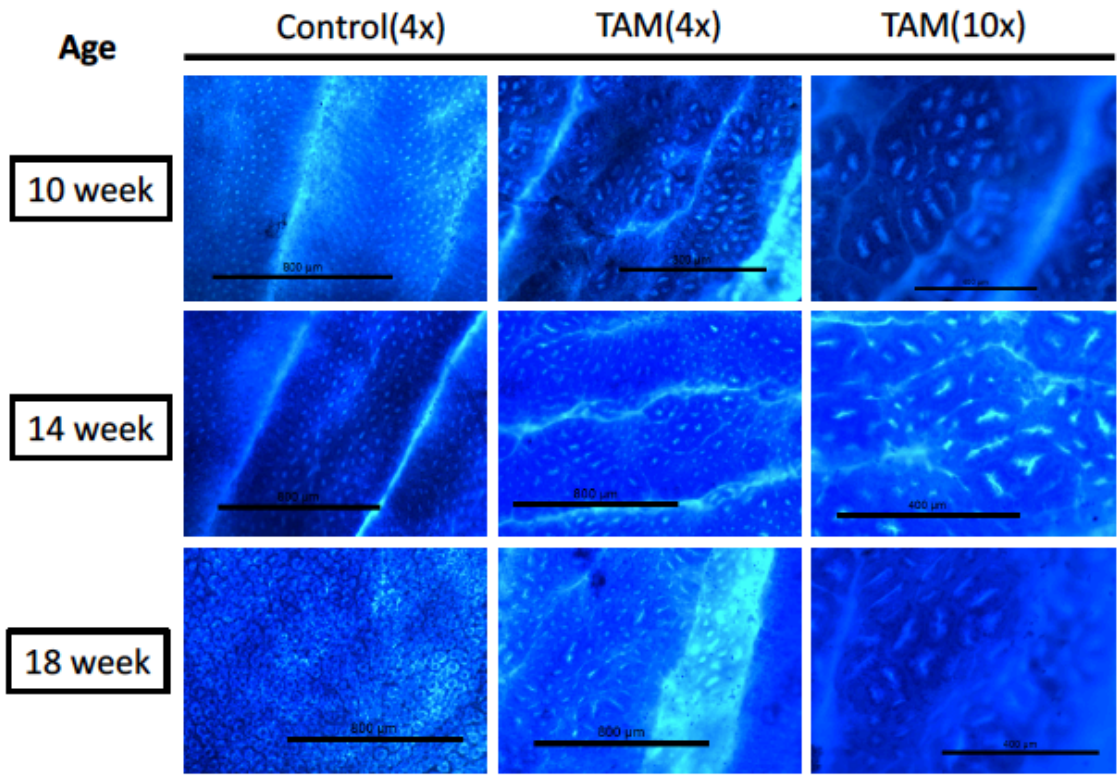

Control
TAM
n=10
Body Weight (g)
28
26
24
22
20
4 weeks
8 weeks
12 weeks

Figure 24. Oncogenic BRAF^{V600E} expression increases phospho-Erk levels in colonic crypts. At 6 weeks of age, Braf-Lgr5 mice were injected with either tamoxifen (200mg/kg) or vehicle control (corn oil) twice in 72 hours. (A) At 10 weeks of age, mouse colonic samples were collected and paraffin embedded. Phospho-Erk IHC staining (indicated by arrows) was performed to assess the status of MAPK signal transduction. (B) Mouse body weight was measured at 4 weeks, 8 weeks or 12 weeks after the last injection of tamoxifen (TAM) or vehicle control (control).

Oncogenic BRAF^{V600E} expression significantly increases the number of colonic ACFs in adult Braf-Lgr5 mice. To determine whether *BRAF^{V600E}* mutation alone could initiate colon tumorigenesis, colon tissues from mice treated with tamoxifen (or controls) were formalin-fixed and colonic epithelium was examined morphologically using methylene blue staining under a light microscope. Compared to the colonic samples from mice in the control group, mice injected with tamoxifen exhibit significant higher numbers of enlarged and darker stained aberrant crypts with slit-shaped luminal openings **(Fig. 25)**. The criteria for characterizing ACF has been described in our previous studies (246) . Importantly, mice sacrificed 3 months post tamoxifen-induced *BRAF^{V600E}* mutation (age of 18 weeks) showed an increase in the size of ACFs compared to mice in the other TAM groups, suggesting that the development of ACF upon oncogenic BRAF^{V600E} expression may be time-dependent **(Fig. 25A)**.

A.


Age
Control(4x)
TAM(4x)
TAM(10x)
10 week
14 week
18 week

B.

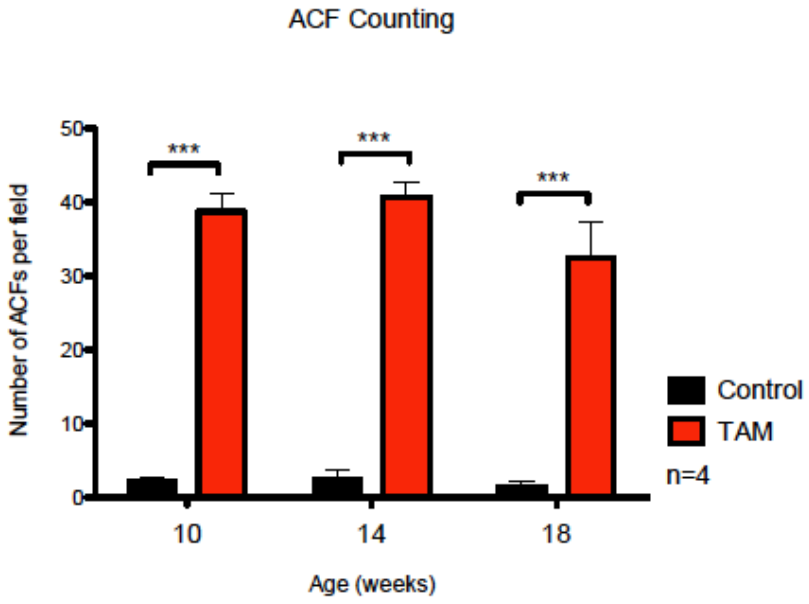

ACF Counting
Number of ACFs per field
50
40
30
20
10
0
***
***
***
10
14
18
Age (weeks)
Control
TAM
n=4

Figure 25. Oncogenic $BRAF^{V600E}$ expression induces development of ACF in the colonic epithelium. At 6 weeks of age, mice were injected twice with either tamoxifen (200mg/kg) or vehicle control (corn oil). At 10, 14 and 18 weeks of age, mice were sacrificed and colons were harvested and prepared for analysis as described under Material and Methods. (A) The morphology of crypts within the colonic mucosa was observed under a dissecting microscope using methylene blue staining. (B) The numbers of ACFs per field were counted in both controls and tamoxifen-treated mice.

Oncogenic BRAF^{V600E} expression induces colon epithelium hyperplasia in Braf-Lgr5 mice. The expression of the *BRAF^{V600E}* mutant gene in Lgr5+ cells led to sporadic crypt hyperplasia within the colon epithelium of tamoxifen-treated mice, as shown by both H&E staining and presence of the proliferation marker, Ki67. Histologically, affected crypts demonstrated significant elongation without dysplasia, as well as widen luminal openings and enrichment of goblet cells **(Fig. 26)**. In addition, sections of tamoxifen treated mice contain higher number of crypts per field than those of the control group. On the other hand, the active proliferating cells are observed in both the bottom and upper half of affected crypts from the TAM group, while in contrast, the cell proliferation is restricted within the base of the crypts in sections from control mice **(Fig. 27)**, suggesting increase in proliferation of colonic epithelial cells upon *BRAF^{V600E}* mutation.

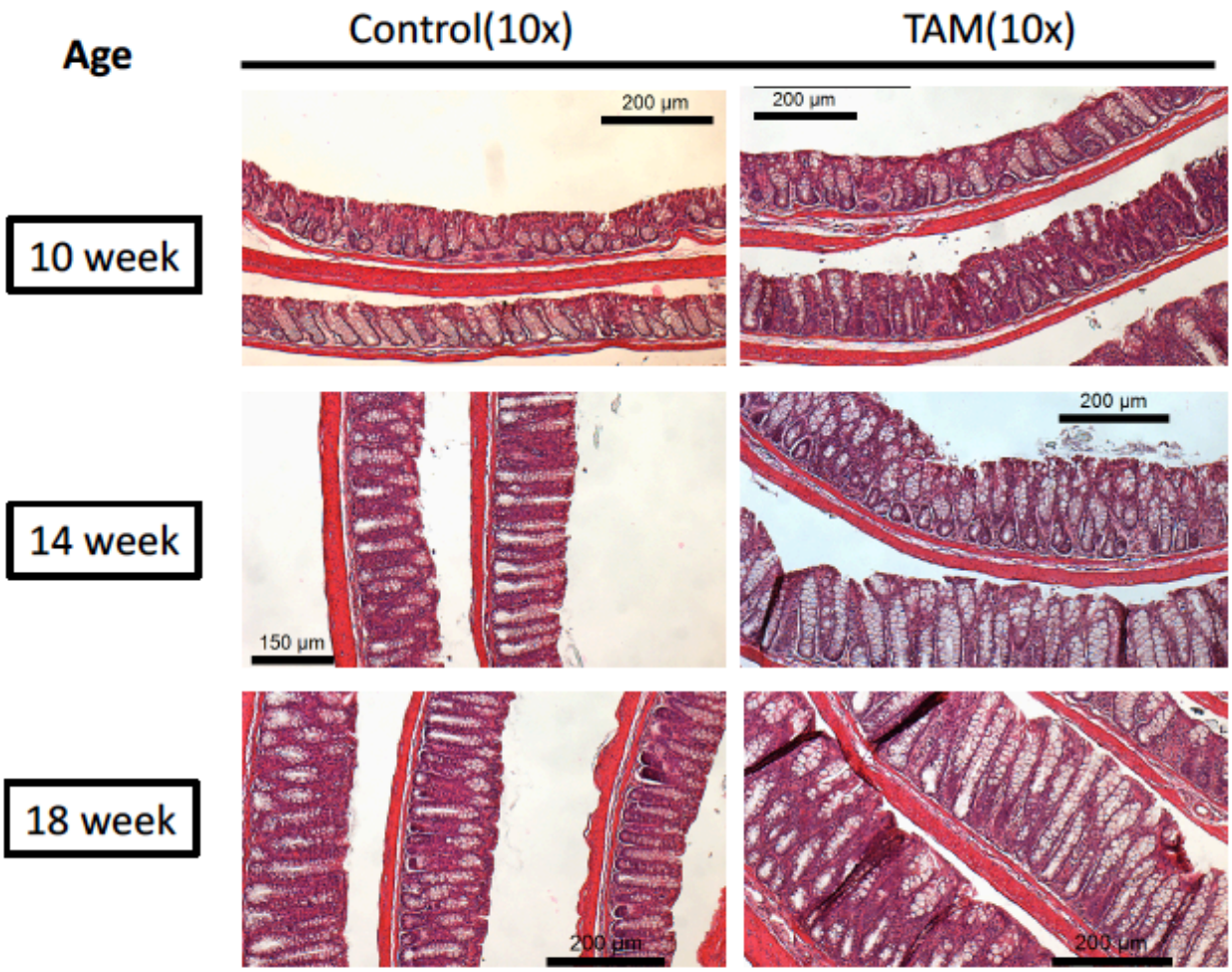


Figure 26. Oncogenic BRAF^{V600E} expression induces colonic hyperplasia in Braf-Lgr5 mice. At 6 weeks of age, mice were injected twice with either tamoxifen (200mg/kg) or vehicle control (corn oil). At 10, 14 and 18 weeks of age, mice were sacrificed and colons were harvested and prepared for analysis as described under Material and Methods Colonic hyperplasia was observed TAM group but not control group by H&E staining of paraffin sections.

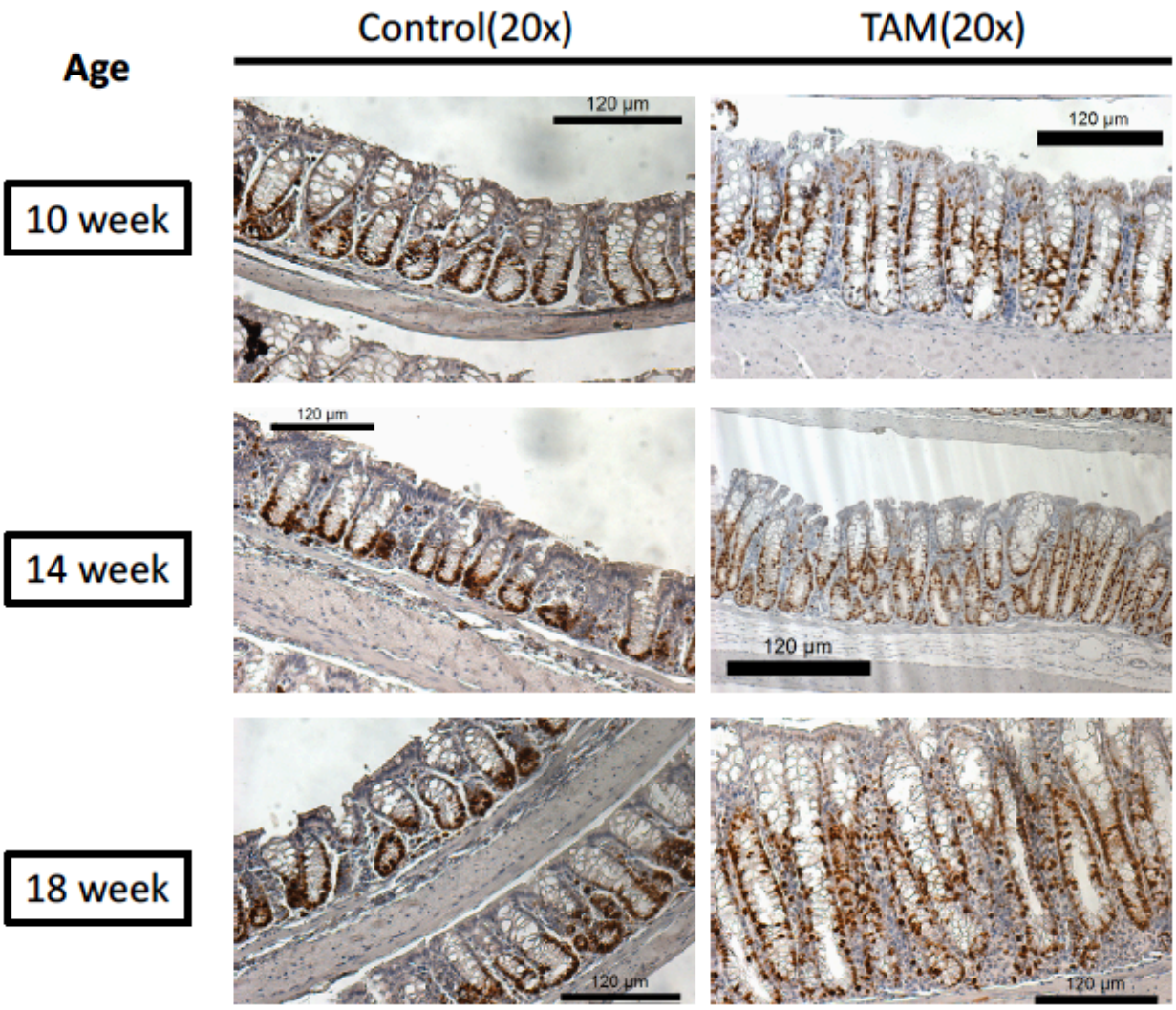
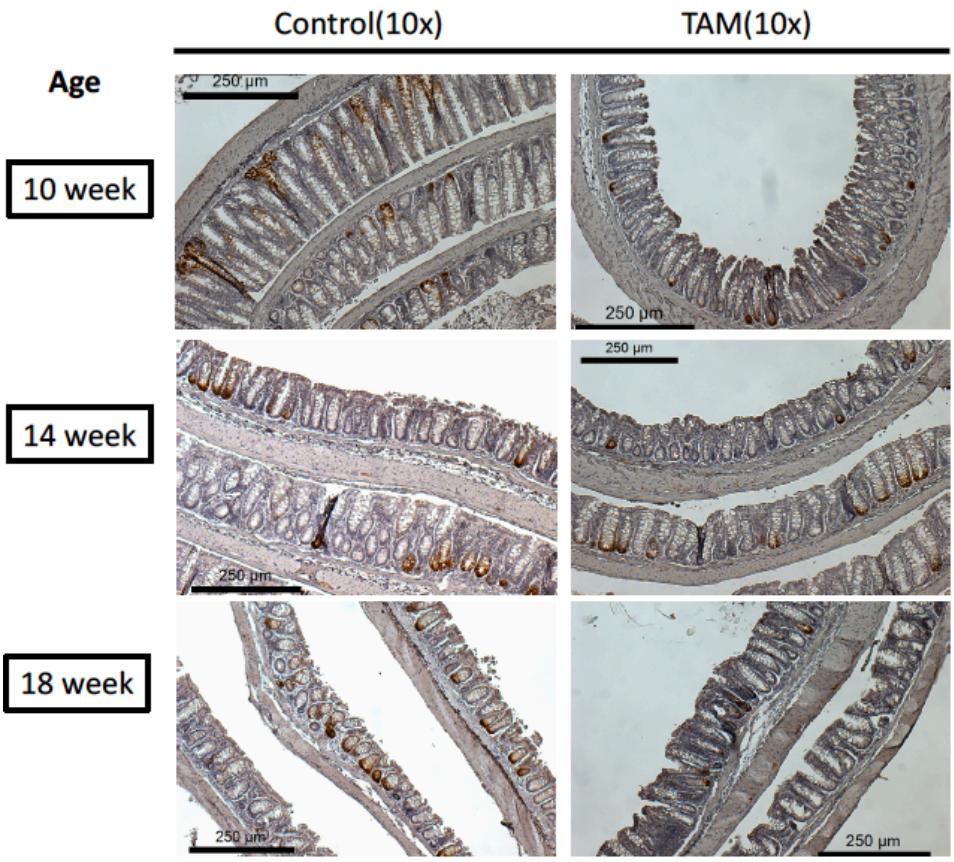


Figure 27. Oncogenic BRAF^{V600E} expression promotes proliferation of colonic epithelial cells in Braf-Lgr5 mice. At 6 weeks of age, mice were injected twice with either tamoxifen (200mg/kg) or vehicle control (corn oil). At 10, 14 and 18 weeks of age, mice were sacrificed and colons were harvested and prepared for analysis as described under Material and Methods. The proliferative cells in colonic crypts were visualized in both TAM and control mice by Ki67 IHC staining in paraffin sections.

Oncogenic BRAF^{V600E} expression causes a loss of intestinal stem cells. Since studies have shown that Lgr5 is exclusively expressed in intestinal stem cells (ISC) in SI and colon (and certain stem cells in other systems), expression of the *BRAF^{V600E}* mutant gene is limited to ISCs in our mouse model. To understand the potential effect of the *BRAF^{V600E}* gene mutation on affected ISCs and the hierarchy of affected crypts, we examined the number of colonic stem cells in both TAM-treated and control mice using GFP staining **(Fig. 28)**. Colon sections from mice treated with tamoxifen exhibited significantly less GFP+ staining per field compared to the control group, suggesting that activation of the *BRAF^{V600E}* gene mutation and an subsequent MAPK pathway activation, significantly decreased the number of Lgr5+ ISCs in mouse colon. This interesting finding is in consistent with the recent discovery by Riemer and colleagues (248) , who found that broad oncogenic *BRAF^{V600K}* mutation rapidly induced the depletion of ISC pool in B6 mice over 3-day time period.

A.


Control(10x)
TAM(10x)
Age
10 week
14 week
18 week
250 µm

B.

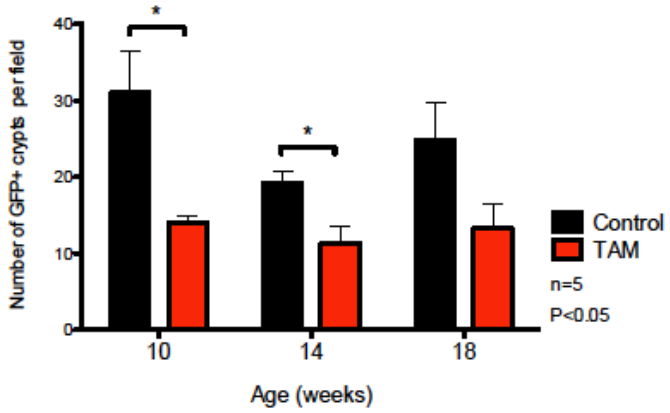

Number of GFP+ crypts per field
40
30
20
10
0
*
*
10
14
18
Age (weeks)
Control
TAM
n=5
P<0.05

Figure 28. Oncogenic $BRAF^{V600E}$ expression causes a loss of Lgr5+ cells. At 6 weeks of age, mice were injected twice with either tamoxifen (200mg/kg) or vehicle control (corn oil). At 10, 14 and 18 weeks of age, mice were sacrificed and colons were harvested and prepared for analysis as described under Material and Methods. (A) The Lgr5+ intestinal stem cells in colonic crypts were visualized in both TAM and control mice by GFP IHC staining. (B) The numbers of crypts containing GFP+ cells per field were counted in both control group and tamoxifen group.

4.4 DISCUSSION

The MAPK signaling pathway is an important mediator in colorectal tumorigenesis. Oncogenic activation of several key modulators in MAPK pathway, including KRAS and BRAF, has been found in human CRC patients. *KRAS* mutations are present in 50% of CRCs while *BRAF* mutations are found in approximately 10% CRC cases. Interestingly, the presence of *KRAS/NRAS* and *BRAF* mutations in human CRC is mutually exclusive, and has been associated with very different molecular pathways of CRC development. *KRAS* mutant CRCs often develop through the traditional "adenoma-carcinoma" pathway and also exhibit loss of *APC* and *TP53* gene, while BRAF mutant CRCs often develop through the alternative pathway – the "serrated" pathway and are associated with high degree of CpG island methylation (CIMP) and/or genome instability (MSI), which is possibly because the $BRAF^{V600E}$ mutation, leads to hyper-methylation of *MLH1* gene promoter and defects the DNA mismatch repair pathway (249) .

In the past decade, clinical studies have classified the *BRAF* mutant CRC as a distinct subtype with very unique clinical characteristics and clinical behavior (28) . *BRAF*-mutant colorectal tumors are primarily located in the proximal colon (right colon) with a mucinous, serrated and poorly differentiated histology. It is also shown that *BARF* mutations are more prevalent in female patients and those of advanced age. Moreover, patients diagnosed with *BRAF*-mutant CRC tend to have poorer prognosis and shorter overall survival time, compared to those with a wild-type *BRAF* gene (10.4months *vs* 34.7 months) (28) . These findings suggest that oncogenic *BRAF* mutations may be associated with the tumorigenesis and clinical outcome of proximal, serrated CRCs.

Recent advances in gene sequencing and genetic modification technologies have facilitated the research on *BRAF* mutations in human CRC. It has been confirmed that $BRAF^{V600E}$ mutation, which is an oncogenic T1799A transversion that encodes constitutively activated BRAF protein, accounts for approximately 90% of *BRAF* mutantions in CRC. In 2013, Rad and colleagues provide the first evidence that $BRAF^{V600E}$ mutation drives intestinal adenoma development using an inducible $BRAF^{V600E}$ knock-in mouse model (244) . Through the broad expression of $BRAF^{V637E}$, the murine counterpart of human $BRAF^{V600E}$, in epithelia of small intestine and colon, they showed $BRAF^{V637E}$ mutation could initiate the formation of serrated colorectal tumor, which could be facilitated to generate an advanced carcinoma by further loss of the tumor suppressor gene, p16. However, $BRAF^{V600E}$ mutations in all intestinal epithelial cells lack translational relevance to the clinical development of serrated CRC in humans, which often develops through somatically acquired sporadic mutations.

In our study, we cross the *LSL-*$BRAF^{V600E}$ mutant mice with *Lgr5-EGFP-IRES-creERT2* mice and generate a mouse model in which the BRAF mutation can only be induced in Lgr5+ cells. Lgr5 has been found exclusively expressed in the ISCs at the base of intestinal crypts, which leads to the wide usage of Lgr5 as imperative ISC marker in numerous stem cell studies (189) . In 2007, Hans Clevers and colleagues generated the first Lgr5 -positive cell specific LacZ expressing mouse model using inducible Cre gene expression system (*Lgr5-EGFP-IRES-creERT2*) to identify and trace the lineage of the Lgr5 expressing ISCs. In our study, by crossing these Lgr5 mice with *LSL-*$BRAF^{V600E}$ mice (245) , we generated the Lgr5-driven BRAF mutation (Lgr5-BRAF) mouse line to induce the expression of mutant BRAF using tamoxifen-mediated Cre expression and activate the MAPK signaling in affected crypts. Instead of adenoma

development induced by germline BRAF mutation, we observed the generation of numerous colonic ACFs induced by BRAF activation in Lgr5+ ISCs at different time points (4 weeks, 8 weeks and 12 weeks) after tamoxifen injections. Based on the histology test (H&E staining), we also observed sporadic hyperplasia of colonic epithelium, which is characterized by elongated crypt length, widen crypt openings and loss of goblet cell differentiation with more proliferative cell (Ki67+ staining) towards the upper side of crypts. Clearly, our results suggest that sporadic $BRAF^{V600E}$ mutation along in ISCs can initiate the hyperplasia in colon mucosa. However, we didn't observe dysplasia (characterized by loss of polarity, dark, elongated nuclei) in our tissue sections. According to the findings from other studies, to achieve the malignant transformation of pre-neoplastic lesions, a second step such as loss of tumor suppressor gene is needed.

Another interesting finding in our study is the observed loss of GFP+ (Lgr+) staining in the colon of mice following activation of $BRAF^{V600E}$ expression. It is believed that most hyperplastic lesions in colonic mucosa, including ACF and hyperplastic polyps, do not have malignant potential. One hypothesis is that the activation of $BRAF^{V600E}$, or activation of MAPK signaling alone, could directly affect metabolism within affected cells and induce the elimination of oncogene-altered crypts *via* the process of oncogene induced senescence, OIS. Recently, a study by Riemer and colleagues (248) showed that inducible, transgenic expression of oncogenic $BRAF^{V600E}$ strongly activates MAPK signaling and induces the differentiation of ISCs to transiently amplifying (TA) progenitor cells, thereby promoting the permanent differentiation of ISCs and affected crypts in the mouse intestine. They also found that the activated BRAF could modulate the expression of cell fate-associated genes *in vitro*, which might explain the impact of BRAF mutations *in vivo*. However, because the $BRAF^{V600E}$ knock-in mouse was

engineered in the embryonic stem cells and caused global intestinal hyperplasia, all mice were sacrificed 4 days after its activation. Thus, it was not possible to observed the long-term effect of a *BRAF* mutation on colonic epithelium *in vivo*. In our study, we were able to maintain the mice for up to 3 months after induction (or even longer) and observed the loss of Lgr5+ cells (in which the $BRAF^{V600E}$ gene was expressed). Our results are consistent with Riemer's findings and could serve as evidence for BRAF/MAPK signaling activation-induced loss of ISCs in mouse intestine.

In summary, we have established a stem cell-specific, inducible $BRAF^{V600E}$ knock-in mouse model and provide preliminary evidence for the promotional effect of $BRAF^{V600E}$ mutation on early stages of colonic neoplasia *in vivo*. We also uncovered an effect of the $BRAF^{V600E}$ mutation on cell fate of ISCs that have sustained BRAF activation. We believe that these changes may explain the potential failure of most of these hyperplastic ACF to progress to advanced neoplasia. Further investigation is needed to better define the molecular mechanisms of the oncogenic *BRAF* mutation and its direct effects on ISCs to gain a better understanding of how crypts evolve during early carcinogenesis. On the other hand, our study provides a valuable model for further studies with a goal towards developing early preventive strategies for proximal serrated CRC, thereby improving the outlook of affected patients.

CHAPTER 5

SUMMARY AND CONCLUSIONS

During the clinical treatment of CRC, the development of acquired drug resistance in tumors after long-term exposure to chemotherapeutic agents can significantly decrease the response rate of CRC to chemotherapy, leading to tumor recurrence and eventually cancer-related deaths (160) . In addition, acquired resistance suppresses the efficacy of conventional chemotherapeutic reagents, such as 5-FU and oxaliplatin, and limits their clinical application. In fact, almost 90% of advanced CRC patients develop acquired resistance to oxaliplatin after approximately 6 months of treatment, which decreases the efficacy of oxaliplatin and eventually leads to tumor metastases and patient death (199) .

Oxaliplatin is a third generation platinum-based drug. Different from other platinum-based drugs (i.e. cisplatin), oxaliplatin features the bidentate ligand in structure and act through different mechanism (unknown) to achieve effective cytotoxicity on colorectal tumor, therefore remains the only FDA-approved first-line platinum compound for CRC (202) . Although several common mechanisms, including modulation of cell apoptosis, deregulation of DNA repair pathways and multidrug resistance (MDR) mechanisms, have been shown as involved in platinum derivative resistance, the key mechanisms of oxaliplatin remains unknown (219) . Recently, several studies have demonstrated the synergistic effects of COX-2 inhibition on

improving the efficacy of oxaliplatin in CRC cell culture and preclinical colon cancer models, suggesting that COX-2 pathway might be involved in the mechanism of oxaliplatin resistance.

The main product of COX-2 pathway, the bioactive pro-inflammatory lipid PGE_2, has been extensively studied for its critical promoting effect on colorectal tumorigenesis in the past three decades. The chemo-preventive benefits of drugs targeting PGE_2, such as NSAIDs and celecoxib, have been confirmed in several large-scale clinical trials (105) . Recently, PGE_2 has been directly associated with chemoresistance in bladder cancer and breast cancer *in vivo* and *in vitro* (165,167) . Our findings have revealed the direct link between deregulation of PGE_2 metabolism and oxaliplatin resistance in CRC, providing the first evidence of PGE_2 being a potent modulator of CRC chemoresistance. In addition, we screened the downstream GPCRs for PGE_2 and confirmed the PGE_2/EP4 receptor signaling as the pathway mediating oxaliplatin resistance. Since PGE_2 affords various critical biological and physiological functions in human body, small molecule antagonist could target EP4 receptor specifically to maintain the beneficial effect on oxaliplatin efficacy while circumventing side effects in clinical use.

As we investigated the impact of EP4 inhibition on human CRC cells, we found that oxaliplatin resistant cells showed increased sensitivity to oxaliplatin upon EP4 blockade, while oxaliplatin cytotoxicity on parental cells was not affected by EP4 inhibition. To further understand the molecular mechanisms of "EP4 addiction" in these resistant cells, we evaluated several mechanisms recently shown to be involved in CRC chemoresistance, including cancer stem cells and modulation of oxidative stress. We found that both mechanisms have been deregulated in favor of oxaliplatin resistance in our HT29 OXR cells, while inhibition of EP4

signaling was able to reverse the de-regulation and suppress oxaliplatin resistance. For example, EP4 blockade significantly reduced the expression of CSC markers CD44 and CD133 in OXR cells and induced the decrease of tumor sphere formation by CSC subpopulation in OXR cells. On the other hand, ROS-mediated cell apoptosis has been shown as key mechanism of oxaliplatin cytotoxicity. We showed that inhibition of EP4 significantly reduced the cellular level and utility of GSH, therefore reduced the clearance of ROS in HT29 OXR cells and induce cell death. It is worth mentioning that the increase in GSH has been shown as a major contributing factor to drug resistance in several malignancies, including ovarian cancer, prostate cancer and melanoma, and GSH depletion has been tested as adjuvant therapy in several clinical trials (234) . Our findings suggest that GSH also plays an important role in mediating oxaliplatin resistance in human CRC. Further studies are needed to decipher the molecular mechanism of GSH regulation by EP4 signaling and assess the effect of EP4 selective antagonist on oxaliplatin resistance in preclinical models and clinical trials.

For decades, the development of human CRC has been assumed to follow the "adenoma to carcinoma" sequence through the classic CIN pathway, and all hyperplastic the lesions in colon mucosa, such including hyperplastic ACFs or polyps, are considered as benign and don't have malignant potential (93) . This opinion has been challenged by the emerging findings of serrated CRC, which develops through a "serrated" pathway and exhibits distinct molecular and histological features, including hyper-methylation of CpG islands, mutations in *BRAF* or *KRAS* instead of *APC* gene, and "saw-tooth" like serrated crypts in polyps and adenomas (236) . In addition, some hyperplastic ACFs/polyps have been found to carry oncogenic *BRAF* mutations and exhibit serrated crypt phenotype, suggesting a strong association between these early lesions

with serrated CRC. Nowadays, all ACFs have been regarded as biomarkers of increased CRC risk and those with dysplastic features or acquired advanced genetic alterations are considered as precursors of CRC with malignant potential. Especially, the ACFs at the proximal (right-sided) colon are more frequently associated with proximal CRCs and could serve as potent target for proximal CRC prevention (93) . However, till now a genetic engineered mouse model is yet to generated for studying proximal premalignant lesions and developing preventive strategies for proximal CRC.

Recent studies have suggested that BRAF, a key modulator of the MAPK signaling pathway, is highly associated with colorectal tumorigenesis (240) . Oncogenic *BRAF* mutations are present in approximately 10% CRC cases and have been found mutually exclusive with KRAS mutations. Interestingly, *BRAF* mutations are extremely rare in left-sided colon cancer and rectal cancers, instead they have been found primarily in proximal cancer, usually associated with high degree of CpG island methylation (CIMP) and/or genome instability (MSI) (236) . These proximal colon tumors usually develop through the "serrated" pathway and exhibit mucinous histology with serrated and poorly differentiated colonic epithelium. Other clinical studies also associate *BRAF* mutant CRC patients with older age, female gender and poorer prognosis compared to those with wild-type *BRAF* gene (239) . These findings suggest that oncogenic *BRAF* mutations might serve as a driver during early tumorigenesis of proximal CRC and affect the prognosis of proximal CRC patients.

Recent studies have shown that broad expression of oncogenic $BRAF^{V600E}$ in intestinal epithelial cells could initiate tumorigenesis and form serrated adenomas in mouse model (244) .

To mimic the sporadic development of *BRAF* mutant CRC and study the impact of $BRAF^{V600E}$ mutations on early tumorigenesis of proximal CRC, we generate the *LSL-*$BRAF^{V600E}$; *Lgr5-EGFP-IRES-creERT2* mice, a novel GEMM in which oncogenic $BRAF^{V600E}$ mutation is inducible in Lgr5+ ISCs at the crypt base in small intestine and colon, *via* tamoxifen-induced cre expression. We found that expression of Mutant $BRAF^{V600E}$ promotes the development of generalized hyperplastic ACFs in large intestine. The hyperplasia in colon epithelium was further confirmed by epithelial cell hyper-proliferation throughout the affect colonic crypts indicated by Ki67+ staining. However, we didn't observe formation of adenoma or dysplasia in *BRAF* mutant mouse colon. As previous studies have shown that the generation of advanced malignant lesions requires further genetic alterations such as loss of *TP53* or *p16*, it is possible that additional silencing of tumor suppressor genes are needed to get dysplasia ACFs or SSAs. Interestingly, we also found that expression of $BRAF^{V600E}$ and activation of MAPK signaling is associated with decreases in Lgr5+ ISCs in *BRAF* mutant mouse. This finding is in consistent with the recent discovery by Riemer and colleagues, who found that inducible expression of oncogenic $BRAF^{V600E}$ for 4 days could activate MAPK pathway and promotes the differentiation of ISCs in mouse intestine, therefore shrink the ISC pool during serrated tumor progression (250) . This effect of mutant BRAF could be antagonized by β–catenin signaling, which has been shown to encourage ISC identity. Our results confirmed the impact of BRAF mutation on the cell fate of ISCs; however, by the end of 3 months post BRAF activation, we still observed plenty of Lgr5+ ISCs in *BRAF* mutant mice. Further investigations are required to understand the molecular mechanisms underlying the maintenance of these *BRAF* mutant ISCs and discover potential targets to prevent the transformation from hyperplastic polyps to SSAs during serrated CRC progression, therefore improve the prognosis of affected patients.

REFERENCES

(1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016 Jan-Feb;66(1):7-30.

(2) Siegel R, Desantis C, Jemal A. Colorectal cancer statistics, 2014. CA Cancer J Clin 2014 Mar-Apr;64(2):104-117.

(3) Amersi F, Agustin M, Ko CY. Colorectal cancer: epidemiology, risk factors, and health services. Clin Colon Rectal Surg 2005 Aug;18(3):133-140.

(4) Winawer SJ, Zauber AG. The advanced adenoma as the primary target of screening. Gastrointest Endosc Clin N Am 2002 Jan;12(1):1-9, v.

(5) Grady WM. Genetic testing for high-risk colon cancer patients. Gastroenterology 2003 May;124(6):1574-1594.

(6) Johnson CM, Wei C, Ensor JE, Smolenski DJ, Amos CI, Levin B, et al. Meta-analyses of colorectal cancer risk factors. Cancer Causes Control 2013 Jun;24(6):1207-1222.

(7) Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterology 2010 Jun;138(6):2044-2058.

(8) Grady WM, Markowitz SD. Hereditary colon cancer genes. Methods Mol Biol 2003;222:59-83.

(9) Stoffel E, Mukherjee B, Raymond VM, Tayob N, Kastrinos F, Sparr J, et al. Calculation of risk of colorectal and endometrial cancer among patients with Lynch syndrome. Gastroenterology 2009 Nov;137(5):1621-1627.

(10) Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med 2003 Mar 6;348(10):919-932.

(11) Bernstein CN, Blanchard JF, Kliewer E, Wajda A. Cancer risk in patients with inflammatory bowel disease: a population-based study. Cancer 2001 Feb 15;91(4):854-862.

(12) Bansal P, Sonnenberg A. Risk factors of colorectal cancer in inflammatory bowel disease. Am J Gastroenterol 1996 Jan;91(1):44-48.

(13) Butler LM, Sinha R, Millikan RC, Martin CF, Newman B, Gammon MD, et al. Heterocyclic amines, meat intake, and association with colon cancer in a population-based study. Am J Epidemiol 2003 Mar 1;157(5):434-445.

(14) Warren GW, Cummings KM. Tobacco and lung cancer: risks, trends, and outcomes in patients with cancer. Am Soc Clin Oncol Educ Book 2013:359-364.

(15) Slattery ML, Potter JD, Friedman GD, Ma KN, Edwards S. Tobacco use and colon cancer. Int J Cancer 1997 Jan 27;70(3):259-264.

(16) American Cancer Society. Colorectal Cancer Facts & Figures 2014-2016. American Cancer Society 2014.

(17) Hari DM, Leung AM, Lee JH, Sim MS, Vuong B, Chiu CG, et al. AJCC Cancer Staging Manual 7th edition criteria for colon cancer: do the complex modifications improve prognostic assessment? J Am Coll Surg 2013 Aug;217(2):181-190.

(18) Iacopetta B. Are there two sides to colorectal cancer? Int J Cancer 2002 Oct 10;101(5):403-408.

(19) Nawa T, Kato J, Kawamoto H, Okada H, Yamamoto H, Kohno H, et al. Differences between right- and left-sided colon cancer in patient characteristics, cancer morphology and histology. J Gastroenterol Hepatol 2008 Mar;23(3):418-423.

(20) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011 Mar 4;144(5):646-674.

(21) Clevers H. Wnt/beta-catenin signaling in development and disease. Cell 2006 Nov 3;127(3):469-480.

(22) Colussi D, Brandi G, Bazzoli F, Ricciardiello L. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. Int J Mol Sci 2013 Aug 7;14(8):16365-16385.

(23) Kwon C, Cheng P, King IN, Andersen P, Shenje L, Nigam V, et al. Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. Nat Cell Biol 2011 Aug 14;13(10):1244-1251.

(24) Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res 1998 Mar 15;58(6):1130-1134.

(25) Firestein R, Bass AJ, Kim SY, Dunn IF, Silver SJ, Guney I, et al. CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. Nature 2008 Sep 25;455(7212):547-551.

(26) Firestein R, Shima K, Nosho K, Irahara N, Baba Y, Bojarski E, et al. CDK8 expression in 470 colorectal cancers in relation to beta-catenin activation, other molecular alterations and patient survival. Int J Cancer 2010 Jun 15;126(12):2863-2873.

(27) Zenonos K, Kyprianou K. RAS signaling pathways, mutations and their role in colorectal cancer. World J Gastrointest Oncol 2013 May 15;5(5):97-101.

(28) Clarke CN, Kopetz ES. BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies. J Gastrointest Oncol 2015 Dec;6(6):660-667.

(29) Guerrero S, Casanova I, Farre L, Mazo A, Capella G, Mangues R. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation or proto-oncogene overexpression. Cancer Res 2000 Dec 1;60(23):6750-6756.

(30) Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Willson JK, et al. P53 Gene Mutations Occur in Combination with 17p Allelic Deletions as Late Events in Colorectal Tumorigenesis. Cancer Res 1990 Dec 1;50(23):7717-7722.

(31) Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.

(32) Li WQ, Kawakami K, Ruszkiewicz A, Bennett G, Moore J, Iacopetta B. BRAF mutations are associated with distinctive clinical, pathological and molecular features of colorectal cancer independently of microsatellite instability status. Mol Cancer 2006 Jan 10;5:2.

(33) Chan TL, Zhao W, Leung SY, Yuen ST, Cancer Genome Project. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res 2003 Aug 15;63(16):4878-4881.

(34) Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, et al. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut 2004 Aug;53(8):1137-1144.

(35) Ogino S, Brahmandam M, Cantor M, Namgyal C, Kawasaki T, Kirkner G, et al. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Mod Pathol 2006 Jan;19(1):59-68.

(36) Kalady MF, Dejulius KL, Sanchez JA, Jarrar A, Liu X, Manilich E, et al. BRAF mutations in colorectal cancer are associated with distinct clinical characteristics and worse prognosis. Dis Colon Rectum 2012 Feb;55(2):128-133.

(37) Kim JH, Bae JM, Cho NY, Kang GH. Distinct features between MLH1-methylated and unmethylated colorectal carcinomas with the CpG island methylator phenotype: implications in the serrated neoplasia pathway. Oncotarget 2016 Mar 22;7(12):14095-14111.

(38) Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, et al. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res 2003 Sep 1;63(17):5209-5212.

(39) Samowitz WS, Sweeney C, Herrick J, Albertsen H, Levin TR, Murtaugh MA, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. Cancer Res 2005 Jul 15;65(14):6063-6069.

(40) Sinicrope FA, Shi Q, Smyrk TC, Thibodeau SN, Dienstmann R, Guinney J, et al. Molecular markers identify subtypes of stage III colon cancer associated with patient outcomes. Gastroenterology 2015 Jan;148(1):88-99.

(41) Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. J Clin Oncol 2010 Feb 20;28(6):1075-1083.

(42) Deming DA, Leystra AA, Nettekoven L, Sievers C, Miller D, Middlebrooks M, et al. PIK3CA and APC mutations are synergistic in the development of intestinal cancers. Oncogene 2014 Apr 24;33(17):2245-2254.

(43) Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 2006 May 25;441(7092):424-430.

(44) Perron MP, Provost P. Protein interactions and complexes in human microRNA biogenesis and function. Front Biosci 2008 Jan 1;13:2537-2547.

(45) Rajewsky N. microRNA target predictions in animals. Nat Genet 2006 Jun;38 Suppl:S8-13.

(46) Liu W, Mao SY, Zhu WY. Impact of tiny miRNAs on cancers. World J Gastroenterol 2007 Jan 28;13(4):497-502.

(47) Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 2003 Oct;1(12):882-891.

(48) Bandres E, Cubedo E, Agirre X, Malumbres R, Zarate R, Ramirez N, et al. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer 2006 Jul 19;5:29.

(49) Motoyama K, Inoue H, Takatsuno Y, Tanaka F, Mimori K, Uetake H, et al. Over- and under-expressed microRNAs in human colorectal cancer. Int J Oncol 2009 Apr;34(4):1069-1075.

(50) Bovell LC, Shanmugam C, Putcha BD, Katkoori VR, Zhang B, Bae S, et al. The prognostic value of microRNAs varies with patient race/ethnicity and stage of colorectal cancer. Clin Cancer Res 2013 Jul 15;19(14):3955-3965.

(51) Gascard P, Tlsty TD. Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. Genes Dev 2016 May 1;30(9):1002-1019.

(52) Ricci-Vitiani L, Fabrizi E, Palio E, De Maria R. Colon cancer stem cells. J Mol Med (Berl) 2009 Nov;87(11):1097-1104.

(53) Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005 Dec 22;353(25):2654-2666.

(54) Ostman A, Augsten M. Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. Curr Opin Genet Dev 2009 Feb;19(1):67-73.

(55) Togo S, Polanska UM, Horimoto Y, Orimo A. Carcinoma-associated fibroblasts are a promising therapeutic target. Cancers (Basel) 2013 Jan 31;5(1):149-169.

(56) Peddareddigari VG, Wang D, Dubois RN. The tumor microenvironment in colorectal carcinogenesis. Cancer Microenviron 2010 Mar 5;3(1):149-166.

(57) Wen S, Niu Y, Yeh S, Chang C. BM-MSCs promote prostate cancer progression via the conversion of normal fibroblasts to cancer-associated fibroblasts. Int J Oncol 2015 Aug;47(2):719-727.

(58) Quante M, Tu SP, Tomita H, Gonda T, Wang SS, Takashi S, et al. Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. Cancer Cell 2011 Feb 15;19(2):257-272.

(59) Xiong Y, McDonald LT, Russell DL, Kelly RR, Wilson KR, Mehrotra M, et al. Hematopoietic stem cell-derived adipocytes and fibroblasts in the tumor microenvironment. World J Stem Cells 2015 Mar 26;7(2):253-265.

(60) Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res 2007 Nov 1;67(21):10123-10128.

(61) Shiga K, Hara M, Nagasaki T, Sato T, Takahashi H, Takeyama H. Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. Cancers (Basel) 2015 Dec 11;7(4):2443-2458.

(62) Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest 2002 Aug;110(3):341-350.

(63) Kalluri R. The biology and function of fibroblasts in cancer. Nat Rev Cancer 2016 Aug 23;16(9):582-598.

(64) Tommelein J, Verset L, Boterberg T, Demetter P, Bracke M, De Wever O. Cancer-associated fibroblasts connect metastasis-promoting communication in colorectal cancer. Front Oncol 2015 Mar 23;5:63.

(65) Hawinkels LJ, Paauwe M, Verspaget HW, Wiercinska E, van der Zon JM, van der Ploeg K, et al. Interaction with colon cancer cells hyperactivates TGF-beta signaling in cancer-associated fibroblasts. Oncogene 2014 Jan 2;33(1):97-107.

(66) Berdiel-Acer M, Sanz-Pamplona R, Calon A, Cuadras D, Berenguer A, Sanjuan X, et al. Differences between CAFs and their paired NCF from adjacent colonic mucosa reveal functional heterogeneity of CAFs, providing prognostic information. Mol Oncol 2014 Oct;8(7):1290-1305.

(67) Wikberg ML, Edin S, Lundberg IV, Van Guelpen B, Dahlin AM, Rutegard J, et al. High intratumoral expression of fibroblast activation protein (FAP) in colon cancer is associated with poorer patient prognosis. Tumour Biol 2013 Apr;34(2):1013-1020.

(68) Huijbers A, Tollenaar RA, v Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol 2013 Jan;24(1):179-185.

(69) Henry LR, Lee HO, Lee JS, Klein-Szanto A, Watts P, Ross EA, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. Clin Cancer Res 2007 Mar 15;13(6):1736-1741.

(70) Rasanen K, Virtanen I, Salmenpera P, Grenman R, Vaheri A. Differences in the nemosis response of normal and cancer-associated fibroblasts from patients with oral squamous cell carcinoma. PLoS One 2009 Sep 1;4(9):e6879.

(71) Sanchez-Lopez E, Flashner-Abramson E, Shalapour S, Zhong Z, Taniguchi K, Levitzki A, et al. Targeting colorectal cancer via its microenvironment by inhibiting IGF-1 receptor-insulin receptor substrate and STAT3 signaling. Oncogene 2016 May 19;35(20):2634-2644.

(72) Vermeulen L, De Sousa E Melo F, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 2010 May;12(5):468-476.

(73) Li F, Zhu YT. HGF-activated colonic fibroblasts mediates carcinogenesis of colonic epithelial cancer cells via PKC-cMET-ERK1/2-COX-2 signaling. Cell Signal 2015 Apr;27(4):860-866.

(74) Nagasaki T, Hara M, Nakanishi H, Takahashi H, Sato M, Takeyama H. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. Br J Cancer 2014 Jan 21;110(2):469-478.

(75) Cheng M, Ho S, Yoo JH, Tran DH, Bakirtzi K, Su B, et al. Cathelicidin suppresses colon cancer development by inhibition of cancer associated fibroblasts. Clin Exp Gastroenterol 2014 Dec 17;8:13-29.

(76) Li M, Li M, Yin T, Shi H, Wen Y, Zhang B, et al. Targeting of cancerassociated fibroblasts enhances the efficacy of cancer chemotherapy by regulating the tumor microenvironment. Mol Med Rep 2016 Mar;13(3):2476-2484.

(77) Hu Y, Yan C, Mu L, Huang K, Li X, Tao D, et al. Fibroblast-Derived Exosomes Contribute to Chemoresistance through Priming Cancer Stem Cells in Colorectal Cancer. PLoS One 2015 May 4;10(5):e0125625.

(78) Binefa G, Rodriguez-Moranta F, Teule A, Medina-Hayas M. Colorectal cancer: from prevention to personalized medicine. World J Gastroenterol 2014 Jun 14;20(22):6786-6808.

(79) Giovannucci E. Modifiable risk factors for colon cancer. Gastroenterol Clin North Am 2002 Dec;31(4):925-943.

(80) Ciccolallo L, Capocaccia R, Coleman MP, Berrino F, Coebergh JW, Damhuis RA, et al. Survival differences between European and US patients with colorectal cancer: role of stage at diagnosis and surgery. Gut 2005 Feb;54(2):268-273.

(81) Kahi CJ, Imperiale TF, Juliar BE, Rex DK. Effect of screening colonoscopy on colorectal cancer incidence and mortality. Clin Gastroenterol Hepatol 2009 Jul;7(7):770-5; quiz 711.

(82) Rabeneck L, Paszat LF, Saskin R, Stukel TA. Association between colonoscopy rates and colorectal cancer mortality. Am J Gastroenterol 2010 Jul;105(7):1627-1632.

(83) Rockey DC, Paulson E, Niedzwiecki D, Davis W, Bosworth HB, Sanders L, et al. Analysis of air contrast barium enema, computed tomographic colonography, and colonoscopy: prospective comparison. Lancet 2005 Jan 22-28;365(9456):305-311.

(84) Baxter NN, Warren JL, Barrett MJ, Stukel TA, Doria-Rose VP. Association between colonoscopy and colorectal cancer mortality in a US cohort according to site of cancer and colonoscopist specialty. J Clin Oncol 2012 Jul 20;30(21):2664-2669.

(85) Burch JA, Soares-Weiser K, St John DJ, Duffy S, Smith S, Kleijnen J, et al. Diagnostic accuracy of faecal occult blood tests used in screening for colorectal cancer: a systematic review. J Med Screen 2007;14(3):132-137.

(86) Pignone M, Campbell MK, Carr C, Phillips C. Meta-analysis of dietary restriction during fecal occult blood testing. Eff Clin Pract 2001 Jul-Aug;4(4):150-156.

(87) Notterman DA, Alon U, Sierk AJ, Levine AJ. Transcriptional gene expression profiles of colorectal adenoma, adenocarcinoma, and normal tissue examined by oligonucleotide arrays. Cancer Res 2001 Apr 1;61(7):3124-3130.

(88) Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME, Colorectal Cancer Study Group. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. N Engl J Med 2004 Dec 23;351(26):2704-2714.

(89) Ahlquist DA, Sargent DJ, Loprinzi CL, Levin TR, Rex DK, Ahnen DJ, et al. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. Ann Intern Med 2008 Oct 7;149(7):441-50, W81.

(90) Link A, Balaguer F, Shen Y, Nagasaka T, Lozano JJ, Boland CR, et al. Fecal MicroRNAs as novel biomarkers for colon cancer screening. Cancer Epidemiol Biomarkers Prev 2010 Jul;19(7):1766-1774.

(91) Brenner H, Hoffmeister M, Arndt V, Stegmaier C, Altenhofen L, Haug U. Protection from right- and left-sided colorectal neoplasms after colonoscopy: population-based study. J Natl Cancer Inst 2010 Jan 20;102(2):89-95.

(92) Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett 1987 Oct 30;37(2):147-151.

(93) Orlando FA, Tan D, Baltodano JD, Khoury T, Gibbs JF, Hassid VJ, et al. Aberrant crypt foci as precursors in colorectal cancer progression. J Surg Oncol 2008 Sep 1;98(3):207-213.

(94) Hurlstone DP, Fujii T. Practical uses of chromoendoscopy and magnification at colonoscopy. Gastrointest Endosc Clin N Am 2005 Oct;15(4):687-702.

(95) Shpitz B, Bomstein Y, Mekori Y, Cohen R, Kaufman Z, Grankin M, et al. Proliferating cell nuclear antigen as a marker of cell kinetics in aberrant crypt foci, hyperplastic polyps, adenomas, and adenocarcinomas of the human colon. Am J Surg 1997 Oct;174(4):425-430.

(96) Nucci MR, Robinson CR, Longo P, Campbell P, Hamilton SR. Phenotypic and genotypic characteristics of aberrant crypt foci in human colorectal mucosa. Hum Pathol 1997 Dec;28(12):1396-1407.

(97) Takayama T, Ohi M, Hayashi T, Miyanishi K, Nobuoka A, Nakajima T, et al. Analysis of K-ras, APC, and beta-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. Gastroenterology 2001 Sep;121(3):599-611.

(98) Smith AJ, Stern HS, Penner M, Hay K, Mitri A, Bapat BV, et al. Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons. Cancer Res 1994 Nov 1;54(21):5527-5530.

(99) Suehiro Y, Hinoda Y. Genetic and epigenetic changes in aberrant crypt foci and serrated polyps. Cancer Sci 2008 Jun;99(6):1071-1076.

(100) Heinen CD, Shivapurkar N, Tang Z, Groden J, Alabaster O. Microsatellite instability in aberrant crypt foci from human colons. Cancer Res 1996 Dec 1;56(23):5339-5341.

(101) Mo A, Jackson S, Varma K, Carpino A, Giardina C, Devers TJ, et al. Distinct Transcriptional Changes and Epithelial-Stromal Interactions Are Altered in Early-Stage Colon Cancer Development. Mol Cancer Res 2016 Sep;14(9):795-804.

(102) Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer 2006 Feb;6(2):130-140.

(103) Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 2003 Mar 6;348(10):883-890.

(104) Kune GA. Colorectal cancer chemoprevention: aspirin, other NSAID and COX-2 inhibitors. Aust N Z J Surg 2000 Jun;70(6):452-455.

(105) Rothwell PM, Wilson M, Elwin CE, Norrving B, Algra A, Warlow CP, et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. Lancet 2010 Nov 20;376(9754):1741-1750.

(106) Nakanishi M, Rosenberg DW. Multifaceted roles of PGE2 in inflammation and cancer. Semin Immunopathol 2013 Mar;35(2):123-137.

(107) Takeuchi K. Prostaglandin EP receptors and their roles in mucosal protection and ulcer healing in the gastrointestinal tract. Adv Clin Chem 2010;51:121-144.

(108) Wallace JL. Prostaglandin biology in inflammatory bowel disease. Gastroenterol Clin North Am 2001 Dec;30(4):971-980.

(109) Pugh S, Thomas GA. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2. Gut 1994 May;35(5):675-678.

(110) Wang D, Xia D, Dubois RN. The Crosstalk of PTGS2 and EGF Signaling Pathways in Colorectal Cancer. Cancers (Basel) 2011 Oct 14;3(4):3894-3908.

(111) Kawamori T, Uchiya N, Sugimura T, Wakabayashi K. Enhancement of colon carcinogenesis by prostaglandin E2 administration. Carcinogenesis 2003 May;24(5):985-990.

(112) Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, et al. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996 Nov 29;87(5):803-809.

(113) Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, et al. Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000 Sep 1;60(17):4705-4708.

(114) Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, et al. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000 Oct 20;275(42):32783-32792.

(115) Nakanishi M, Menoret A, Tanaka T, Miyamoto S, Montrose DC, Vella AT, et al. Selective PGE(2) suppression inhibits colon carcinogenesis and modifies local mucosal immunity. Cancer Prev Res (Phila) 2011 Aug;4(8):1198-1208.

(116) Nakanishi M, Montrose DC, Clark P, Nambiar PR, Belinsky GS, Claffey KP, et al. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. Cancer Res 2008 May 1;68(9):3251-3259.

(117) Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature 2011 May 19;473(7347):298-307.

(118) Fong GH. Mechanisms of adaptive angiogenesis to tissue hypoxia. Angiogenesis 2008;11(2):121-140.

(119) Salvado MD, Alfranca A, Haeggstrom JZ, Redondo JM. Prostanoids in tumor angiogenesis: therapeutic intervention beyond COX-2. Trends Mol Med 2012 Apr;18(4):233-243.

(120) Chang SH, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, et al. Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. Proc Natl Acad Sci U S A 2004 Jan 13;101(2):591-596.

(121) Spinella F, Rosano L, Di Castro V, Natali PG, Bagnato A. Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. J Biol Chem 2004 Nov 5;279(45):46700-46705.

(122) Jain S, Chakraborty G, Raja R, Kale S, Kundu GC. Prostaglandin E2 regulates tumor angiogenesis in prostate cancer. Cancer Res 2008 Oct 1;68(19):7750-7759.

(123) Seno H, Oshima M, Ishikawa TO, Oshima H, Takaku K, Chiba T, et al. Cyclooxygenase 2- and prostaglandin E(2) receptor EP(2)-dependent angiogenesis in Apc(Delta716) mouse intestinal polyps. Cancer Res 2002 Jan 15;62(2):506-511.

(124) Mutoh M, Watanabe K, Kitamura T, Shoji Y, Takahashi M, Kawamori T, et al. Involvement of prostaglandin E receptor subtype EP(4) in colon carcinogenesis. Cancer Res 2002 Jan 1;62(1):28-32.

(125) Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis 2009 Mar;30(3):377-386.

(126) Ahmadi M, Emery DC, Morgan DJ. Prevention of both direct and cross-priming of antitumor CD8+ T-cell responses following overproduction of prostaglandin E2 by tumor cells in vivo. Cancer Res 2008 Sep 15;68(18):7520-7529.

(127) Yang L, Yamagata N, Yadav R, Brandon S, Courtney RL, Morrow JD, et al. Cancer-associated immunodeficiency and dendritic cell abnormalities mediated by the prostaglandin EP2 receptor. J Clin Invest 2003 Mar;111(5):727-735.

(128) Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J Immunol 1997 Jul 1;159(1):28-35.

(129) De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell 2013 Mar 18;23(3):277-286.

(130) Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. Cancer Res 2007 May 1;67(9):4507-4513.

(131) Zhu Y, Hua P, Lance P. Cyclooxygenase-2 expression and prostanoid biogenesis reflect clinical phenotype in human colorectal fibroblast strains. Cancer Res 2003 Jan 15;63(2):522-526.

(132) Adegboyega PA, Ololade O, Saada J, Mifflin R, Di Mari JF, Powell DW. Subepithelial myofibroblasts express cyclooxygenase-2 in colorectal tubular adenomas. Clin Cancer Res 2004 Sep 1;10(17):5870-5879.

(133) Mo A, Jackson S, Varma K, Carpino A, Giardina C, Devers TJ, et al. Distinct Transcriptional Changes and Epithelial-Stromal Interactions Are Altered in Early-Stage Colon Cancer Development. Mol Cancer Res 2016 Sep;14(9):795-804.

(134) Konstantinopoulos PA, Vandoros GP, Karamouzis MV, Gkermpesi M, Sotiropoulou-Bonikou G, Papavassiliou AG. EGF-R is expressed and AP-1 and NF-kappaB are activated in stromal myofibroblasts surrounding colon adenocarcinomas paralleling expression of COX-2 and VEGF. Cell Oncol 2007;29(6):477-482.

(135) Kim EC, Zhu Y, Andersen V, Sciaky D, Cao HJ, Meekins H, et al. Cytokine-mediated PGE2 expression in human colonic fibroblasts. Am J Physiol 1998 Oct;275(4 Pt 1):C988-94.

(136) Zhu Y, Zhu M, Lance P. IL1beta-mediated Stromal COX-2 signaling mediates proliferation and invasiveness of colonic epithelial cancer cells. Exp Cell Res 2012 Nov 15;318(19):2520-2530.

(137) Zhu Y, Zhu M, Lance P. Stromal COX-2 signaling activated by deoxycholic acid mediates proliferation and invasiveness of colorectal epithelial cancer cells. Biochem Biophys Res Commun 2012 Aug 31;425(3):607-612.

(138) Zhu M, Zhu Y, Lance P. TNFalpha-activated stromal COX-2 signalling promotes proliferative and invasive potential of colon cancer epithelial cells. Cell Prolif 2013 Aug;46(4):374-381.

(139) Park SW, Kim HS, Choi MS, Jeong WJ, Heo DS, Kim KH, et al. The effects of the stromal cell-derived cyclooxygenase-2 metabolite prostaglandin E2 on the proliferation of colon cancer cells. J Pharmacol Exp Ther 2011 Feb;336(2):516-523.

(140) Nakagawa H, Liyanarachchi S, Davuluri RV, Auer H, Martin EW,Jr, de la Chapelle A, et al. Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. Oncogene 2004 Sep 23;23(44):7366-7377.

(141) Patrignani P, Tacconelli S, Bruno A, Sostres C, Lanas A. Managing the adverse effects of nonsteroidal anti-inflammatory drugs. Expert Rev Clin Pharmacol 2011 Sep;4(5):605-621.

(142) Cannon CP, Cannon PJ. Physiology. COX-2 inhibitors and cardiovascular risk. Science 2012 Jun 15;336(6087):1386-1387.

(143) Tytherleigh MG, Warren BF, Mortensen NJ. Management of early rectal cancer. Br J Surg 2008 Apr;95(4):409-423.

(144) West NP, Hohenberger W, Weber K, Perrakis A, Finan PJ, Quirke P. Complete mesocolic excision with central vascular ligation produces an oncologically superior specimen compared with standard surgery for carcinoma of the colon. J Clin Oncol 2010 Jan 10;28(2):272-278.

(145) Pulitano C, Bodingbauer M, Aldrighetti L, de Jong MC, Castillo F, Schulick RD, et al. Liver resection for colorectal metastases in presence of extrahepatic disease: results from an international multi-institutional analysis. Ann Surg Oncol 2011 May;18(5):1380-1388.

(146) Sauer R, Becker H, Hohenberger W, Rodel C, Wittekind C, Fietkau R, et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. N Engl J Med 2004 Oct 21;351(17):1731-1740.

(147) Kelland L. The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer 2007 Aug;7(8):573-584.

(148) Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. Lancet 1995 Apr 15;345(8955):939-944.

(149) Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004 Jan 1;22(1):23-30.

(150) Maughan TS, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, et al. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. Lancet 2011 Jun 18;377(9783):2103-2114.

(151) Farrell A. A close look at cancer. Nat Med 2011 Mar;17(3):262-265.

(152) Rebucci M, Michiels C. Molecular aspects of cancer cell resistance to chemotherapy. Biochem Pharmacol 2013 May 1;85(9):1219-1226.

(153) Alberts SR, Horvath WL, Sternfeld WC, Goldberg RM, Mahoney MR, Dakhil SR, et al. Oxaliplatin, fluorouracil, and leucovorin for patients with unresectable liver-only metastases from colorectal cancer: a North Central Cancer Treatment Group phase II study. J Clin Oncol 2005 Dec 20;23(36):9243-9249.

(154) Raguz S, Yague E. Resistance to chemotherapy: new treatments and novel insights into an old problem. Br J Cancer 2008 Aug 5;99(3):387-391.

(155) Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, et al. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 2004 Aug;6(2):117-127.

(156) Lage H. An overview of cancer multidrug resistance: a still unsolved problem. Cell Mol Life Sci 2008 Oct;65(20):3145-3167.

(157) Wu CP, Hsieh CH, Wu YS. The emergence of drug transporter-mediated multidrug resistance to cancer chemotherapy. Mol Pharm 2011 Dec 5;8(6):1996-2011.

(158) Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002 Jan;2(1):48-58.

(159) Hilgeroth A, Hemmer M, Coburger C. The impact of the induction of multidrug resistance transporters in therapies by used drugs: recent studies. Mini Rev Med Chem 2012 Oct;12(11):1127-1134.

(160) Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med 2013 Jan 17;2(1):3-1326-2-3.

(161) Haraguchi N, Inoue H, Tanaka F, Mimori K, Utsunomiya T, Sasaki A, et al. Cancer stem cells in human gastrointestinal cancers. Hum Cell 2006 Feb;19(1):24-29.

(162) Boman BM, Huang E. Human colon cancer stem cells: a new paradigm in gastrointestinal oncology. J Clin Oncol 2008 Jun 10;26(17):2828-2838.

(163) Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell 2007 Oct 11;1(4):389-402.

(164) Larsson K, Jakobsson PJ. Inhibition of microsomal prostaglandin E synthase-1 as targeted therapy in cancer treatment. Prostaglandins Other Lipid Mediat 2015 Jul;120:161-165.

(165) Kurtova AV, Xiao J, Mo Q, Pazhanisamy S, Krasnow R, Lerner SP, et al. Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. Nature 2015 Jan 8;517(7533):209-213.

(166) Xu L, Stevens J, Hilton MB, Seaman S, Conrads TP, Veenstra TD, et al. COX-2 inhibition potentiates antiangiogenic cancer therapy and prevents metastasis in preclinical models. Sci Transl Med 2014 Jun 25;6(242):242ra84.

(167) Falandry C, Canney PA, Freyer G, Dirix LY. Role of combination therapy with aromatase and cyclooxygenase-2 inhibitors in patients with metastatic breast cancer. Ann Oncol 2009 Apr;20(4):615-620.

(168) Zhang DQ, Guo Q, Zhu JH, Chen WC. Increase of cyclooxygenase-2 inhibition with celecoxib combined with 5-FU enhances tumor cell apoptosis and antitumor efficacy in a subcutaneous implantation tumor model of human colon cancer. World J Surg Oncol 2013 Jan 24;11:16-7819-11-16.

(169) Lin J, Hsiao PW, Chiu TH, Chao JI. Combination of cyclooxygenase-2 inhibitors and oxaliplatin increases the growth inhibition and death in human colon cancer cells. Biochem Pharmacol 2005 Sep 1;70(5):658-667.

(170) Zhao S, Cai J, Bian H, Gui L, Zhao F. Synergistic inhibition effect of tumor growth by using celecoxib in combination with oxaliplatin. Cancer Invest 2009 Jul;27(6):636-640.

(171) Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS. Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 2005 Dec 2;310(5753):1504-1510.

(172) Leone V, di Palma A, Ricchi P, Acquaviva F, Giannouli M, Di Prisco AM, et al. PGE2 inhibits apoptosis in human adenocarcinoma Caco-2 cell line through Ras-PI3K association and cAMP-dependent kinase A activation. Am J Physiol Gastrointest Liver Physiol 2007 Oct;293(4):G673-81.

(173) Tessner TG, Muhale F, Riehl TE, Anant S, Stenson WF. Prostaglandin E2 reduces radiation-induced epithelial apoptosis through a mechanism involving AKT activation and bax translocation. J Clin Invest 2004 Dec;114(11):1676-1685.

(174) Elwood PC, Morgan G, Pickering JE, Galante J, Weightman AL, Morris D, et al. Aspirin in the Treatment of Cancer: Reductions in Metastatic Spread and in Mortality: A Systematic Review and Meta-Analyses of Published Studies. PLoS One 2016 Apr 20;11(4):e0152402.

(175) Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. N Engl J Med 2012 Oct 25;367(17):1596-1606.

(176) Ng K, Meyerhardt JA, Chan AT, Sato K, Chan JA, Niedzwiecki D, et al. Aspirin and COX-2 inhibitor use in patients with stage III colon cancer. J Natl Cancer Inst 2014 Nov 27;107(1):345.

(177) Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 1990 Jan 19;247(4940):322-324.

(178) Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992 May 1;256(5057):668-670.

(179) Roper J, Hung KE. Priceless GEMMs: genetically engineered mouse models for colorectal cancer drug development. Trends Pharmacol Sci 2012 Aug;33(8):449-455.

(180) Moser AR, Shoemaker AR, Connelly CS, Clipson L, Gould KA, Luongo C, et al. Homozygosity for the Min allele of Apc results in disruption of mouse development prior to gastrulation. Dev Dyn 1995 Aug;203(4):422-433.

(181) Shao J, Washington MK, Saxena R, Sheng H. Heterozygous disruption of the PTEN promotes intestinal neoplasia in APCmin/+ mouse: roles of osteopontin. Carcinogenesis 2007 Dec;28(12):2476-2483.

(182) Corpet DE, Pierre F. Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. Cancer Epidemiol Biomarkers Prev 2003 May;12(5):391-400.

(183) Dragatsis I, Zeitlin S. A method for the generation of conditional gene repair mutations in mice. Nucleic Acids Res 2001 Feb 1;29(3):E10.

(184) Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, et al. Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. Science 1997 Oct 3;278(5335):120-123.

(185) Hung KE, Maricevich MA, Richard LG, Chen WY, Richardson MP, Kunin A, et al. Development of a mouse model for sporadic and metastatic colon tumors and its use in assessing drug treatment. Proc Natl Acad Sci U S A 2010 Jan 26;107(4):1565-1570.

(186) el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 2004 Jul;39(3):186-193.

(187) Munoz NM, Upton M, Rojas A, Washington MK, Lin L, Chytil A, et al. Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. Cancer Res 2006 Oct 15;66(20):9837-9844.

(188) Haigis KM, Kendall KR, Wang Y, Cheung A, Haigis MC, Glickman JN, et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nat Genet 2008 May;40(5):600-608.

(189) Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007 Oct 25;449(7165):1003-1007.

(190) Byun AJ, Hung KE, Fleet JC, Bronson RT, Mason JB, Garcia PE, et al. Colon-specific tumorigenesis in mice driven by Cre-mediated inactivation of Apc and activation of mutant Kras. Cancer Lett 2014 Jun 1;347(2):191-195.

(191) Sasaki Y, Kamei D, Ishikawa Y, Ishii T, Uematsu S, Akira S, et al. Microsomal prostaglandin E synthase-1 is involved in multiple steps of colon carcinogenesis. Oncogene 2012 Jun 14;31(24):2943-2952.

(192) O'Callaghan G, Houston A. Prostaglandin E2 and the EP receptors in malignancy: possible therapeutic targets? Br J Pharmacol 2015 Nov;172(22):5239-5250.

(193) Rundhaug JE, Simper MS, Surh I, Fischer SM. The role of the EP receptors for prostaglandin E2 in skin and skin cancer. Cancer Metastasis Rev 2011 Dec;30(3-4):465-480.

(194) Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, et al. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. Nat Med 2001 Sep;7(9):1048-1051.

(195) Shoji Y, Takahashi M, Kitamura T, Watanabe K, Kawamori T, Maruyama T, et al. Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development. Gut 2004 Aug;53(8):1151-1158.

(196) Yokoyama U, Iwatsubo K, Umemura M, Fujita T, Ishikawa Y. The prostanoid EP4 receptor and its signaling pathway. Pharmacol Rev 2013 Jun 17;65(3):1010-1052.

(197) Chang J, Vacher J, Yao B, Fan X, Zhang B, Harris RC, et al. Prostaglandin E receptor 4 (EP4) promotes colonic tumorigenesis. Oncotarget 2015 Oct 20;6(32):33500-33511.

(198) Hawcroft G, Ko CW, Hull MA. Prostaglandin E2-EP4 receptor signalling promotes tumorigenic behaviour of HT-29 human colorectal cancer cells. Oncogene 2007 May 10;26(21):3006-3019.

(199) Yang AD, Fan F, Camp ER, van Buren G, Liu W, Somcio R, et al. Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. Clin Cancer Res 2006 Jul 15;12(14 Pt 1):4147-4153.

(200) Young L, Sung J, Stacey G, Masters JR. Detection of Mycoplasma in cell cultures. Nat Protoc 2010 May;5(5):929-934.

(201) Arango D, Wilson AJ, Shi Q, Corner GA, Aranes MJ, Nicholas C, et al. Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. Br J Cancer 2004 Nov 29;91(11):1931-1946.

(202) Alcindor T, Beauger N. Oxaliplatin: a review in the era of molecularly targeted therapy. Curr Oncol 2011 Jan;18(1):18-25.

(203) Reti A, Barna G, Pap E, Adleff V, L Komlosi V, Jeney A, et al. Enhancement of 5-fluorouracil efficacy on high COX-2 expressing HCA-7 cells by low dose indomethacin and NS-398 but not on low COX-2 expressing HT-29 cells. Pathol Oncol Res 2009 Sep;15(3):335-344.

(204) Flis S, Splwinski J. Inhibitory effects of 5-fluorouracil and oxaliplatin on human colorectal cancer cell survival are synergistically enhanced by sulindac sulfide. Anticancer Res 2009 Jan;29(1):435-441.

(205) Liu B, Qu L, Tao H. Cyclo-oxygenase 2 up-regulates the effect of multidrug resistance. Cell Biol Int 2009 Dec 16;34(1):21-25.

(206) Sui H, Zhou S, Wang Y, Liu X, Zhou L, Yin P, et al. COX-2 contributes to P-glycoprotein-mediated multidrug resistance via phosphorylation of c-Jun at Ser63/73 in colorectal cancer. Carcinogenesis 2011 May;32(5):667-675.

(207) Yu Y, Ricciotti E, Scalia R, Tang SY, Grant G, Yu Z, et al. Vascular COX-2 modulates blood pressure and thrombosis in mice. Sci Transl Med 2012 May 2;4(132):132ra54.

(208) Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, et al. Celecoxib for the prevention of sporadic colorectal adenomas. N Engl J Med 2006 Aug 31;355(9):873-884.

(209) Backlund MG, Mann JR, Holla VR, Buchanan FG, Tai HH, Musiek ES, et al. 15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer. J Biol Chem 2005 Feb 4;280(5):3217-3223.

(210) Virag P, Fischer-Fodor E, Perde-Schrepler M, Brie I, Tatomir C, Balacescu L, et al. Oxaliplatin induces different cellular and molecular chemoresistance patterns in colorectal cancer cell lines of identical origins. BMC Genomics 2013 Jul 16;14:480-2164-14-480.

(211) Sheng H, Shao J, Washington MK, DuBois RN. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. J Biol Chem 2001 May 25;276(21):18075-18081.

(212) Nemoto S, Nakamura M, Osawa Y, Kono S, Itoh Y, Okano Y, et al. Sphingosine kinase isoforms regulate oxaliplatin sensitivity of human colon cancer cells through ceramide accumulation and Akt activation. J Biol Chem 2009 Apr 17;284(16):10422-10432.

(213) Xiao ZM, Wang XY, Wang AM. Periostin induces chemoresistance in colon cancer cells through activation of the PI3K/Akt/survivin pathway. Biotechnol Appl Biochem 2015 May-Jun;62(3):401-406.

(214) Sugimoto Y, Narumiya S. Prostaglandin E receptors. J Biol Chem 2007 Apr 20;282(16):11613-11617.

(215) Watanabe K, Kawamori T, Nakatsugi S, Ohta T, Ohuchida S, Yamamoto H, et al. Role of the prostaglandin E receptor subtype EP1 in colon carcinogenesis. Cancer Res 1999 Oct 15;59(20):5093-5096.

(216) Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 2002 Mar;8(3):289-293.

(217) Rothenberg ML. Efficacy of oxaliplatin in the treatment of colorectal cancer. Oncology (Williston Park) 2000 Dec;14(12 Suppl 11):9-14.

(218) Martinez-Cardus A, Martinez-Balibrea E, Bandres E, Malumbres R, Gines A, Manzano JL, et al. Pharmacogenomic approach for the identification of novel determinants of acquired resistance to oxaliplatin in colorectal cancer. Mol Cancer Ther 2009 Jan;8(1):194-202.

(219) Martin LP, Hamilton TC, Schilder RJ. Platinum resistance: the role of DNA repair pathways. Clin Cancer Res 2008 Mar 1;14(5):1291-1295.

(220) Dallas NA, Xia L, Fan F, Gray MJ, Gaur P, van Buren G,2nd, et al. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. Cancer Res 2009 Mar 1;69(5):1951-1957.

(221) Vinogradov S, Wei X. Cancer stem cells and drug resistance: the potential of nanomedicine. Nanomedicine (Lond) 2012 Apr;7(4):597-615.

(222) Sullivan LB, Chandel NS. Mitochondrial reactive oxygen species and cancer. Cancer Metab 2014 Nov 28;2:17-3002-2-17. eCollection 2014.

(223) Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov 2009 Jul;8(7):579-591.

(224) Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nat Rev Drug Discov 2013 Dec;12(12):931-947.

(225) Shi Y, Tang B, Yu PW, Tang B, Hao YX, Lei X, et al. Autophagy protects against oxaliplatin-induced cell death via ER stress and ROS in Caco-2 cells. PLoS One 2012;7(11):e51076.

(226) Lin S, Lei K, Du W, Yang L, Shi H, Gao Y, et al. Enhancement of oxaliplatin sensitivity in human colorectal cancer by hypericin mediated photodynamic therapy via ROS-related mechanism. Int J Biochem Cell Biol 2016 Feb;71:24-34.

(227) Kopetz S, Lesslie DP, Dallas NA, Park SI, Johnson M, Parikh NU, et al. Synergistic activity of the SRC family kinase inhibitor dasatinib and oxaliplatin in colon carcinoma cells is mediated by oxidative stress. Cancer Res 2009 May 1;69(9):3842-3849.

(228) Mo C, Zhao R, Vallejo J, Igwe O, Bonewald L, Wetmore L, et al. Prostaglandin E2 promotes proliferation of skeletal muscle myoblasts via EP4 receptor activation. Cell Cycle 2015;14(10):1507-1516.

(229) Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003 May 15;17(10):1253-1270.

(230) Santoro V, Jia R, Thompson H, Nijhuis A, Jeffery R, Kiakos K, et al. Role of Reactive Oxygen Species in the Abrogation of Oxaliplatin Activity by Cetuximab in Colorectal Cancer. J Natl Cancer Inst 2015 Dec 30;108(6):djv394.

(231) Wang D, Fu L, Sun H, Guo L, DuBois RN. Prostaglandin E2 Promotes Colorectal Cancer Stem Cell Expansion and Metastasis in Mice. Gastroenterology 2015 Dec;149(7):1884-1895.e4.

(232) Malkomes P, Lunger I, Luetticke A, Oppermann E, Haetscher N, Serve H, et al. Selective AKT Inhibition by MK-2206 Represses Colorectal Cancer-Initiating Stem Cells. Ann Surg Oncol 2016;23:2849-2857.

(233) Xia P, Xu XY. PI3K/Akt/mTOR signaling pathway in cancer stem cells: from basic research to clinical application. Am J Cancer Res 2015;5(5):1602-1609.

(234) Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, et al. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev 2013;2013:972913.

(235) Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990 Jun 1;61(5):759-767.

(236) Bettington M, Walker N, Clouston A, Brown I, Leggett B, Whitehall V. The serrated pathway to colorectal carcinoma: current concepts and challenges. Histopathology 2013 Feb;62(3):367-386.

(237) Torlakovic E, Snover DC. Serrated adenomatous polyposis in humans. Gastroenterology 1996 Mar;110(3):748-755.

(238) Bosman FT, World Health Organization, International Agency for Research on Cancer. World Health Organization classification of tumours of the degistive system. 2010.

(239) Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007 Jan;50(1):113-130.

(240) Barras D. BRAF Mutation in Colorectal Cancer: An Update. Biomark Cancer 2015 Sep 6;7(Suppl 1):9-12.

(241) Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002 Aug 29;418(6901):934.

(242) Thiel A, Ristimaki A. Toward a Molecular Classification of Colorectal Cancer: The Role of BRAF. Front Oncol 2013 Nov 15;3:281.

(243) Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004 Mar 19;116(6):855-867.

(244) Rad R, Cadinanos J, Rad L, Varela I, Strong A, Kriegl L, et al. A genetic progression model of Braf(V600E)-induced intestinal tumorigenesis reveals targets for therapeutic intervention. Cancer Cell 2013 Jul 8;24(1):15-29.

(245) Mercer K, Giblett S, Green S, Lloyd D, DaRocha Dias S, Plumb M, et al. Expression of endogenous oncogenic V600EB-raf induces proliferation and developmental defects in mice and transformation of primary fibroblasts. Cancer Res 2005 Dec 15;65(24):11493-11500.

(246) Nambiar PR, Nakanishi M, Gupta R, Cheung E, Firouzi A, Ma XJ, et al. Genetic signatures of high- and low-risk aberrant crypt foci in a mouse model of sporadic colon cancer. Cancer Res 2004 Sep 15;64(18):6394-6401.

(247) Boivin GP, Washington K, Yang K, Ward JM, Pretlow TP, Russell R, et al. Pathology of mouse models of intestinal cancer: consensus report and recommendations. Gastroenterology 2003 Mar;124(3):762-777.

(248) Riemer P, Sreekumar A, Reinke S, Rad R, Schafer R, Sers C, et al. Transgenic expression of oncogenic BRAF induces loss of stem cells in the mouse intestine, which is antagonized by beta-catenin activity. Oncogene 2015 Jun 11;34(24):3164-3175.

(249) Oliveira C, Pinto M, Duval A, Brennetot C, Domingo E, Espin E, et al. BRAF mutations characterize colon but not gastric cancer with mismatch repair deficiency. Oncogene 2003 Dec 11;22(57):9192-9196.

(250) Riemer P, Sreekumar A, Reinke S, Rad R, Schafer R, Sers C, et al. Transgenic expression of oncogenic BRAF induces loss of stem cells in the mouse intestine, which is antagonized by beta-catenin activity. Oncogene 2015 Jun 11;34(24):3164-3175.