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THE ROLE OF THE MIR-200 FAMILY ON THE TUMOR SUPPRESSOR RASSF2
AND THE EFFECT ON MAPK PATHWAY ACTIVITY IN COLORECTAL CANCER

By

Jane V. Carter

M.B. Ch.B - University of Bristol, United Kingdom, 2007

MRCS - Royal College of Surgeons of England, United Kingdom, 2010

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A Dissertation Approved on

November 22nd, 2016

by the following Dissertation Committee:

Susan Galandiuk, M.D., Dissertation Director

Irving G. Joshua, Ph.D., Co-Advisor

Aruni Bhatnagar, Ph.D.

Claudio Maldonado, Ph.D.

Shesh N. Rai, Ph.D.

Dale Schuschke, Ph.D.

DEDICATION

This dissertation is dedicated to my parents

Gill and Cliff Carter.

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my mentor Dr. Susan Galandiuk, Professor of Surgery and Program Director of the Section of Colon and Rectal Surgery, for the continuous support of my PhD study and related research. Without her time, guidance, motivation, and immense knowledge, much of what I have achieved would not have been possible. I could not have imagined having a better mentor for my PhD study.

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To Dr. Hiram C. Polk Jr. for his constructive criticism to my work and for his teaching in all aspects of surgery, both clinical and scientific. I am very fortunate to have learnt from his vast wealth of knowledge and experience.

I would like to give very special thanks to the people within our lab, Robert Eichenberger, James Burton and Campbell Bishop. Their hands on help, support, and

guidance allowed me to complete the most significant achievement in my career. To Robert for getting me started within the lab and his assistance with planning of experiments. His general support of all fellows goes above and beyond his job role and his help in settling and finding my feet in Louisville is something I will always be grateful for. To James, my right-hand man, it has been a pleasure to get to know you. I wish you the very best of luck with medical school and your future career. Thank you for being there for me throughout my time in the lab.

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I also thank my friends, the “Cliftonites”, for their support and friendship throughout my time here. Their endless entertainment from over 4,000 miles away has kept me smiling and I look forward to being together again soon. To all those who have visited me here, you will never know how much that means to me. Thank you.

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Lastly, I would like to thank my family for all their love and encouragement. For my hard-working parents who have sacrificed their lives for myself and provided me with the opportunities I have had. Their unconditional love, care, and support of my pursuits is so appreciated, and I would not have made it this far without them. Thank you.

ABSTRACT

THE ROLE OF THE MIR-200 FAMILY ON THE TUMOR SUPPRESSOR RASSF2 AND THE EFFECT ON MAPK PATHWAY ACTIVITY IN COLORECTAL CANCER

Jane V. Carter

November 22nd 2016

This dissertation investigated the role of the miR-200 family in normal colon epithelial (CCD 841) and Dukes' C (HT-29) colorectal cancer (CRC) cell lines. Our aim was to characterize expression of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) in colorectal cell lines, study their effect on the tumor suppressor Ras Associated Domain-Containing Protein (RASSF) 2 and on subsequent activity within the mitogen-activated protein kinase (MAPK) signaling pathway. We wanted to determine whether regulation of miR-200 family members could change cell behavior towards more "cancer-like" in a normal colon epithelium (CCD 841) cell line, or less "cancer-like" in a Dukes' C (HT-29) CRC cell line.

We found the following:

1. All miR-200 family members were highly expressed in colorectal cancer cell lines compared to a normal colon epithelial cell line.

2. RASSF2 mRNA and protein expression was downregulated in all CRC cell lines compared to the normal colon epithelial (CCD 841) cell line.
3. Overexpression of miR-200 family members in a normal colon epithelial (CCD 841) cell line decreased expression of both RASSF2 mRNA and protein.
4. Inhibition of miR-200 family members in a Dukes' C (HT-29) CRC cell line increased expression of both RASSF2 mRNA and protein.
5. Total K-Ras expression and phosphorylation of ERK 1/2 increased following overexpression of miR-200 family members in a normal colon epithelial (CCD 841) cell line, indicating increased activity within the MAPK pathway resulting in increased cell proliferation.
6. MAPK pathway activity decreased, as measured by reduced ERK 1/2 phosphorylation and reduced cell proliferation in a Dukes' C (HT-29) CRC cell line following inhibition of miR-200 family members.

These findings demonstrate a novel association of the miR-200 family, the tumor suppressor RASSF2, and the MAPK signaling pathway in CRC. In contrast to the previous understanding that miR-200 family dysregulation is considered to exhibit tumor suppressive behavior by blocking epithelial to mesenchymal transition, we refute this in the case of CRC and propose the miR-200 family contribute to CRC tumorigenesis. This improved understanding of the miR-200 family may have the potential to be developed as a therapeutic intervention in CRC.

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PREFACE

During my general surgical registrar training in the South West of England I was fortunate to have the opportunity to conduct research at the Price Institute of Surgical Research under the mentorship of Dr. Susan Galandiuk, Louisville, Kentucky.

I was introduced to a laboratory working with colorectal cancer cell lines and microRNAs as biomarkers for the detection of colorectal cancer. It was here I became interested in the differential expression of miRNAs between cancers that were locally confined (surgically curable) and those that had begun to metastasize (less likely to be cured by surgery). As a way of learning laboratory techniques and cell culture I performed miRNA screening studies on four colorectal cancer cell lines, two locally confined and two metastatic, and one normal colon epithelium, to determine whether there were differences in miRNA expression. This resulted in the observation that the miR-200 family members were highly expressed in colorectal cancer. Research into the miR-200 family identified an association with the tumor suppressor RASSF2 and after confirmatory studies, a hypothesis was generated.

Based upon the screening results and my thesis committee's advice, a Dukes' C (HT-29) colorectal cancer cell line and a normal colon epithelial (CCD 841) cell line were selected for further study. A commonly dysregulated pathway in colorectal cancer is the MAPK signaling pathway. This pathway is a major regulator of cell proliferation.

For this reason we chose to study the effect of the miR-200 family on both proliferation in the Dukes' C (HT-29) colorectal cancer cell line and the normal colon epithelial (CCD 841) cell line.

Another goal was to perform gain and loss of function studies of the miR-200 family with the idea that colorectal cancer cells would adopt less malignant behavior, whereas normal colon epithelial cells would have more malignant behavior.

I have enjoyed learning and understanding the processes involved in scientific research and I have deepened my knowledge on aspects contributing to surgical disease. This has set the foundations on which I will take back with me to the United Kingdom to develop my own practice and begin my career in academic surgery.

CHAPTER I

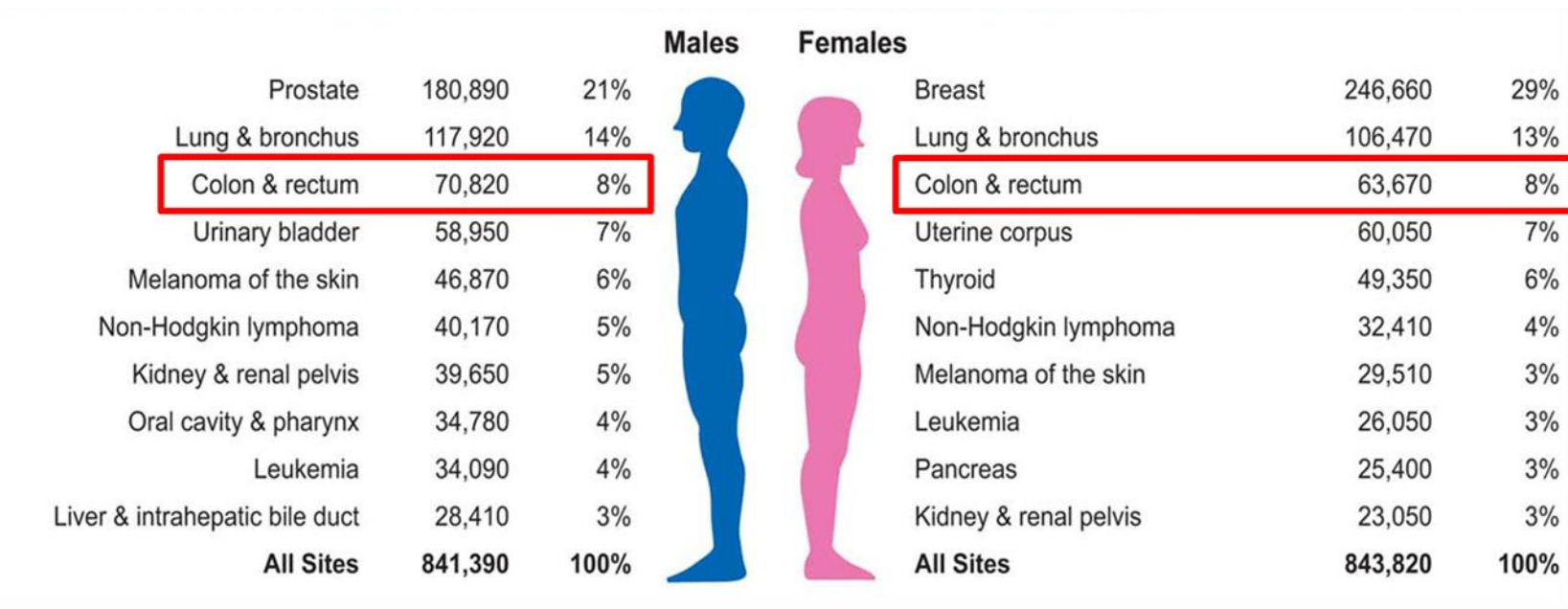
INTRODUCTION

a) Epidemiology and Risk Factors for the Development of Colorectal Cancer

Colorectal cancer (CRC) is common worldwide and associated with significant mortality. In the United States alone in 2015, CRC was the third most common cancer in men and women with approximately 135,000 new cases diagnosed (Figure 1). It was also accountable as the third most leading cause of cancer deaths continuing to make CRC an enormous public health burden (Figure 2).(1) Most patients present with late stage disease and the 5-year survival for patients with newly diagnosed colon cancer with distant metastases is less than 15% (Figure 3).(2) Overall, the current lifetime risk of developing CRC is approximately 1 in 21 (4.7%) for men and 1 in 23 (4.4%) for women.(2)

The underlying causes of CRC are complex and heterogeneous. Most sporadic CRC develops from precursor polyps, namely advanced adenomas and hyperplastic/serrated polyps. Adenocarcinomas form more than 95% of all colorectal cancers. Other less common types of tumors to develop in the colon and rectum include carcinoid tumors, gastrointestinal stromal tumors, lymphomas and sarcomas.

Figure 1. Estimated New Cases of Colorectal Cancer in the United States 2015



2

Figure 2. Estimated Colorectal Cancer Deaths in the United States 2015

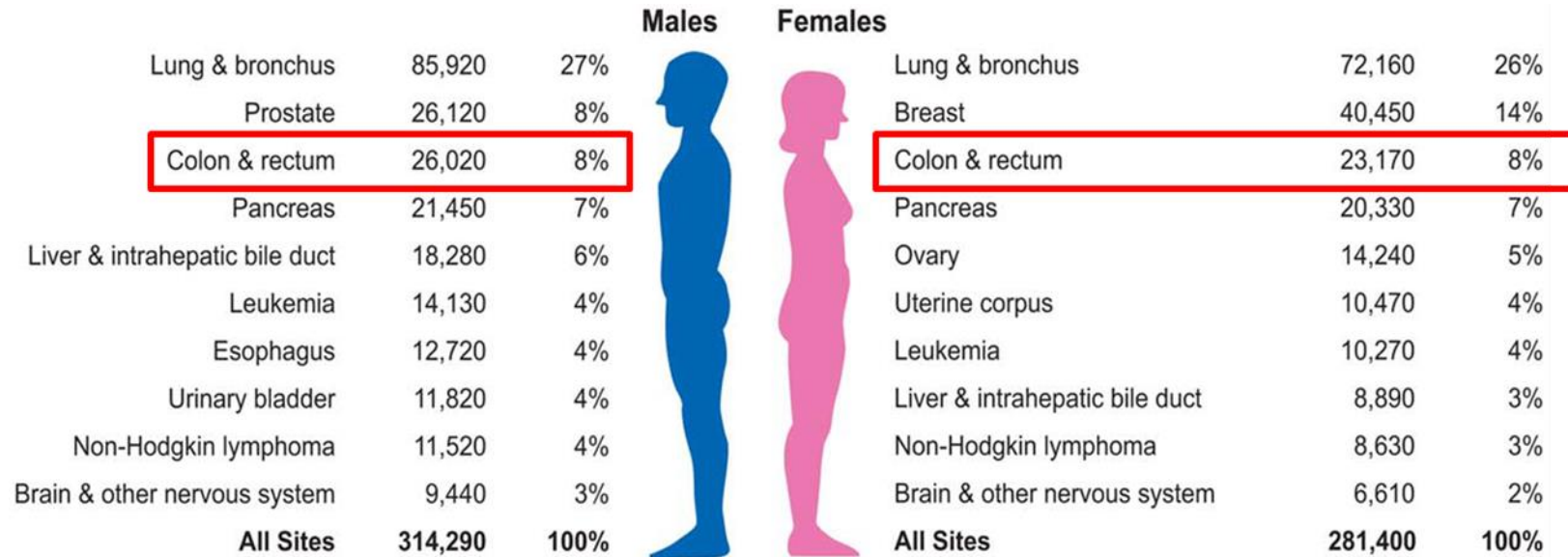
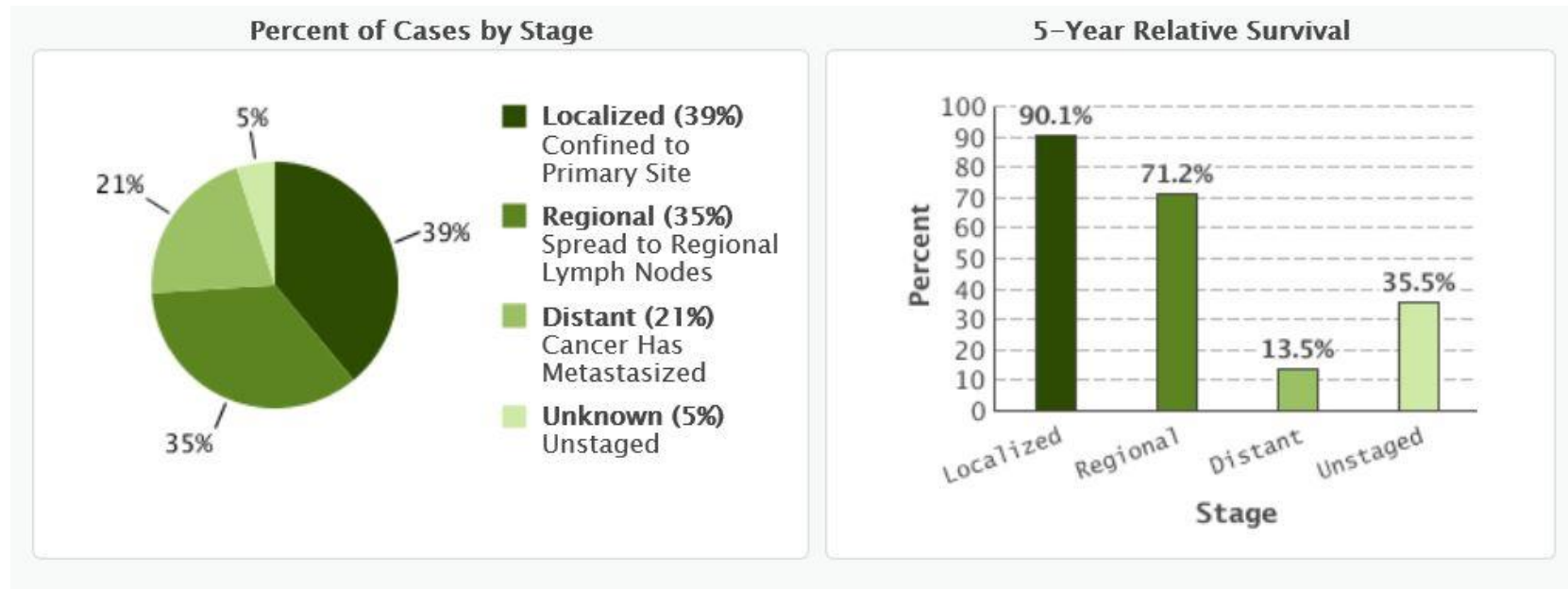


Figure 3. Percent of Cases and 5-Year Relative Survival by Stage at Diagnosis for Colorectal Cancer



Sheets SSF. Colon and Rectum Cancer. Cancer Statistics: Statistical Summaries National Cancer Institute. 2014. Available from: <http://seercancer.gov/statfacts/html/colorecthtml> [Accessed 28th September 2016].

Both environmental and inherited risk factors have also been identified to contribute to the development of CRC. Modifiable environmental factors include a high fat/high red meat diet, excess alcohol consumption, obesity, smoking, and lack of physical activity. Non-modifiable risk factors such as an older age, a personal history of colorectal polyps or inflammatory bowel disease, a family history of colorectal cancer in a first-degree relative, or having an inherited syndrome can all contribute to the development of CRC.

b) Diagnosis of Colorectal Cancer

As stated, the majority of colorectal cancer present at a late stage. This is due, in part, to a delay in presentation of symptoms and signs associated with CRC. In addition, many of these general symptoms, such as abdominal discomfort, bloating, and irregular bowel movements, are often caused by other conditions than cancer. “Red flag” signs include rectal bleeding, blood within the stool, unintentional weight loss, fatigue and unexplained iron-deficiency anemia.

Colonoscopy is the current “gold-standard” for screening and diagnosis of colorectal neoplasia and has >95% sensitivity and 90% specificity.(3) It allows for removal of precancerous polyps, and according to case-control and cohort studies, decreases both CRC incidence and CRC related mortality.(4-8) Colonoscopy is; however, expensive, invasive, has a risk of complications such as bowel perforation, and has a relatively high rate of patient non-compliance. The screening interval of 10 years for colonoscopy has a detection rate of early CRC of only 18-35%.(9-12) Despite these

shortcomings, the broad use of colonoscopy for the last 3 years in the U.S. has been associated with a significant decrease in frequency of CRC.(13)

Flexible sigmoidoscopy has been shown to decrease both CRC incidence as well as the mortality of distal CRC.(14) Other “imaging” tests for cancer screening include barium enema and virtual colonoscopy. Disadvantages of such screening include 1) high patient non-compliance, 2) the invasive nature of such procedures, 3) the need for sedation for colonoscopy, 4) rare patient morbidity such as colon perforation,(15) 5) the need for vigorous bowel cleansing, and 6) expense.(16)

Available less-invasive tests include stool-based assays, such as the guaiac and immunochemical fecal occult blood tests (FOBTs) and DNA-based tests.(17) Although immunochemical FOBTs are superior to guaiac FOBTs, their ability to detect premalignant colorectal adenomas is limited. A recent German prospective screening study assessed the two best-performing immunohistochemical FOBTs and determined the sensitivity for detection of advanced adenomas of 25% and 27%.(18) Stool-based DNA testing identified 54% of patients with adenomas >1 cm in size with 90% specificity.(19) Due to the nature of the tested substance, stool-based testing is not popular among patients, physicians, or lab personnel. A stool-based bowel-screening program in the United Kingdom found that only 50% participated in screening; and of those with an abnormal guaiac FOBT, only 83% underwent subsequent colonoscopy.(20)

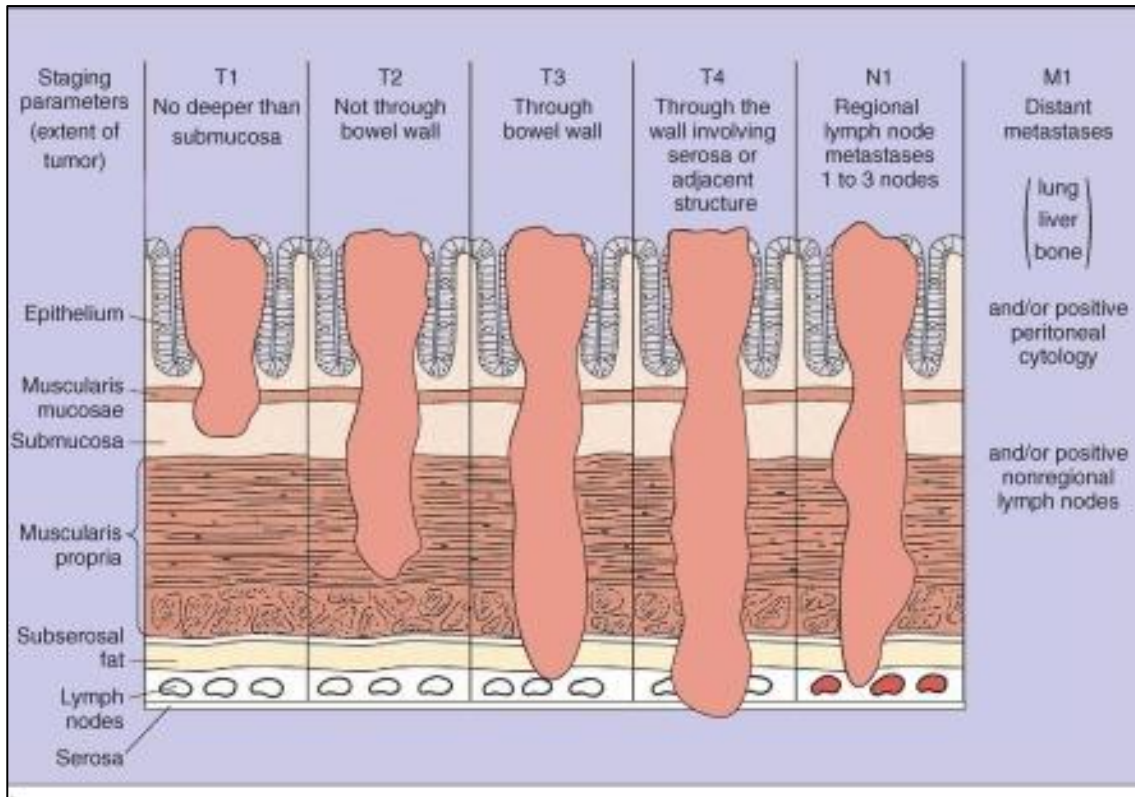
The only plasma-based assay that has been regularly available for clinical monitoring, and, in some cases, for CRC screening, has been the carcinoembryonic antigen (CEA) assay, which is also used for post-operative surveillance and for

monitoring response to therapy. CEA lacks sufficient sensitivity and specificity (36-74% and 87% respectively) for use as a population screening tool or for detecting CRC recurrence.(21, 22) Carbohydrate antigen 19-9 (CA 19-9) has also been used as a prognostic tumor marker, but it is even less sensitive than CEA for CRC.(23) Recently, CA11-19, has been identified as a promising serologic tumor marker which has been reported to detect early CRC with a sensitivity of 98% and specificity of 84%.(24)

c) Colorectal Cancer Staging

Cancer staging is performed for diagnostic purposes and to also determine the best treatment for patients. Staging in CRC refers to the extent of local invasion, the degree of lymph node involvement, and whether there is distant metastasis. The most common and accepted staging system from the American Joint Committee on Cancer (AJCC) is called the Tumor Node Metastases (TNM) system. This system is comprised of three categories, where by “T” represents the degree of invasion of cancer into the bowel wall, “N” the amount of lymph node involvement, and “M” to the presence of metastasis (Figure 4) (Table 1). The older Dukes’ staging of colorectal cancer, described by a British pathologist in the 1930’s and more commonly used within Europe, has largely been replaced by the TNM staging system to ensure a standardized practice of reporting. The initial Dukes’ classification was divided into three categories, Dukes’ A, B, and C with each stage representing more advanced disease (Figure 5). Modifications to the Dukes’ classification have been developed, such as the Astler-Coller classification, which subdivided Dukes’ B and C stages, and added a D stage for metastatic disease, to allow for more detailed information for the prognosis and management of the cancer.

Figure 4. Tumor Node Metastases (TNM) Staging of Colon Cancer



T represents how far the primary tumor has grown into the intestinal wall and whether it has invaded nearby surrounding tissues

N denotes the number of regional lymph nodes infiltrated by disease

M indicates if the cancer has metastasized to other organs of the body

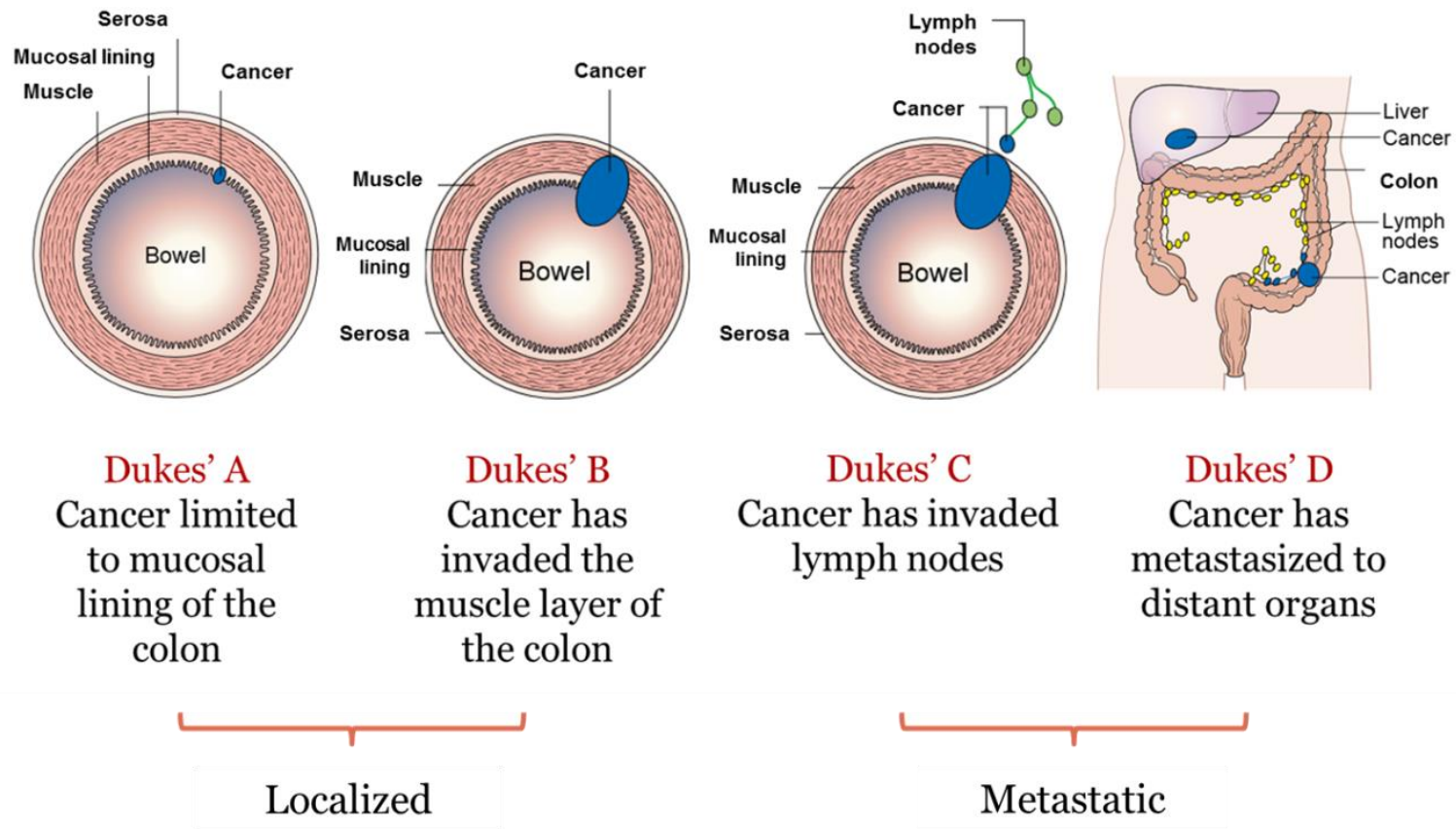
Kasper D, Fauci A, Hauser S, Longo D, Jameson J. 2015. Harrison's Principles of Internal Medicine. 19th ed. New York: McGraw-Hill Education.

Table 1. Modified AJCC TNM Staging and Corresponding Dukes' Classification

Stage	Primary Tumor (T)	Regional Lymph Nodes (N)	Distant Metastases (M)	Dukes' Stage	Primary Tumor	Regional Lymph Nodes	Distant Metastasis
0	Tis	N0	M0	-	Tis – Cancer in situ	N0 – No regional lymph nodes	M0 – No metastasis
I	T1/2	N0	M0	A	T1 – Invasion into submucosa	N1 – Metastasis in 1-3 pericolic nodes	M1 – Distant metastasis
IIA	T3	N0	M0	B	T2 – Invasion into muscularis propria	N2 – Metastasis into ≥ 4 pericolic nodes	
IIB	T4a	N0	M0		T3 – Invasion into serosa		
IIC	T4b	N0	M0		T4 – Invasion into adjacent structures		
IIIA	T1/2	N1	M0	C			
IIIB	T3/4	N1	M0				
IIIC	Any T	N2	M0				
IV	Any T	Any N	M1	D			

Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol. 2010;17(6):1471-4.

Figure 5. Dukes' Classification of Colon Cancer



d) Treatment for Colorectal Cancer

For the majority of cases, the mainstay of treatment for colorectal cancer is excision of the tumor either surgically or endoscopically. Other adjunct agents include chemotherapy, radiation therapy, or targeted therapy. Typically precursor colorectal adenomas can be removed via colonoscopy and very early stage cancers can be removed by local excision. Early stage disease is best treated surgically with resection of the tumor and surrounding tissue to ensure adequate removal of the cancer as demonstrated by the absence of tumor cells at the resected surgical margin. Chemotherapy, either adjuvant or neoadjuvant, may be indicated in patients presenting with more advanced disease defined in the case of colon cancer by lymph node metastasis. Chemotherapy can be administered systemically, regionally, or by hepatic artery infusion in cases where the cancer has metastasized to the liver. Commonly used chemotherapeutic agents include 5-fluorouracil, leucovorin, capecitabine, oxaliplatin and irinotecan. Often, two or more of these drugs are given in combination to increase their efficacy.

Not all CRCs respond to traditional chemotherapy. As research has advanced to understand the genetic and protein changes involved in the development of CRC, newer drugs have been developed to target these changes. Classes of targeted drugs include those that 1) act to stop blood vessel formation by targeting vascular endothelial growth factor, a protein involved in angiogenesis, 2) drugs that target epidermal growth factor receptor, a protein present on the cell surface that is involved in the growth of cancer cells, and 3) drugs that inhibit kinase activity, proteins on or near the surface of cells that transmit signals to the control center of cells.

In patients with isolated or few metastases to the liver or lung, it is possible to surgically remove these with the primary tumor. For other forms of advanced disease, newer techniques such as ablation and embolization therapy can be used to destroy the tumor. These methods of treatment are often reserved for patients who are unable to have surgery for other reasons, or whose disease burden is so great it cannot be removed surgically. Palliative care is a form of medical care used for symptom control in advanced and end stage disease in patients. Palliative care can be operative or non-operative depending on the symptoms experienced with the overall aim to improve a patient's quality of life.

CHAPTER II

MOLECULAR MECHANISMS OF COLORECTAL CARCINOGENESIS

Colorectal cancer presents in one of three major forms: inherited, sporadic, and familial. Inherited and familial forms of CRC are from germline mutations, with inherited CRC accounting for <10% and familial accounting for approximately 25% of all CRC cases. Common inherited conditions leading to the development of CRC include Familial adenomatous polyposis (FAP), its variant Gardner syndrome, Lynch syndrome (hereditary non-polyposis colon cancer), and Peutz-Jeghers syndrome. Each of these syndromes are caused by inherited mutations in genes associated with cell growth or DNA repair. Sporadic CRC, derived from somatic or acquired gene mutations, is by far the most common presentation of CRC, accounting for up to 70% of all cases. It is not associated with family history.

In CRC, three distinct pathways of genomic instability underlie the development of sporadic and inherited colorectal carcinogenesis: chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) pathway. This thesis will focus only on sporadic CRC and the CIN pathway.

The CIN pathway, also known as the adenoma-carcinoma sequence, follows a predictable progression of genetic mutations and corresponding histologic

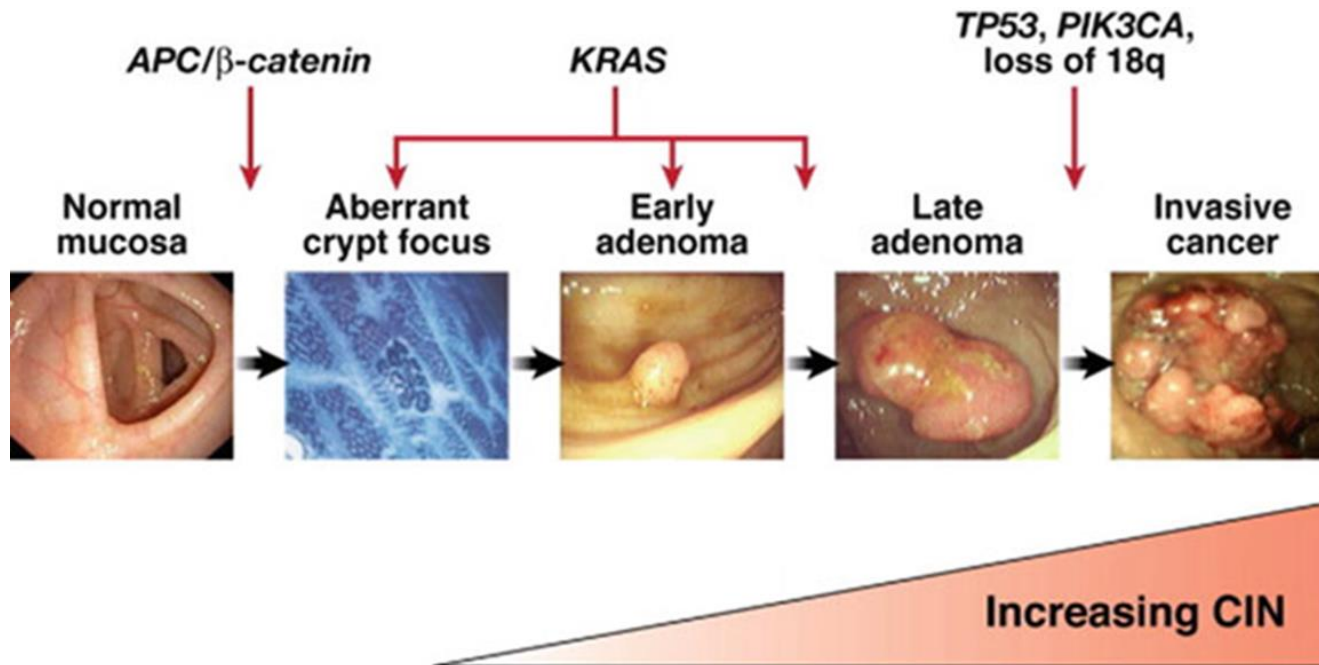
changes. It is well recognized that the majority of sporadic CRC's develop from adenomas or polyps. Genetic changes drive normal colonic epithelium to invasive cancer through a four-step progression: firstly, transformation of normal epithelium to an adenoma or polyp, proceeding to an in-situ carcinoma, and ultimately to an invasive and metastatic adenocarcinoma. The adenoma-carcinoma sequence is the most common colorectal polyp pathway accounting for around 80% of cases, the other 20% being via the serrated polyp pathway.

In this adenoma-carcinoma sequence, normal mechanisms regulating epithelial renewal are disrupted. Under normal conditions, surface cells are lost due to apoptosis, cell proliferation occurs at the crypt base, and at the luminal surface cells cease proliferation and terminally differentiate. As adenoma size increases, this normally ordered process is increasingly disrupted, the cells become dysplastic and develop invasive potential. Since the 1970's indirect evidence such as pathologic, epidemiologic and observational data has supported this sequence of events. Firstly, residual benign adenomatous tissues have been found in resected carcinoma specimens. Secondly, malignant foci have been observed in advanced adenomas. Thirdly, longitudinal studies have shown that benign polyps develop into invasive CRC over time. Stryker et al. observed 226 patients with colonic polyps >1 cm in diameter who declined surgical or endoscopic resection. At 20-year follow-up, patients had a 24% risk of developing invasive adenocarcinoma at the site of the index polyp, and a 35% risk of carcinoma at any colonic site. (25) The National Polyp Study also confirmed the hypothesis that CRC arises from adenomas by showing that endoscopic polypectomy reduced the risk of developing a subsequent CRC. (5)

There are well-defined molecular genetic steps within the CIN pathway that contribute to the development of invasive carcinoma whereby mutational activation of oncogenes and inactivation of tumor suppressor genes occur. (26) The initial step in the CIN pathway is identification of a dysplastic aberrant crypt focus, a microscopic mucosal lesion that precedes the development of a polyp. (27, 28) At this stage, mutations in the adenomatous polyposis coli (APC) gene cause activation of the Wnt signaling pathway. Progression to late adenoma and early stage carcinoma requires the activation of the KRAS proto-oncogene. Additional malignant transformation is caused by mutations in TP53, PIK3CA, and TGF- β pathway genes (Figure 6). (29-31) Key principles defining the CIN pathway include multiple genetic mutations, the step-wise progression from normal mucosa to invasive carcinoma, and aberrant crypt foci of the colon as the precursor to adenoma formation. (28, 32)

Recent evidence within the last 15 years defines an alternative route to the development of CRC. The serrated polyp pathway is responsible for ~20% of all sporadic colorectal cancers. Hyperplastic polyps were previously regarded as benign lesions with little or no malignant potential. It is now recognized that these polyps form part of a heterogeneous group, termed serrated polyps, which are characterized by their saw-toothed appearance. This group contains three premalignant lesions: the hyperplastic polyp, the traditional serrated adenoma, and the sessile serrated adenoma. It is believed that the sessile serrated adenoma is the main precursor lesion within this pathway leading to the development of CRC (Figure 7). Similar to the CIN pathway, a series of molecular mutations contribute to the development of sporadic CRC. Such mutations include BRAF

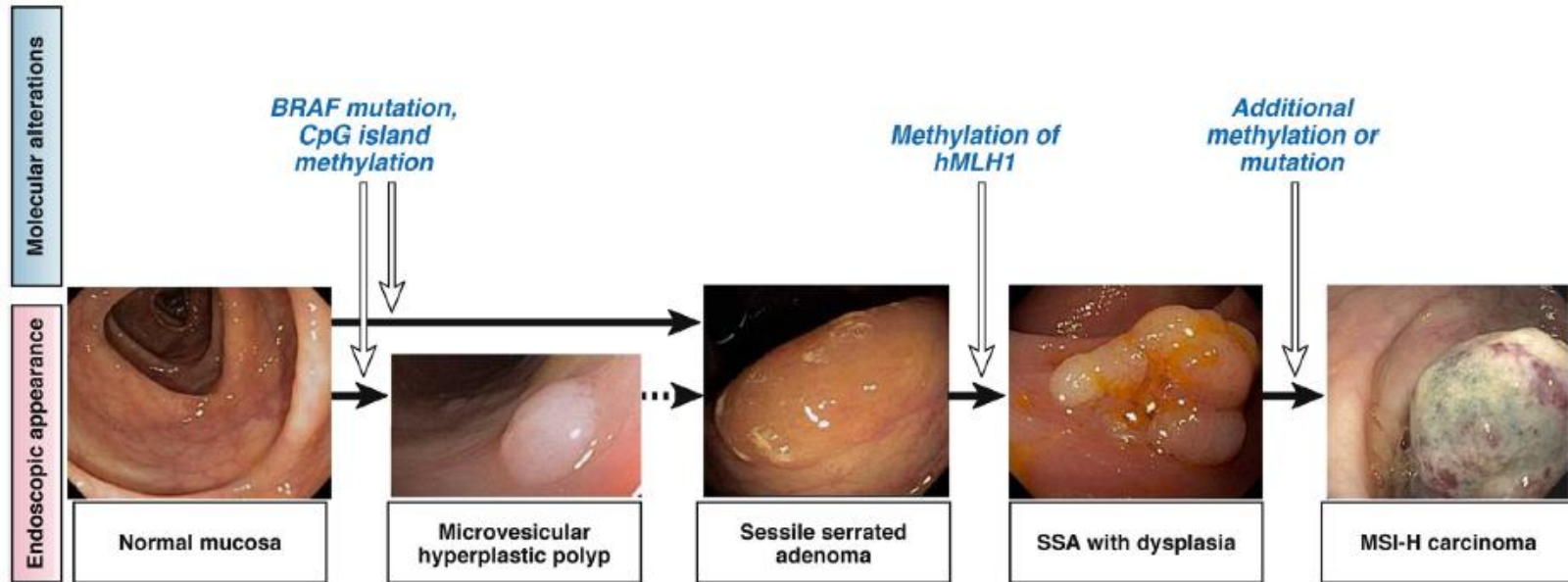
Figure 6. The Chromosomal Instability Pathway (CIN) in Colon Cancer



Multistep genetic model of colorectal carcinogenesis. The initial step is formation of aberrant crypt foci following mutations in the APC gene. Progression to larger adenomas and early carcinoma require activating mutations in KRAS oncogene TP52 and loss of heterozygosity at chromosome 18q Chromosomal instability increases with tumor progression.

Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology*. 2010;138(6):2059-72.

Figure 7. The Serrated Polyp Pathway in Colon Cancer



17

Proposed serrated polyp pathway to MSI colorectal cancer. Characteristic crypt alterations, and epigenetic and genetic alterations

Crockett SD, Snover DC, Ahnen DJ, Baron JA. Sessile serrated adenomas: an evidence-based guide to management. Clin Gastroenterol Hepatol. 2015;13(1):11-26 e1.

mutation and CpG island methylation which causes inactivation of the MLH1 mismatch repair gene resulting in microsatellite instability (MSI). Sessile serrated adenomas are also believed to be a significant contributor to development of CIMP and microsatellite stable (MSS) carcinomas.(33, 34)

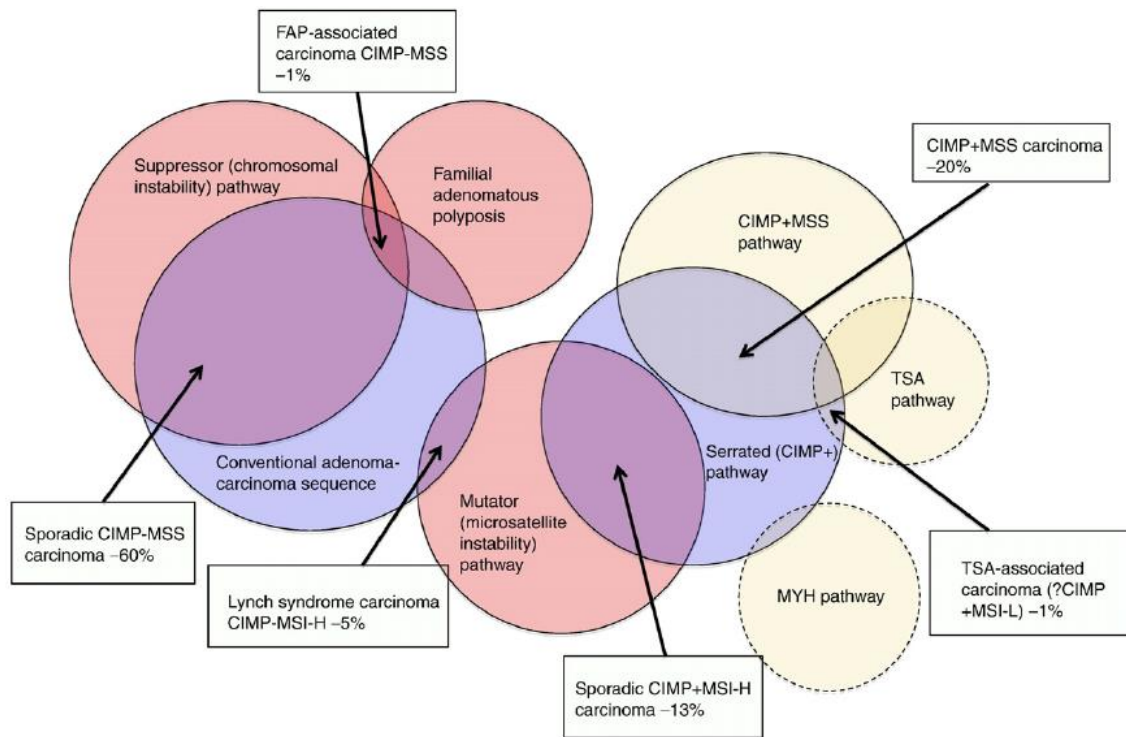
Defects in mismatch repair genes (MMR) lead to MSI carcinomas. MSI colorectal carcinomas account for ~15% of all CRC tumors. The term microsatellite refers to repeated short sequences of DNA. In normal conditions within cells MMR enzymes correct spontaneous errors within these regions during DNA replication. Defects in MMR genes result in a reduced capacity of these cells to repair specific types of DNA damage. The majority of MMR defects in sporadic CRC are due to epigenetic silencing of Mut L homologue 1 (MLH1) gene expression by promoter hypermethylation.(35) Two phenotypes of MSI exist. The first is MSI-High, defined as the presence of instability in greater than 30% of markers and characteristically cause silencing of MLH1. Patients with MSI-High colorectal cancer tend to be younger, and despite the histological features of poor differentiation, patients tend to have a better long-term prognosis than those with MSS cancers. MSI-low cancers, defined as the presence of instability in 10-29% of markers, do not differ from microsatellite stable tumors and typically arise from the CIN pathway.(26) Hereditary Non-Polyposis Colorectal Cancer (HNPCC), as part of Lynch Syndrome, is an autosomal dominant disease caused by germline mutations in MMR genes. MSI resulting from this genetic defect is seen in more than 95% of all patients with HNPCC. HNPCC accounts for up to 5% of all CRC cases.

CpG island methylated phenotype (CIMP) pathway is the third, and most recently described, major molecular pathway involved in colorectal carcinogenesis. A CpG site is

a region of DNA where cytosine occurs next to guanine. DNA methylation occurs at these sites and 80-90% of all CpG are methylated. The remaining 10% form cluster “islands” in the promoter region and are unmethylated in the germ line. When vast hypermethylation of the promoter CpG island sites occur, tumor suppressor genes are silenced and cancer develops. The precursor lesion is considered to be a sessile serrated adenoma.(36)

Understanding the molecular and genetic differences involved in each pathway in colorectal tumorigenesis, as described above, can be difficult. Figure 8 shows a schematic representation of several of the overlapping pathways involved in the development of colorectal carcinoma.(37) In conclusion sporadic CRC develops via a multistep process of specific genetic changes driving tumorigenesis. Single, specific germline mutations underlie inherited syndromes, whereas sporadic CRC results from accumulation of somatic mutations. The three major molecular carcinogenic pathways are the chromosomal instability, microsatellite instability, and CpG Island methylated phenotype pathways. Abnormalities underlying familial CRC remain poorly understood.

Figure 8. Schematic representation of several overlapping pathways involved in the development of colorectal cancer



Red circles represent mechanisms based on tumor suppressor and mutator pathways.

Blue circles represent mechanisms based on the precursor lesion (CIN and Serrated polyp pathways).

Yellow circles represent currently poorly characterized pathways.

Snover DC. Update on the serrated pathway to colorectal carcinoma. *Hum Pathol.* 2011;42(1):1-10.

Kirsten rat sarcoma viral oncogene homolog (KRAS) in Colorectal Cancer

1) History

Ras is a family of small guanosine-nucleotide-binding proteins (G-proteins) involved in transmitting signals within cells. In humans, the three most clinically notable members of the Ras family of genes are HRAS, KRAS and NRAS. KRAS and HRAS, the first of the two Ras genes were identified from studies of cancer-causing viruses, namely Kirsten and Harvey sarcoma virus, respectively. (38) These viruses were originally discovered in rats by Jennifer Harvey and Werner Kirsten in the 1960's, coining the name Rat sarcoma. (39, 40) Discovery was made in 1982 that a single nucleotide substitution within KRAS gene was responsible for activating mutation in human cells and transformation of the subsequent protein resulted in various malignancies. (41-44)

2) Function

The protein product of the normal KRAS gene performs an essential function in normal tissue signaling. Mutation within the gene is implicated in the development of many cancers, in particular, colorectal cancer. KRAS mutations have been observed in some 10% of colorectal adenomas, up to 50% of colorectal adenomas displaying severe dysplasia, and 35-45% of colorectal carcinomas.(45-47) Located on human chromosome 12, KRAS is a membrane-bound guanosine triphosphate/guanosine diphosphate (GTP/GDP)-binding protein. It contains four coding exons and one 5' non-coding exon. Single nucleotide point mutations in KRAS occur in codons 12 and 13 of exon 2, in codon 146 in exon 4, and rarely in codon 61 of exon 3. (48, 49)

KRAS acts as a molecular on/off switch downstream of growth factor receptors such as the epidermal growth factor receptor (EGFR). Under normal physiologic conditions, activation of KRAS occurs from upstream signaling by the exchange of bound GDP for GTP. KRAS is a membrane-bound GTPase that cycles between the active GTP-bound form and the inactive GDP-bound form. This process is transient due to GTPase-activating protein (GAP) mediated GTP hydrolysis. Upon conversion of GTP to GDP, KRAS is turned off. When mutations occur, the intrinsic activity of KRAS GTPase is impaired which prevent GAPs from GTP hydrolysis, locking the KRAS protein in its active GTP-bound form. This results in over activation of downstream proliferative signaling pathways. (50) The consequence of this includes increased cell proliferation, reduced apoptosis, altered cell metabolism and changes in the tumor microenvironment.

Mitogen-Activated Protein Kinase (MAPK) Pathway

The Mitogen-activated protein kinase (MAPK) pathway is a chain of proteins within a cell that communicates signals from cell surface membrane receptors to the DNA in the nucleus of a cell. The communication from the cell surface to the nucleus occurs through the addition of phosphate groups to neighboring proteins. These steps act as “on” or “off” switches, however; when a protein within the pathway is mutated, it can cause the pathway to become stuck in either the “on” or “off” position. This is typically a necessary step in the development of cancer and is frequently dysregulated in approximately one-third of all cancers.

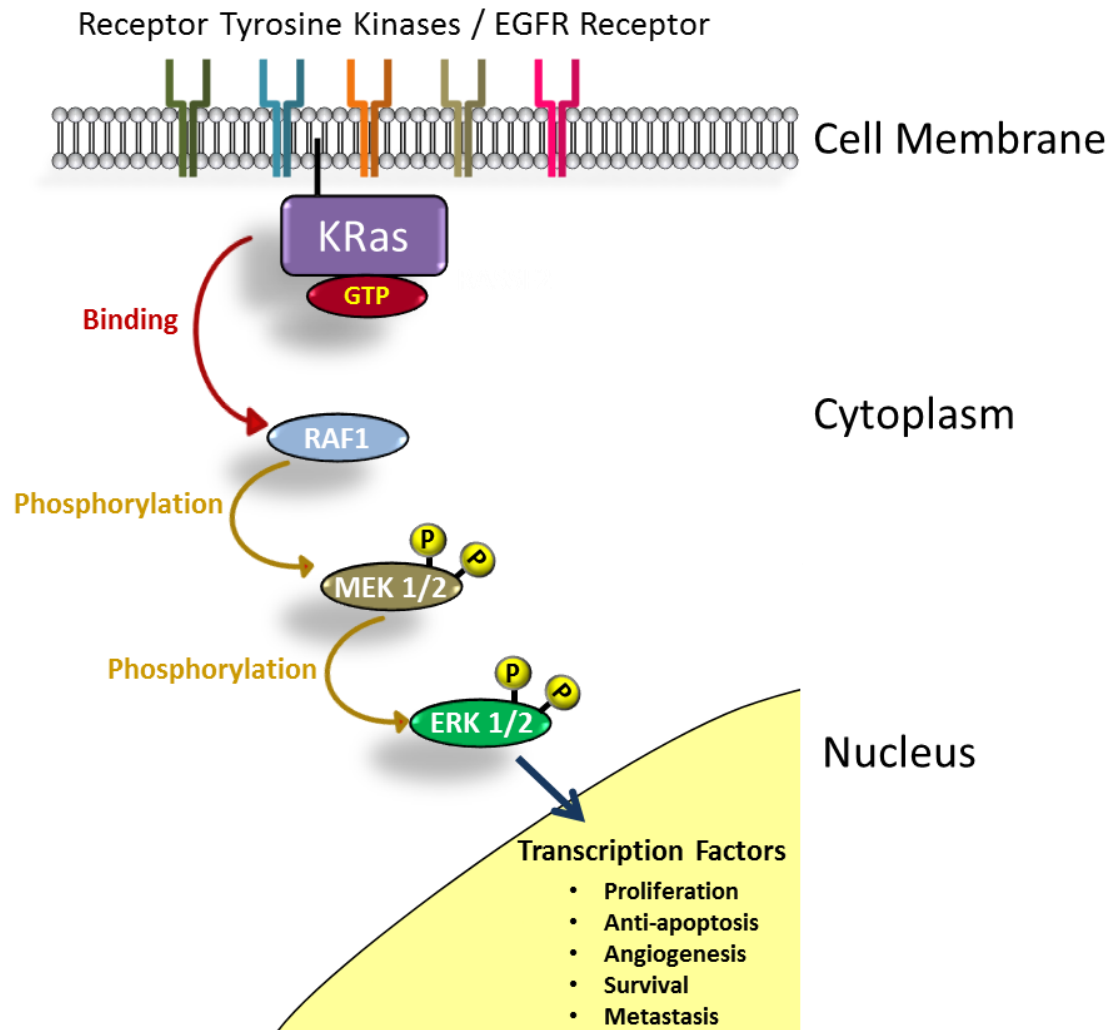
There are three major subfamilies, namely the extracellular-signal-regulated kinases (ERK MAPK, Ras/Raf1/MEK/ERK), the c-jun N-terminal kinase or stress-

activated protein kinases (JNK or SAPK), and MAPK14. They are located downstream of growth-factor receptors, including epidermal growth factor receptors. Overexpression and activation of this receptor is commonly observed in CRC. Of these sub families, the ERK MAPK pathway is one of the most important for cell proliferation. Several proto-oncogenes transduce the signals that promote cell growth and differentiation within this cascade. (51) It is for these reasons that the ERK MAPK pathway is believed to be implicated in the pathogenesis, progression, and oncogenic behavior of CRC. (52) For this reason, kinases within the ERK MAPK pathway have the potential to be used as targets for the treatment of CRC.

The MAPK pathway comprises of three-tiers of protein kinases, MAPK1 - 3, in which the top tier activates the subsequent level, which in turn activates the next level. In order for activation of the downstream MAPK within the pathway, it is phosphorylated by mitogen-activated protein kinase kinase (MAP2K), which in turn has been activated by mitogen-activate protein kinase kinase kinase (MAP3K).

The initial step to activate the ERK MAPK pathway is through extracellular signals transmitted via protein kinase C (PKC) or Ras. PKC activity promotes binding of GTP to members of the Ras family, leading to activation of Raf. Raf (MAP3K) phosphorylates MEK1 and MEK2 (MAP2K), which in turn phosphorylates ERK 1/2 (MAP1K). MEK1 and MEK2, also known as ERK kinases, are dual-specificity protein kinases that function to control cell growth and differentiation within the MAPK cascade. Activation of ERK results in migration of enzymes to the nucleus of the cell, where phosphorylation of transcription factors regulate genes that increase cell proliferation and reduce apoptosis (Figure 9).(53)

Figure 9. Schematic Overview of the ERK MAPK Pathway



Extracellular signal proteins bind to receptor tyrosine kinases. K-Ras is activated by binding to GTP. Active K-Ras binds to Raf1 and initiates a phosphorylation cascade. Downstream transcription factors are affected leading to altered gene expression.

Raf = MAP3K

MEK 1/2 = MAP2K

ERK 1/2 = MAP1K

CHAPTER III

MICRORNAs AND COLORECTAL CANCER

a) Overview and Biogenesis

microRNAs (miRNAs) are recognized to play a crucial role in the regulation of cell function through specific interactions with their messenger RNA (mRNA) counterparts. They are small, naturally occurring, non-protein coding RNA molecules that downregulate gene expression and affect subsequent protein expression. A single miRNA can bind to and regulate many different mRNA targets and, conversely, several different miRNAs can bind to and jointly control a single mRNA.

miRNAs are transcribed in the nucleus as large RNA precursors called primary-miRNAs (pri-miRNAs) (54). These pri-miRNAs are enzymatically cleaved in the nucleus by the enzyme Drosha into precursor miRNA (pre-miRNA). (55) The resulting approximately 70-nucleotide pre-miRNA are folded into stem-loop structures. These pre-miRNAs are then exported into the cytoplasm by the GTP-dependent transport protein exportin 5. (56) Once in the cytoplasm, they undergo additional processing by the enzyme Dicer to generate the mature double-stranded miRNA approximately 22 nucleotides in length. Dicer also initiates the formation of the RNA-induced silencing complex (RISC). (57) It is in this final part of miRNA biogenesis that the leading miRNA

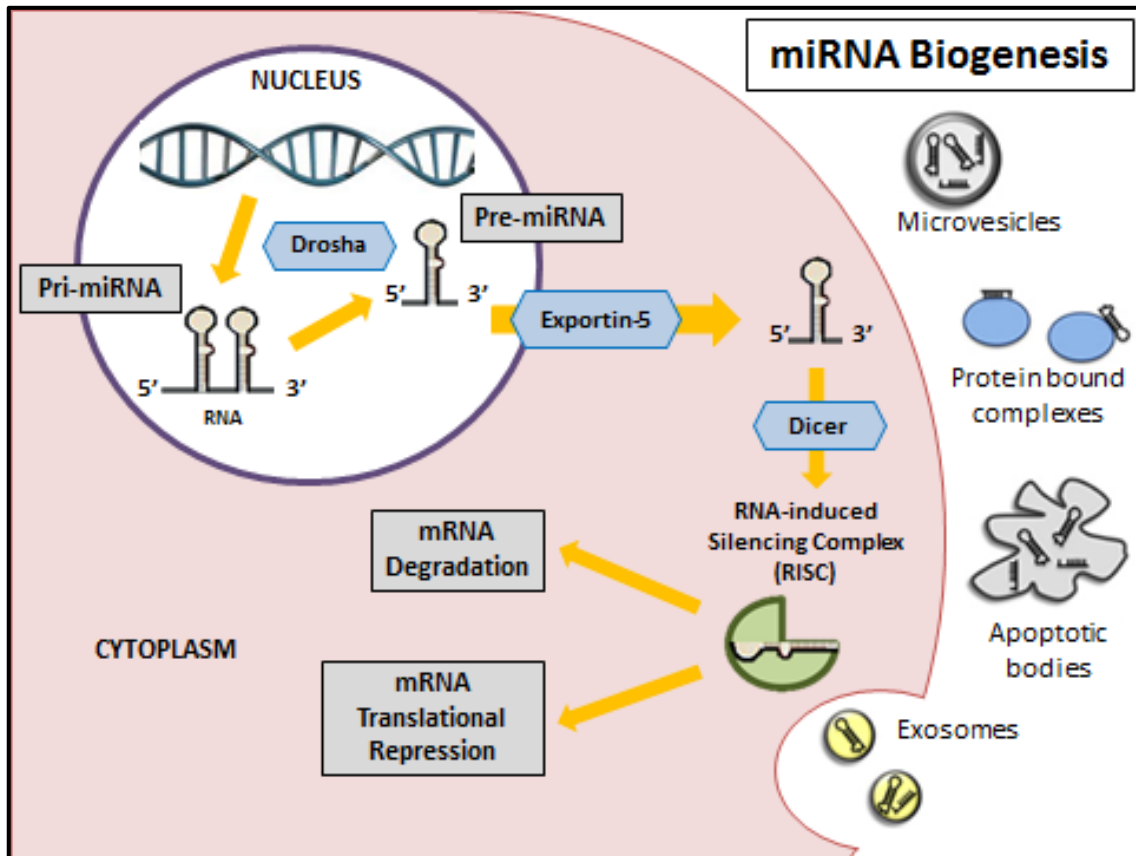
strand is incorporated into the RISC complex, which is then guided into the complementary 3' or 5' untranslated region of the target mRNA. The RISC is responsible for the gene silencing observed due to miRNA expression and RNA interference (Figure 10). (58) miRNAs cause translational repression, target degradation or gene silencing and therefore affect subsequent protein expression.

miRNAs exhibit a variety of crucial regulatory functions related to cell growth, development, and differentiation. (59, 60) miRNAs can function as either tumor suppressor genes or oncogenes. Overexpression of miRNAs in cancer may function as oncogenes and promote oncogenesis by down-regulating tumor suppressors or other genes involved in cell differentiation. In contrast, miRNAs in cancer may function as tumor suppressors by inhibiting oncogenesis via down-regulation of proteins with oncogenic qualities. (61, 62) Studying the specific function of miRNAs in human carcinogenesis will help to identify new targets for cancer research, diagnosis and treatment.

b) microRNAs as Biomarkers for Diagnosis of Colorectal Cancer

Biological markers or biomarkers are defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention”. (63) Biomarkers, such as blood, urine and cerebrospinal fluid, can be used for screening, diagnosis, and predicting prognosis in human diseases. (64) Plasma and serum are components of blood and are commonly used in diagnostic assays. Serum is of similar

Figure 10. microRNA Biogenesis



MicroRNAs are transcribed from DNA into pri-microRNA and are then processed by Drosha into the pre-microRNA. The pre-microRNA is transported into the cytoplasm by Exportin-5 and processed by Dicer and integrated into the RNA-induced silencing complex (RISC). The complementarity of the microRNA-seed sequence determines the effect on the target mRNA: a 100% match results in the degradation of the mRNA whereas an 80% match inhibits protein production. Drosha and Dicer are RNA-processing enzyme located in the nucleus or cytoplasm, respectively. miRNAs circulate in various secreted extracellular vesicles, such as microvesicles, apoptotic bodies and exosomes, or bound to proteins.

composition to plasma except for lacking clotting factors. Both plasma and serum contain hormones, glucose, electrolytes, antibodies, antigens and nutrients making them ideal media in diagnostic testing. (65) The most commonly used blood-based CRC biomarker, carcinoembryonic antigen (CEA), which is used for post-operative surveillance and for monitoring response to therapy, lacks sensitivity and specificity for screening or for the detection of recurrent CRC (see also pg. 6-7). (21)

Significant advances have occurred in the field of tumor-associated miRNAs since their first discovery in plasma as the number of studies investigating their expression has markedly increased in recent years. (66, 67) Although colonoscopy remains the gold standard screening test for the diagnosis of CRC, the evaluation of miRNA within plasma and/or serum of circulating blood has provided a new area for biomarker research. (4, 6, 7, 68)

miRNAs are stable in extracellular fluid since they are protected from RNases by virtue of being bound to argonaute proteins. (69) Discovery of dysregulated miRNA expression has been identified in colorectal, esophageal, lung, liver, pancreatic, bladder, ovarian, and gastric cancers.(70, 71) miRNAs have been identified in numerous body fluids, such as plasma, saliva, feces, and urine. (67, 72) These factors suggest that miRNAs can be used as biomarkers for cancer and other disease states. (73)

The miR-17-92 cluster, also known as oncomiR-1 was the first miRNA “oncogene” to be described. (74) Six miRNAs form this miR-17-92 cluster: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1 and are known to be dysregulated in many cancers. (75) miR-17-92 is overexpressed in both tissue and serum

of CRC patients. (76, 77) In a recent systematic review, 5 of the 6 members of this cluster, with the exception of miR-19b-1, were significantly upregulated in the serum or plasma of CRC patients as compared to controls in 8 studies. (77-84) The most predictive miRNA was miR-92a with area under the curve (AUC) values ranging from 0.786 to 0.890, 65.5 to 89% sensitivity and 70 to 82.5% specificity. The remaining member of the cluster, miR-19b-1, was also significantly upregulated in CRC patients when combined as a 2 miRNA panel together with miR-19a, with an AUC of 0.82, 78.57% sensitivity and 77.36% specificity. (85) These data support the oncogenic role of the miR-17-92 cluster in CRC.

miR-21 has been identified in multiple studies to be upregulated in CRC patients as compared to healthy controls. Kanaan *et al.* identified miR-21 as a biomarker for CRC. (72) Validated CRC tissue miRNAs were evaluated in a plasma test set consisting of 30 CRC patients and 30 healthy controls. The most dysregulated tissue miRNAs were then validated in different cohort of 20 CRC patients with 20 age- and race-matched subjects without CRC. In the plasma test group, miR-21 differentiated CRC patients from controls with an AUC 0.910 and a 90% specificity and sensitivity. (72) Following this, 6 groups reproduced similar results with AUC's ranging from 0.647 – 0.927, 65 – 87.5% sensitivity and 74.4 – 93.2% specificity. Although these data were promising, dysregulation of miR-21 has been described in many other cancers. (86-93) There is therefore need for a plasma miRNA profile specific for CRC.

miR-29a is another miRNA that has been reported to be upregulated in CRC patients in 3 studies. (78, 80, 94) miR-29a is part of the miR-29 family (miR-29a, miR-29b, miR-29c). Interestingly, another member of the miR-29 family, miR-29b, was

observed to be downregulated in CRC compared to control subjects in 2 studies. (95, 96) Members of the miR-29 family exhibit differential regulation, implying that their functional relevance may not be identical. (97) miR-29b has been shown to function as a tumor suppressor in various cancers and diseases (98), while miR-29a has been shown to act as a tumor suppressor in lung cancer (99), but as an oncogene in CRC, ovarian and breast cancer. (100, 101)

In order to reduce CRC associated mortality, the ideal biomarker would be able to identify individuals with both early stage CRC and its precursor lesion, the colorectal advanced adenoma (CAA), from healthy control subjects. CAA are defined as adenomas with a villous component, those with high grade dysplasia, or size greater than 0.75cm size in diameter. (102) In the studies included in the same recent review, 7 studies evaluated miRNAs found to be significantly dysregulated in CRC patients, and in 420 patients with CAA. Five of these 7 studies reported miRNA panels significantly upregulated in CAA as compared to healthy controls. (78, 81, 82, 103, 104) One study also identified a panel of 5 miRNAs that distinguished between CRC and CAA with an AUC of 0.856. (103) The remaining 2 studies found no significant differences in miRNA profile between CAA and healthy controls. (85, 105)

Identification of numerous miRNAs associated with colorectal carcinogenesis demonstrates that miRNAs can distinguish patients with CRC from healthy controls with high sensitivity and specificity comparable to that of other common, currently used invasive screening methods for CRC.

However, no single miRNA has yet been identified as an ideal biomarker for the diagnosis of CRC. With deaths from colorectal cancer amongst the leading cause of cancer deaths worldwide, there is an emphasis to increase screening, and therefore identify early, less invasive stage disease, and to improve survival. For this purpose, miRNAs have the potential in the future to be used as a relatively non-invasive, inexpensive, blood-based marker for the detection of CRC.

c) miRNAs and Colorectal Cancer Prognosis

Currently, clinicopathologic tumor staging based on the tumor–node–metastasis (TNM) system is the basic prognostic marker for CRC clinical outcomes. The TNM system describes the degree to which the tumor has invaded the bowel wall and spread to the regional lymph nodes as well as metastasized to distant organs. Although the TNM staging system is the mainstay of assessing prognosis, this classification has weaknesses. Inadequate examination of lymph nodes may lead to under staging of the tumor and subsequent inadequate treatment.(106) Additionally, histologically identical CRC patients may have different genetic and epigenetic backgrounds that lead to distinct disease progression and clinical outcomes. Unfortunately, no prognostic marker is currently available for identifying the patients who would benefit from more-aggressive treatments. Recent epigenetic studies have suggested that miRNAs may help to better categorize CRC subtypes and predict outcomes.

miR-21 is one of the most studied and most promising prognostic miRNAs in CRC. Increased levels of miR-21 have been correlated with CRC cell proliferation, invasion, lymph node metastases, and advanced clinical stage.(107-111)

Members of the miR-29 family exhibit differential regulation, implying that their functional relevance may not be identical. miR-29a has been identified to be significantly elevated in patients with liver metastases in multiple studies,(78, 112, 113) however; in other studies, upregulation of miR-29a was associated with a better 12 month survival.(114, 115) In addition, elevated levels of another member of the miR-29 family, miR-29b, was associated with higher 5-year disease free and overall survival.(116)

Other miRNAs suggestive of poor prognosis include miR-34b/c, miR-155 and miR-130b. Increased expression of each of these miRNAs in CRC tissue is associated with poor prognosis and more advanced stage tumors.(117-119) Downregulation of miR-139-3p in CRC tissue samples was associated with poor survival, especially in those patients with early stage (TNM I and II) CRC. (120)

In order to develop these miRNAs as prognostic markers in CRC, further studies validating these miRNAs are required. It is also important to understand the underlying mechanisms of each prognostic biomarker, and its respective molecular function, both in order to support their clinical use and provide therapeutic targets.

d) miRNAs and Response to Chemotherapy in Colorectal Cancer

Primary treatment of stage I to III CRC's is surgical resection of the primary tumor together with the regional lymph nodes. In patients with high risk stage II and stage III disease, adjuvant chemotherapy should be considered. A common chemotherapy agent used to treat CRC is 5-fluorouracil (5-FU) a pyrimidine analog. 5-FU metabolism is complex, requiring multiple enzymes, including thymidylate synthase (TS). TS catalyzes the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate

(dTMP), which is one of the three nucleotides that forms thymine (121). Thymine is a nucleic acid that is necessary for DNA replication. 5-FU acts to irreversibly inhibit TS, essentially starving rapidly dividing cells by halting DNA production.

In addition to the potential use of miRNAs as biomarkers for detection and for prognosis in CRC patients, several studies have identified miRNAs to predict the sensitivity of CRC to chemotherapy. The key to successful treatment is predicting response to chemotherapy. It has been suggested that 5-FU could alter miRNA expression in colon cancer cells after exposure and treatment thereby affecting the mechanisms involved in 5-FU metabolism. (122)

A recent review by Hollis *et al.* identified ten miRNAs to be associated with chemosensitivity. Of these, upregulation of miR-21, miR-153, miR-19a, miR-106a, miR-130b, and miR-484 was associated with increased resistance to 5-FU and other common chemotherapy agents. Resistance to chemotherapy was also observed when the following four miRNAs, miR-129, miR-15b, miR-1915, and miR-122 were down-regulated.

CHAPTER IV

COLORECTAL CANCER CELL LINE SCREENING

a) Introduction

Cell lines are a common experimental model used to study biological mechanisms involved in various diseases. *In-vitro* cell line studies allow for the investigation of signaling pathways, functional processes, identification of molecular markers of disease, and testing of cancer therapeutics. As previously described, cancer occurs when a somatic cell undergoes a mutation disrupting normal cell cycling resulting in uncontrolled proliferation. Immortalized cell lines are a population of cells that can be grown for prolonged periods due to similar mutations that enable cells to continually proliferate. It is for this reason that cell lines provide an almost unlimited supply of cells with similar genotypes and phenotypes for study. The main advantage of this is consistency, reproducibility, and the associated relative low-cost. However; questions often arise over to what extent the cells actually resemble their tissue of origin after years in culture. The most common way to generate an immortalized cell line is by isolating cells from a naturally occurring cancer. Whilst cell lines generated in this fashion are known to have originated from a specific tissue type, the cells have undergone significant mutation allowing for indefinite proliferation. However; for these reasons, caution must be applied

when interpreting results as the biology of the cell can be altered.

By utilizing well established immortalized CRC cell lines, we are able to manipulate cancer pathology in order to gain a better understanding of cancer biology. We have chosen to characterize the differential expression of miRNAs in CRC as compared to a normal colon epithelial cell line in order to identify potential therapeutic targets for intervention.

b) miRNA Screening of Colorectal Cancer Cell Lines

Five immortalized colorectal cell lines, four sporadic colorectal cancer cell lines, representing each stage of the Modified Dukes' classification, and one normal colon epithelial cell line used for comparison were studied. Our aim was to characterize the differential expression of miRNAs in CRC cell lines as compared to the normal colon epithelial cell line in order to identify potential therapeutic targets for intervention (see pg. 59 for methods).

Comparison of miRNA expression between combined localized stage cancer (Dukes' A and B) and normal colon epithelium, and metastatic stage cancer (Dukes' C and D) and normal colon epithelium was performed (n=4). In order to achieve adequate power and group size of four, each sporadic colorectal cancer cell line, SW1116, SW480, HT-29 and T84 (Dukes' A – D, respectively), was screened twice for miRNA expression and CCD841, normal colon epithelium, was screened four times.

We identified 190 miRNA to be significantly dysregulated, of which 36 were up-regulated and 154 were downregulated in localized stage CRC (Dukes' A and B) compared to normal colon epithelium. Similarly, 231 miRNAs were significantly

dysregulated in metastatic stage CRC (Dukes' C and D) compared to normal colon epithelium, 39 up- and 192 and downregulated, respectively (Table 2). Fold change was calculated to assess the magnitude of change between the two groups compared. In addition to the *p*-value, a change was deemed significant when the fold change was > 2-fold up or down. The twenty most significantly up-regulated and downregulated miRNA for each comparison (localized vs. normal and metastatic vs. normal) with their corresponding fold change are shown in tables 3 & 4. Representative waterfall plots are shown in figures 11 & 12.

Of interest, five of the top eight most significantly up-regulated miRNAs in localized stage CRC compared to normal colon epithelium were miR-200a, miR-429, miR-141, miR-200b, and miR-200c. Similarly, in metastatic stage CRC compared to normal colon epithelium, five of the top six most significantly up-regulated miRNAs were these same five miRNAs (Table 5).

Single assay miRNA expression was performed in each of the cell lines to confirm our findings from screening. Here, miRNA expression in each individual colorectal cancer cell line was compared to the normal colon epithelial cell line.

We confirmed miRNA expression of these five miRNAs to be significantly up-regulated in all colorectal cancer cell lines compared to the normal colon epithelial cell line (Table 6). The Dukes' C CRC cell line had the largest up-regulation of all five miR-200 family members compared to the normal colon epithelial cell line than any of the other Dukes' stage cancer cell lines, and were selected for further study.

Table 2. Significantly Dysregulated miRNAs in Colorectal Cancer Cell Lines Compared to a Normal Colon Epithelial Cell Line

Cell Line Comparison vs. Normal Colon Epithelium	No. of Dysregulated miRNA*	Up-Regulated (n)	Downregulated (n)
Localized Stage Cancer (Dukes' A SW1116 and B SW480)	190	36	154
Metastatic Stage Cancer (Dukes' C HT-29 and D T84)	231	39	192

37

* $p < 0.05$ Student t-test

Table 3. The Most Significantly Up-regulated and Downregulated miRNAs in Localized Colorectal Cancer Cell Lines (Dukes' A SW1116 and B SW480) Compared to the Normal Colon Epithelial Cell Line (CCD 841) and Corresponding Fold Change

38

Up-regulated			Downregulated		
miRNA	Fold Change	<i>p</i> -value	miRNA	Fold Change	<i>p</i> -value
miR-200a	18830	<0.0001	miR-143	-4545	<0.0001
miR-429	11414	<0.0001	miR-329	-5000	<0.0001
miR-203	7406	<0.0001	miR-654-3p	-5556	<0.0001
miR-135b	6566	<0.0001	miR-134	-7143	<0.0001
miR-141	6478	<0.0001	miR-193a-5p	-10000	<0.0001
miR-200b	3756	<0.0001	miR-214	-10000	<0.0001
miR-95	3380	<0.0001	miR-541	-11111	<0.0001
miR-200c	2688	<0.0001	miR-485-3p	-16667	<0.0001
miR-518f	1289	0.041	miR-485-5p	-16667	<0.0001
miR-9	1165	<0.0001	miR-296	-25000	<0.0001
miR-182	1129	<0.0001	miR-409-5p	-33333	<0.0001
miR-183	546	<0.0001	miR-654	-33333	<0.0001
miR-375	532	<0.0001	miR-379	-33333	<0.0001
miR-302a	368	0.003	miR-199a	-50000	<0.0001
miR-142-3p	348	0.008	miR-410	-50000	<0.0001
miR-372	272	0.045	miR-493	-50000	<0.0001
miR-96	151	<0.0001	miR-495	-50000	<0.0001
miR-192	124	0.009	miR-127	-100000	<0.0001
miR-135a	119	<0.0001	miR-369-5p	-100000	<0.0001
miR-196b	105	0.003	miR-655	-100000	<0.0001

Table 4. The Most Significantly Up-regulated and Downregulated miRNAs in Metastatic Colorectal Cancer Cell Lines (Dukes' C HT-29 and D T84) Compared to the Normal Colon Epithelial Cell Line (CCD 841) and Corresponding Fold Change

39

Up-regulated			Downregulated		
miRNA	Fold Change	<i>p</i> -value	miRNA	Fold Change	<i>p</i> -value
miR-200a	35668	<0.0001	miR-486-3p	-250	<0.0001
miR-429	30476	<0.0001	miR-504	-250	0.002
miR-200b	8862	<0.0001	miR-34c	-333	<0.0001
miR-141	6531	<0.0001	miR-542-3p	-333	<0.0001
miR-203	6347	<0.0001	miR-99a	-333	<0.0001
miR-200c	4171	<0.0001	miR-100	-500	<0.0001
miR-135b	3377	<0.0001	miR-424	-500	0.002
miR-375	2490	<0.0001	miR-450a	-500	0.001
miR-183	759	<0.0001	miR-541	-500	<0.0001
miR-182	754	<0.0001	miR-130a	-1000	<0.0001
miR-142-3p	577	0.019	miR-199a	-1000	<0.0001
miR-215	454	0.001	miR-299-5p	-1000	0.001
miR-196b	407	<0.0001	miR-323-3p	-1000	<0.0001
miR-192	296	<0.0001	miR-369-3p	-1000	<0.0001
miR-194	179	<0.0001	miR-409-5p	-1000	<0.0001
miR-96	109	0.001	miR-485-3p	-1000	<0.0001
miR-582-5p	95	<0.0001	miR-485-5p	-1000	<0.0001
miR-95	88	0.022	miR-654	-1000	<0.0001
miR-135a	77	<0.0001	miR-654-3p	-1000	<0.0001
miR-10b	42	0.009	miR-137	-1000	0.004

Figure 11. Waterfall Plot of the Significantly Dysregulated miRNAs in Localized Colorectal Cancer Cell Lines (Dukes' A SW1116 and B SW480) as Compared to the Normal Colon Epithelial Cell Line (CCD 841)

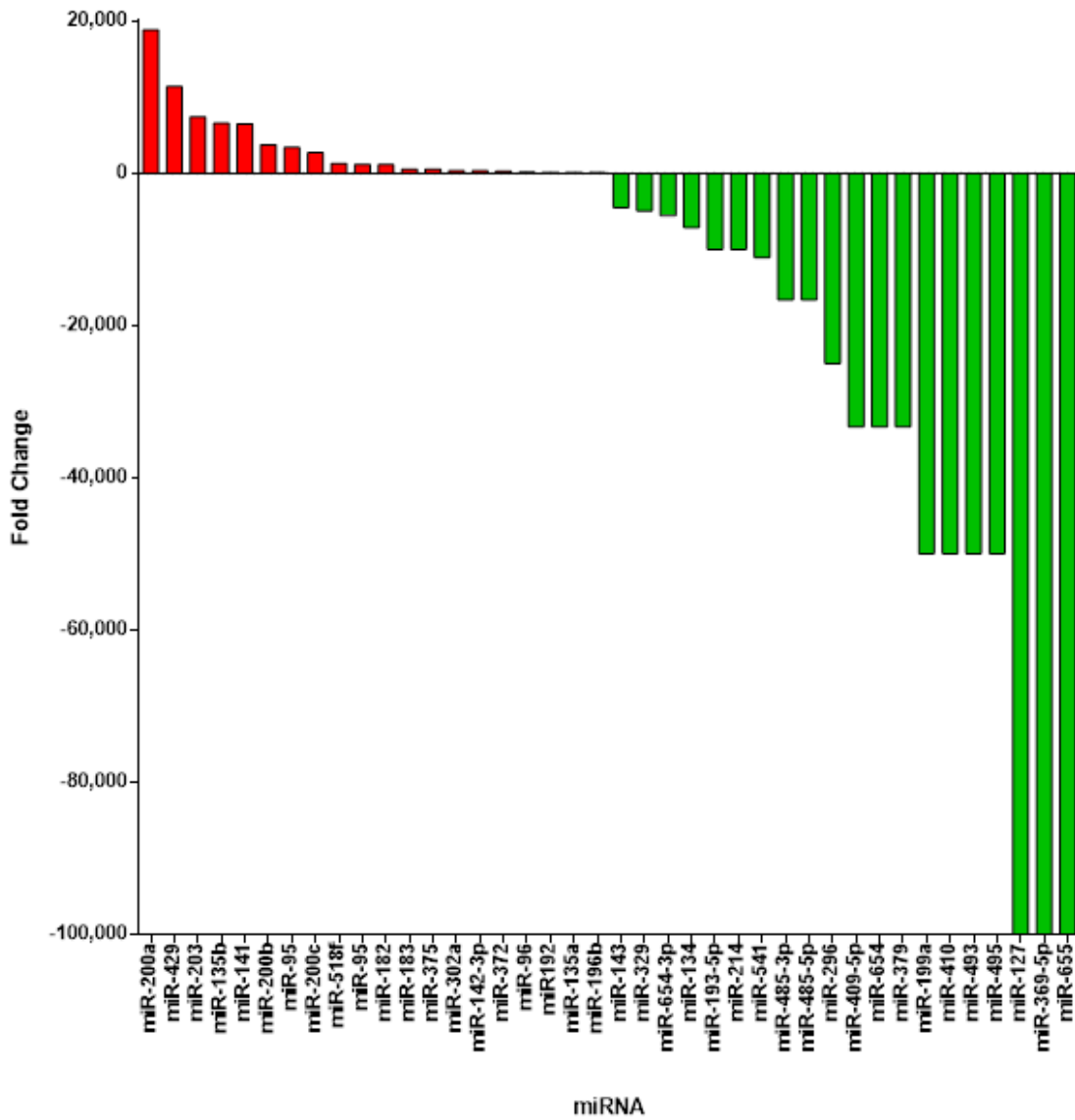


Figure 12. Waterfall Plot of the Significantly Dysregulated miRNAs in Metastatic Colorectal Cancer Cell Lines (Dukes' C HT-29 and D T84) as Compared to the Normal Colon Epithelial Cell Line (CCD 841)

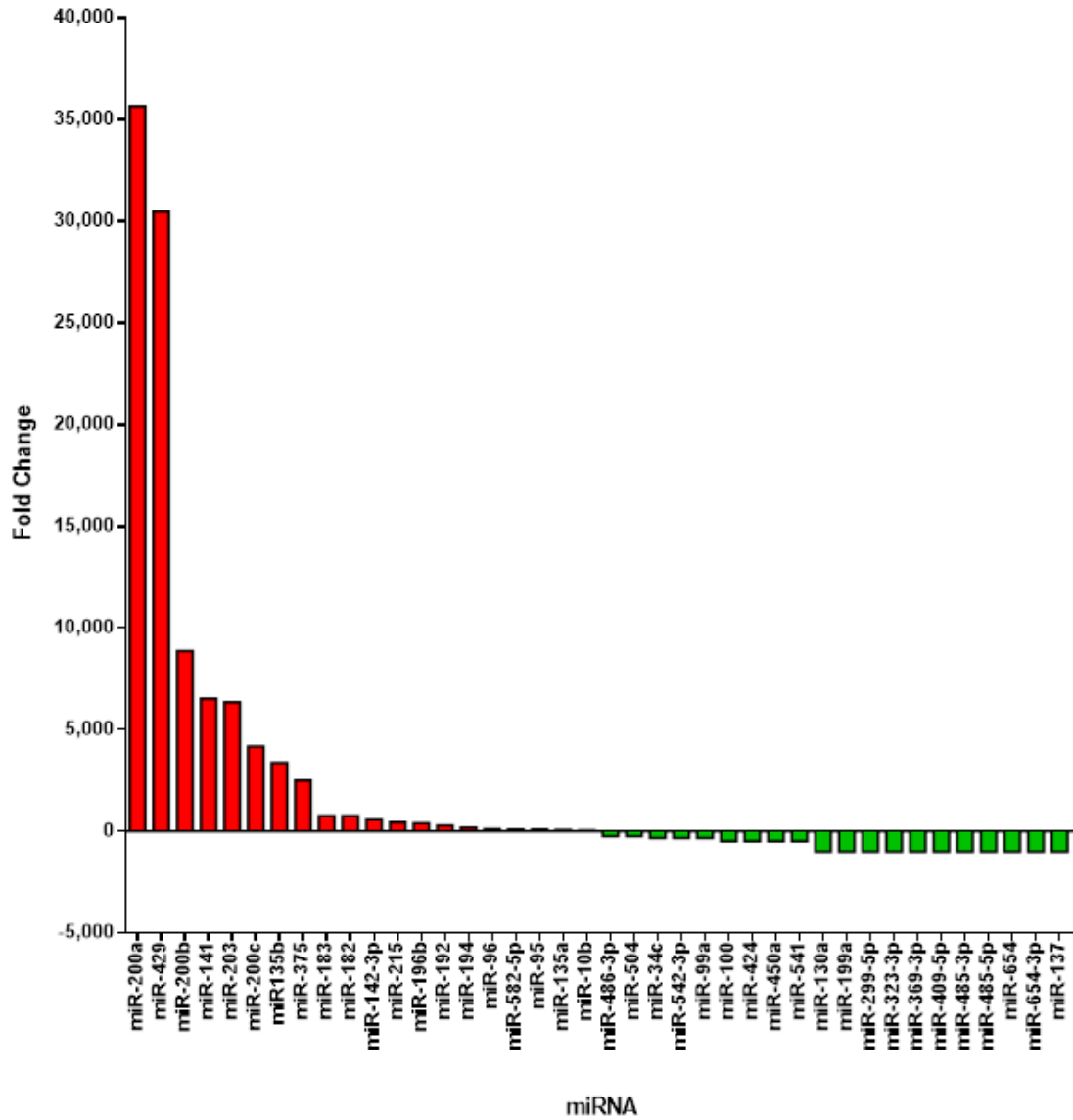


Table 5. miR-200 Family Expression in Colorectal Cancer Cell Line Screening

Cell Line Comparison vs. Normal Colon Epithelium	miRNA	Fold Change	<i>p</i>-value
Localized Stage Cancer (Dukes' A SW1116 and B SW480)	miR-200a	18830	<0.0001
	miR-200b	3756	<0.0001
	miR-200c	2688	<0.0001
	miR-141	6478	<0.0001
	miR-429	11414	<0.0001
Metastatic Stage Cancer (Dukes' C HT-29 and D T84)	miR-200a	35668	<0.0001
	miR-200b	8862	<0.0001
	miR-200c	4171	<0.0001
	miR-141	6531	<0.0001
	miR-429	30476	<0.0001

Table 6. Single Assay miRNA Validation of miR-200 Family in Colorectal Cancer Cell Lines Compared to a Normal Colon Epithelial Cell Line (CCD 841)

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Cell Line Comparison vs. Normal Colon Epithelium								
miRNA	Dukes' A SW1116		Dukes' B SW480		Dukes' C HT-29		Dukes' D T84	
	Fold Change	<i>p</i> -value	Fold Change	<i>p</i> -value	Fold Change	<i>p</i> -value	Fold Change	<i>p</i> -value
miR-200a	1541	<0.0001	1186	<0.0001	15104	<0.0001	1410	<0.0001
miR-200b	4238	<0.0001	3989	<0.0001	22039	<0.0001	3340	<0.0001
miR-200c	3303	<0.0001	3752	<0.0001	7323	<0.0001	5216	<0.0001
miR-141	23207	<0.0001	27067	<0.0001	35026	<0.0001	19135	<0.0001
miR-429	4729	<0.0001	3171	<0.0001	50262	<0.0001	7193	<0.0001

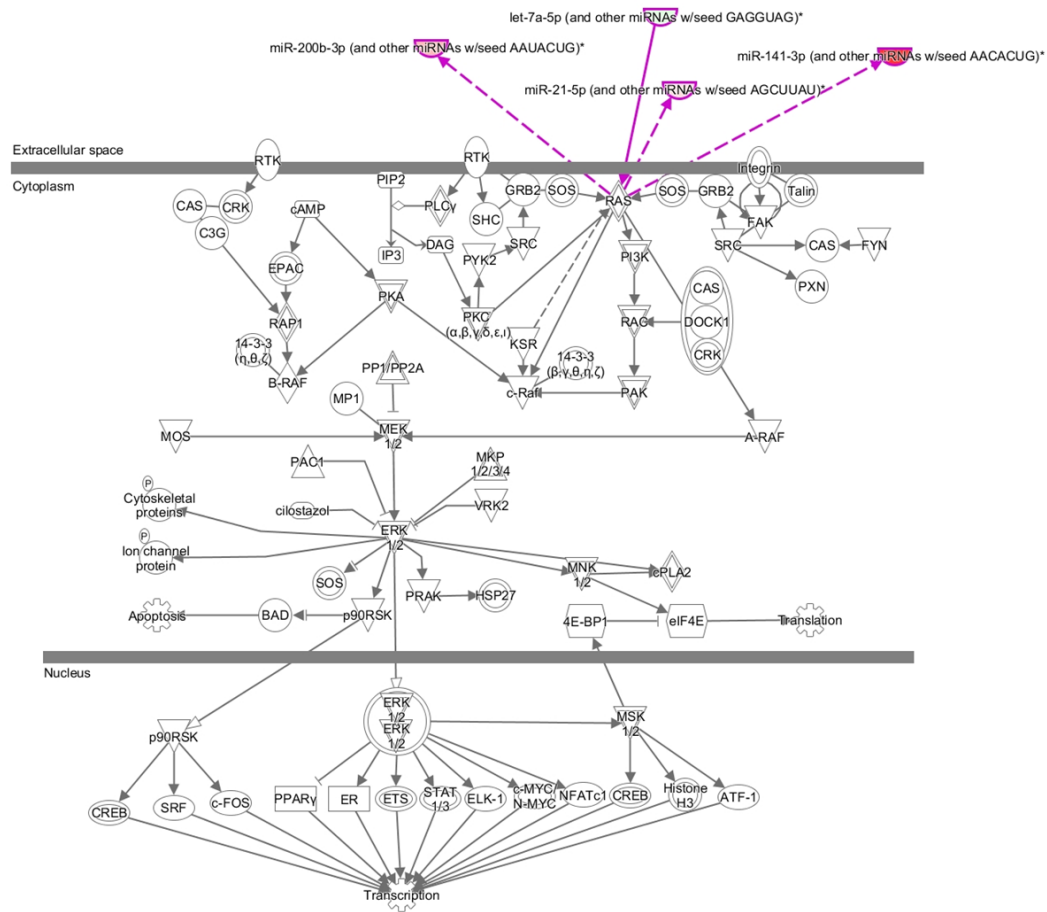
c) Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) is an intuitive web-based application for quickly analyzing and accurately interpreting the biological meaning in your genomic data. It allows for the input of sequencing, Real-Time PCR, and microarray data. Among many of its features, IPA is able to combine filtering tools and microRNA-mRNA content to provide insight into the biological effects of identified miRNAs. It also identifies genes that are shown to be activated in a pathological condition and highlights canonical and non-canonical pathways associated with biological processes. IPA allows for identification of promising targets for therapeutic development.

From our microarray screening, IPA was able to identify miRNAs and the associated pathways they targeted, relevant to our data. The signaling pathway most closely linked to the colorectal cancer cell line screening data was the ERK MAPK pathway as previously described. miR-200b and miR-141 were identified as being strongly upregulated within this pathway (Figure 13).

We used a miRNA target prediction program to identify targets associated to these dysregulated miRNAs. miRTarBase is a database of miRNA-target interactions that are experimentally validated by reporter assay, western blot, microarray and next-generation sequencing experiments. It provides up to date information and combines the largest amount of validated miRNA-target interactions by comparing content from other similar target databases. miRTarBase identified all five miRNAs (miR-200a, miR-200b, miR-200c, miR-141 and miR-429)

Figure 13. ERK MAPK Signaling and miRNAs Results from Ingenuity Pathway Analysis



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The networks were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Figure 14. miR-200a Targets Identified by miRTarBase

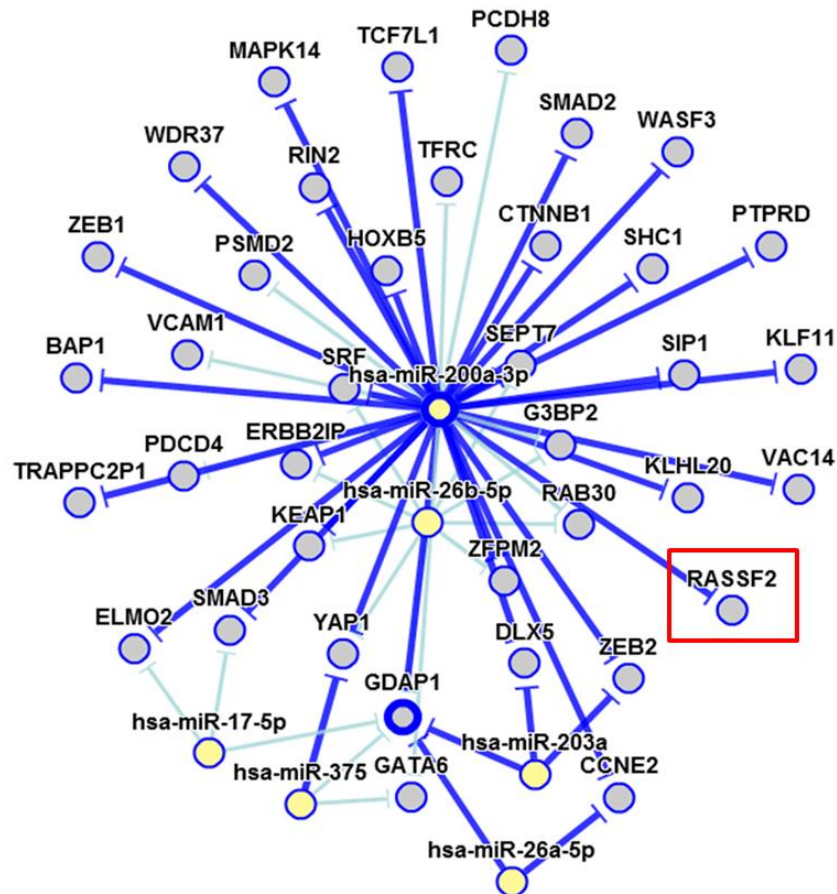
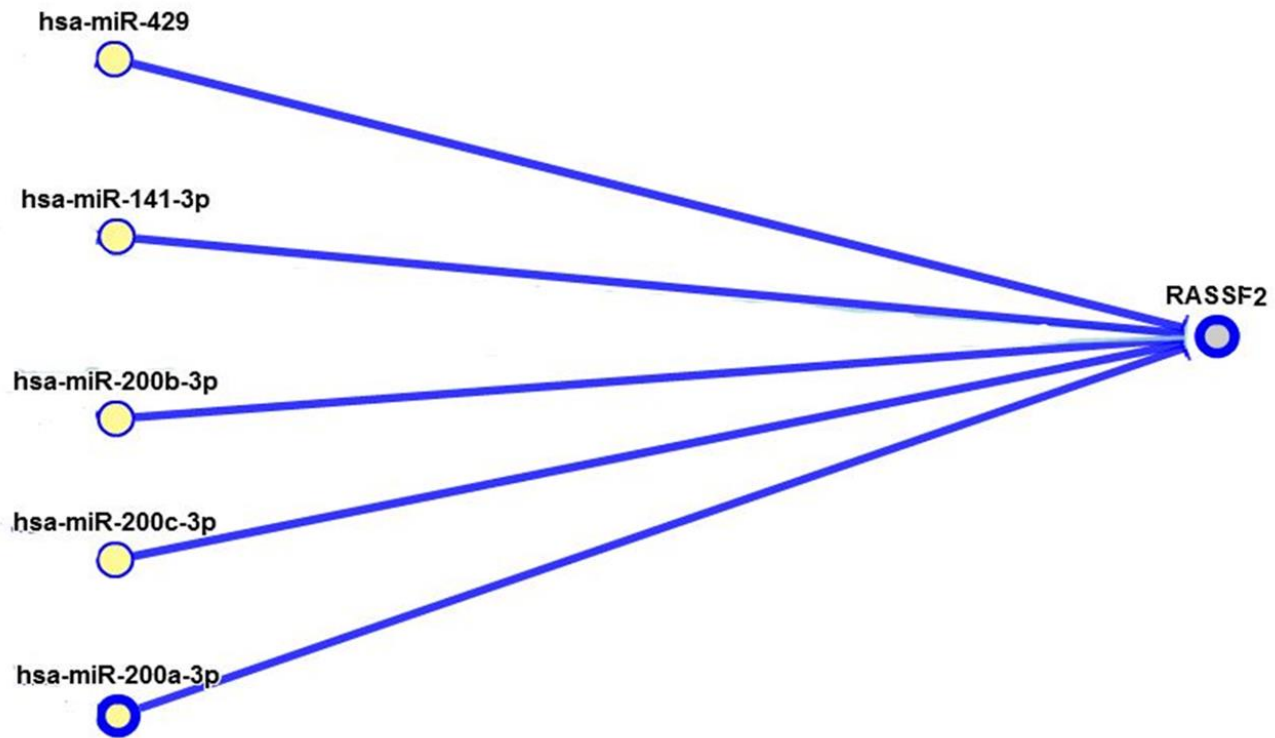


Figure 15. miR-200 Family and its Target RASSF2 as Identified by miRTarBase



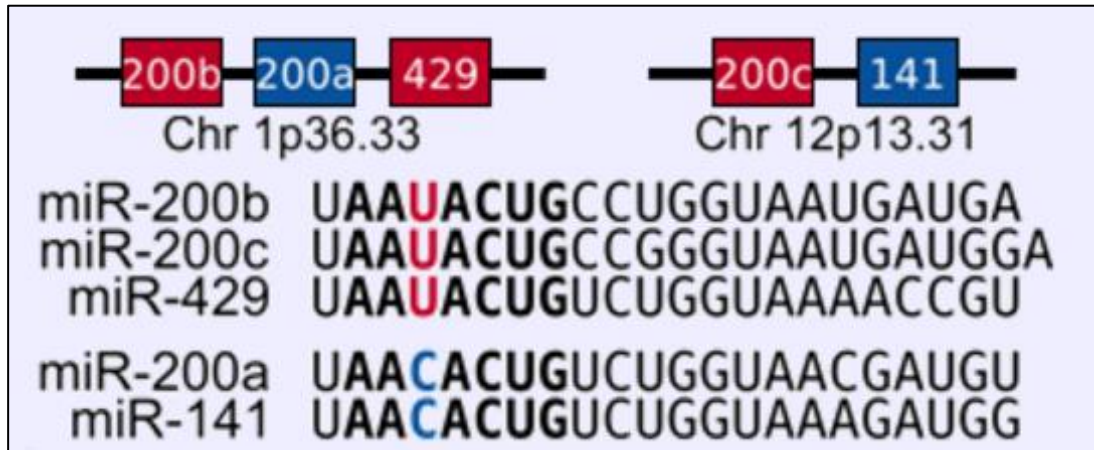
significantly dysregulated in our colorectal cancer cell lines compared to normal colon epithelium to have multiple targets. Of interest, all five of these miRNAs were validated targets of the same gene, Ras associated domain-containing protein 2 (RASFF2) (Figure 14 & 15).

d) microRNA-200 Family

The miR-200 family consists of five members, miR-200a, miR-200b, miR-200c, miR-141, and miR-429. They form two clusters located in two different genomic regions. Cluster 1, miR-200a, miR-200b, and miR-429, is located on chromosome 1, whereas miR-200c, and miR-141 is located on chromosome 12. The seed sequence of a miRNA is the second to eighth nucleotide region at the 5' end of the mature miRNA, that generates the specificity of each miRNA to its target mRNA. Additionally, the miR-200 family can be divided into two functional groups, based on their seed sequences. Functional group 1, miR-200b, miR-200c, and miR-429, and functional group 2, miR-200a and miR-141, with each functional group sharing the same seed sequence (Figure 16). The miR-200 family has been widely investigated in regards to its role in cancer, and is known to be well associated with epithelial to mesenchymal transition (EMT).

EMT is the process by which epithelial cells lose cell polarity, cell to cell adhesion, and gain migratory and invasive properties.(123) These cells transform into mesenchymal stem cells (Figure 17). EMT is essential for numerous development processes, and plays a role in wound healing, fibrosis, and cancer. EMT was recognized as a feature of embryogenesis, and its reverse process, mesenchymal-epithelial transition

Figure 16. Genomic Location of miR-200 Family and Seed Sequence of Functional Groups 1 and 2.



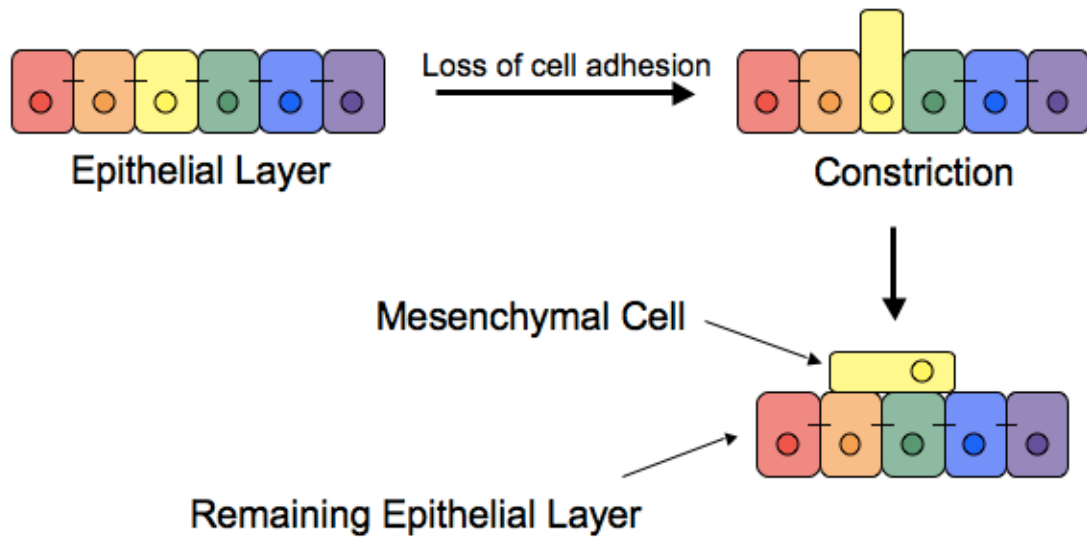
miR-200a, miR-200b and miR-429 located on Chromosome 1

miR-200c and miR-141 located on Chromosome 12

Red = Functional group 1, Blue = Functional group 2. The two functional groups only differ in the seed sequence by one nucleotide, U for Group 1 and C for Group 2.

Jurmeister S, Uhlmann S, Sahin Ö. MIR200C (microRNA 200c). Atlas Genet Cytogenet Oncol Haematol. 2012;16(2):92-99

Figure 17. Epithelial to Mesenchymal Transition



Epithelial to Mesenchymal Cell Transition – loss of cell adhesion leads to constriction and extrusion of newly mesenchymal cell.

Thiery JP, Sleeman JP. (2006). "Complex networks orchestrate epithelial-mesenchymal transitions". *Nature Reviews Molecular Cell Biology* 7: 131–142

(MET), is critical for development of many tissues and organs in the developing embryo. Epithelial cells are stationary, expressing high levels of E-cadherin, whereas mesenchymal cells are able to migrate and invade and express N-cadherin, fibronectin, and vimentin. The loss of E-cadherin is considered a fundamental event in EMT, either direct or indirect. Direct loss occurs when Zinc finger E-box-binding homeobox (ZEB) 1 / ZEB2 binds to E-cadherin and causes repression of transcription. Several signaling pathways may induce EMT. TGF- β activates the expression of ZEB and SNAIL to regulate EMT in cancer. The p53 tumor suppressor inhibits EMT by activating the expression of various miRNAs. EMT is involved in cancer progression and metastasis. The loss of E-cadherin causes cells to lose adhesion which break through the basement membrane, increasing the invasive properties of cells.

Differential expression of the miR-200 family is involved in initiation and progression of malignant transformation. The miR-200 family regulates epithelial to mesenchymal transition by targeting ZEB1 and ZEB2.(124) Low miR-200 levels were identified in breast cancer cells that underwent EMT in response to TGF- β , conversely, increased miR-200 expression prevented TGF- β induced EMT. Induction of EMT occurred with inhibition of the miR-200 family and subsequent upregulation of ZEB1 and ZEB2. Hence, it was concluded that downregulation of the miR-200 family was an important step in tumor progression. Another study identified miR-200 members were downregulated in colorectal adenocarcinomas that had invaded beyond the basement membrane, however; lymph node metastases showed high expression of miR-200. On the other hand, colorectal adenomas and adenocarcinomas with intact basement membranes

had high expression of miR-200 members. Their conclusions supported the involvement of the miR-200 family in EMT and cancer progression.(125)

Significant work has been performed investigating the role of the miR-200 family in many processes. The miR-200 family has been implicated in cell transformation and tumorigenesis, cancer metastasis, tumor growth, angiogenesis, invasion and migration, tumor cell survival in circulation, intra and extravasation, and as a potential diagnostic and prognostic tool.(126) The majority of research with the miR-200 family focuses on EMT of cancer cells where dysregulation is considered to exhibit tumor suppressive behavior by blocking EMT. However; increased levels of miR-200c, in particular, in various cancers contradicts the tumor suppressive role of this miRNA.(127) miR-200 family levels are enriched in epithelial tissues. This is supported by lower expression of miR-200c in metastatic colorectal cancer cell lines, SW620, LOVO, and RKO compared to epithelial colorectal cancer cell lines, SW480, HT-29, and T84.(128-130) Multiple studies have also confirmed the findings of enhanced levels of miR-200c in CRC tissue compared to healthy control tissue.(131-133) Overexpression of miR-200c induced cell proliferation in SW620 CRC cell lines (129), whereas another study observed reduced proliferation in SW480 and SW620 CRC cell lines.(130) Downregulation of miR-200c in HT-29 and T84 CRC cell lines increased cell apoptosis and decreased tumor growth.(134)

Since the data regarding the miR-200 family is contradictory more work is needed to gain a better understanding on the role this family plays in cancer, and to understand the mechanisms involved in cancer initiation and progression. Much of what we already know relates to the individual miRNAs, miR-200b and miR-200c, hence more research

needs to be conducted on the remaining members, miR-200a, miR-141, and miR-429. It is important to be able to distinguish which miR-200 family members act as tumor suppressors and which may promote cancer progression.

e) Ras Associated Domain-Containing Protein (RASSF2)

The Ras family of onco-proteins regulates a wide range of biological processes by interacting with a variety of effector proteins, the most well characterized being BRAF and PI3K. Activated forms of Ras are associated with oncogenesis usually due to mutation, however; they can also exhibit growth-antagonistic effects such as senescence, cell cycle arrest, differentiation, and apoptosis.(135, 136) Previously, a novel Ras effector, RASSF1, was identified as a mediator of apoptosis and cell cycle arrest, with the protein displaying many properties associated with tumor suppressors.(137) Ras Associated Domain-Containing Protein (RASSF2) is one of ten in the RASSF family of proteins encoded by RASSF genes 1-10, and subsequent to the discovery of RASSF1, was identified as a novel K-Ras specific effector and tumor suppressor.(138) RASSF2 was found to be a negative regulator of Ras, by binding directly to K-Ras in a GTP-dependent manner via the Ras effector domain. The interaction of RASSF2 and Ras proteins was found to be specific to K-Ras, rather than H-Ras or N-Ras. RASSF2 was found to promote apoptosis and cell-cycle arrest, and was frequently downregulated in human lung cancer cell lines. In addition, overexpression of RASSF2 inhibited growth of lung cancer cells suggesting that RASSF2 shared the properties, similar to other identified RASSF proteins, in being a potential tumor suppressor. Other groups confirmed the findings that RASSF2 and K-Ras form an endogenous complex, and demonstrated tumor suppressor properties by the loss of expression causing enhanced cell

proliferation and invasion in cell lines.(139) RASSF2 is silenced by aberrant DNA promoter methylation in CRC, and expression has been found to be frequently downregulated in CRC cell lines. This is supported by studies investigating the properties and methylation status of RASSF2 in CRC tissue and colorectal adenoma samples. Multiple studies have reported the methylation of RASSF2 between 42% - 73% in CRC samples, and one study reported the methylation status of RASSF2 of 100% in colorectal adenoma samples.(140-142)

It is widely accepted that RASSF members belong to a recently identified family of tumor suppressor Ras effectors for which epigenetic silencing by promoter methylation has been shown to occur throughout the progression of cancer including CRC.(143-145) Clarification of cellular mechanisms that regulate the signaling pathways RASSFs are involved in is further needed, however; RASSFs may have the potential to be developed as a molecular biomarker or targeted therapy for CRC.

CHAPTER V

HYPOTHESIS, SPECIFIC AIMS, AND EXPERIMENTAL PLAN

a) Key Objective

To investigate the role of the miR-200 family on the tumor suppressor RASSF2, and determine the effect it has on activity within the mitogen-activated protein kinase (MAPK) signaling pathway, in a normal colon epithelial (CCD 841) cell line and a Dukes' C (HT-29) colorectal cancer cell line.

b) Hypothesis

The MAPK pathway is a major regulator of cell proliferation and is frequently mutated in CRC. miRNAs from the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, miR-429) have been shown to promote cell proliferation when transfected into various cell lines. It has been shown that the miR-200 family targets RASSF2, a negative regulator of K-Ras, however; the relationship between miR-200, RASSF2, K-Ras, and subsequent activity within the MAPK pathway in colorectal cancer, has not yet been fully described.

We hypothesize that miR-200 family expression differs between normal colon epithelial (CCD 841) and Dukes' C (HT-29) colorectal cancer cells and manipulation of

their expression could modify cancer cell behavior. In doing so, the miR-200 family may have the potential to be developed as a therapeutic intervention in CRC (Figure 18).

c) Specific Aims

1: To determine the expression of the miR-200 family and RASSF2 in both a normal colon epithelial (CCD 841) and a sporadic Dukes' C (HT-29) colorectal cancer cell line.

2: To examine the gain and loss of function of the miR-200 family and the resulting effect on RASSF2 expression, in both a normal colon epithelial (CCD 841) and a sporadic Dukes' C (HT-29) colorectal cancer cell line, respectively.

3: To demonstrate the effect of the miR-200 family on MAPK signaling pathway activity as measured by activation of pathway proteins and resulting cell proliferation.

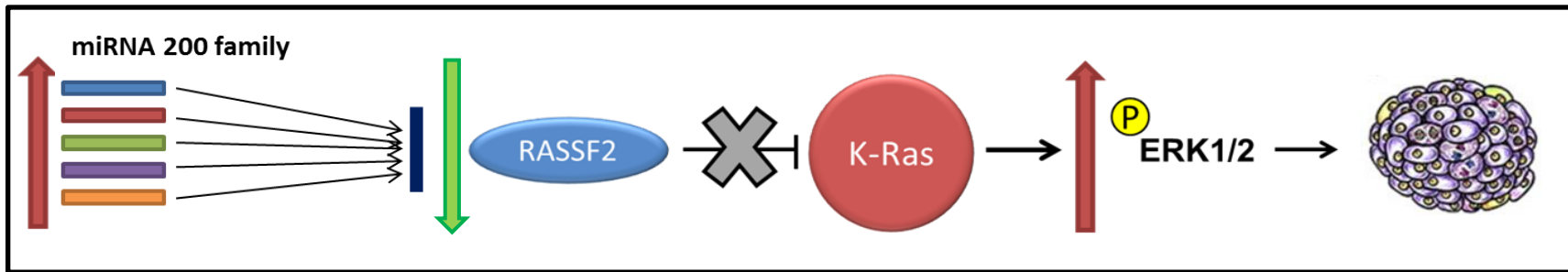
d) Experimental Plan

The overall goal of this project is to investigate the role of the miR-200 family in relation to activity of the MAPK signaling pathway and effect upon cell proliferation mediated via the tumor suppressor RASSF2. We will first evaluate expression of the miR-200 family in a normal colon epithelial (CCD 841) cell line and compare it to the expression in Dukes' stage CRC cell lines. If a difference is observed, RASSF2, a target of the miR-200 family, will be investigated, along with the associated signaling pathway. Secondly, the miR-200 family will be manipulated using miRNA mimics and antagomirs to increase or decrease their expression in the respective cell lines, and RASSF2 mRNA

and protein expression then measured. Finally, MAPK signaling pathway activation will be assessed and cell proliferation, the major resulting functional outcome, will be measured in both normal colon epithelial and Dukes' C CRC cells. We will thus determine whether manipulation of the miR-200 family can influence CRC cell behavior and thereby investigate the potential use of the miR-200 family as a therapeutic target in CRC.

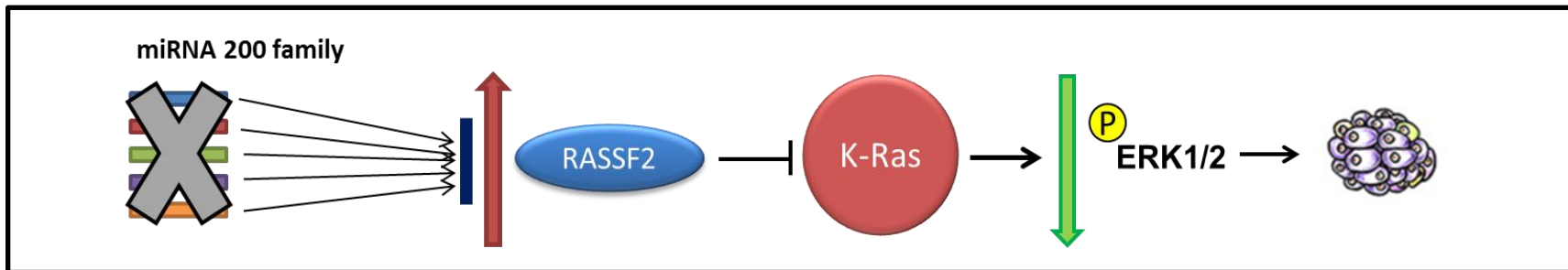
Figure 18. Hypothesis Overview.

miR-200 Family and Normal Colon Epithelium (CCD 841)



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miR-200 Family and Dukes' C (HT-29) Colorectal Cancer



CHAPTER VI

MATERIALS AND METHODS

a. Cell Lines

For the purposes of this study, five cell lines representing the four stages of modified Dukes' A to D colorectal cancer and normal colon epithelium were acquired from American Type Culture Collection (ATCC[®], Manassas, Virginia). Sporadic colorectal cancer cell lines were selected for study. KRAS mutation and microsatellite instability status is shown in Table 7. Cells were cryopreserved in liquid nitrogen at -80°C until being initiated and allowed to grow for future testing. All cell lines were adherent in nature, cultured in cell culture flasks and incubated at 37°C and 5% CO₂. Cells were grown to 70-90% confluence before being harvested for further studies.

1. CCD 841 CoN (ATCC[®] CRL-1790™)

Normal epithelial colon cells harvested from a human female fetus at 21 weeks gestation period, and cultured in Eagle's Minimum Essential Medium (EMEM) with 15% fetal bovine serum (FBS), and supplemented with a 0.5% mixture of penicillin, streptomycin, amphotericin B, and 0.5% L-glutamine (Figure 19).

2. SW1116 (ATCC[®] CCL-233[™])

Dukes' A, grade III, primary colorectal adenocarcinoma epithelial cells were harvested from a 73 year-old man. Cells were cultured in Dulbecco's Modified Eagle (DMEM) medium with 10% FBS, and supplemented with a 0.5% mixture of penicillin, streptomycin, amphotericin B, and 0.5% L-glutamine. This cell line was microsatellite stable and positive for expression of mutated K-Ras oncogene (Figure 20).

3. SW480 (ATCC[®] CCL-228[™])

Dukes' B, primary colorectal adenocarcinoma epithelial cells were harvested from a 50 year-old man. Cells were cultured in Dulbecco's Modified Eagle (DMEM) medium with 10% FBS, and supplemented with a 0.5% mixture of penicillin, streptomycin, amphotericin B, and 0.5% L-glutamine. This cell line was microsatellite stable and positive for expression of K-Ras oncogene with a mutation in codon 12 of the ras oncogene (Figure 21).

4. HT-29 (ATCC[®] HTB-38[™])

Dukes' C, primary colorectal adenocarcinoma epithelial cells were harvested from a 44 year-old woman. The cells were cultured in McCoy's 5A medium with 10% FBS, and supplemented with a 0.5% mixture of penicillin, streptomycin, amphotericin B, and 0.5% L-glutamine. This cell line was microsatellite stable and positive for expression of wild type K-Ras oncogene (Figure 22).

5. T84 (ATCC[®] CCL-248[™])

Dukes' D, secondary colorectal adenocarcinoma epithelial cells derived from a metastatic lung site were harvested from a 72 year-old man. The cells were

cultured in Dulbecco's Modified Eagle's medium and Ham's F-12 (DMEM/F-12) with 10% FBS, and supplemented with a 0.5% mixture of penicillin, streptomycin, amphotericin B, and 0.5% L-glutamine. This cell line was microsatellite stable and positive for expression of mutated K-Ras oncogene (Figure 23).

b. Maintenance of Cell Lines

All cell lines were incubated at 37°C and 5% CO₂ until being transferred to a sterile cell culture hood for subsequent use. Gloved hands were cleansed with 70% ethanol (EtOH) prior to handling and transferring cell culture flasks from the incubator to the hood. All other items transferred into the culture hood were cleansed with 70% EtOH prior to use. Once cells were returned to the incubator, the cell culture hood was cleansed with 70% EtOH to maintain sterility. Only one cell line at a time was used in the culture hood to avoid cross contamination.

1. Establishment of Cell Lines from Cryopreservation

Cell line specific medium was removed from refrigeration and warmed to room temperature in a water bath. For each cell line, 8mL of culture medium was added to a 15mL tube. Another 8mLs of culture medium was then added to a 25cm³ flask. Care was taken not to wet the flask neck in order to avoid contamination. The cell line name, medium, %FBS, passage number of cells, date and initials were written on each flask. Each cryopreserved cell line vial was removed from liquid nitrogen and immediately thawed in a 37°C water bath. The O-ring and cap were suspended above the water to avoid contamination. Once thawed, the contents were transferred to the 15mL tube using a 1000µL pipette. This tube was centrifuged at 830 rpm, at 37°C for 7 minutes, until a

cell pellet was formed. The medium was then aspirated and discarded and the cell pellet re-suspended with 1mL culture medium. This re-suspended cell mixture was then transferred to the labelled 25cm³ culture flask. Cells were visualized within the culture flask under a microscope before transferring to the incubator for growth.

2. Sub-culturing and Splitting of Cells

Cells were visualized daily under a microscope (10X) to monitor growth. Once the cells were deemed to be 70 – 90% confluent they were divided. Appropriate culture medium and a 6mL aliquot of trypsin-ethylenediaminetetraacetic acid (EDTA) were warmed and thawed, respectively, in a water-bath to room temperature before being cleansed with 70% EtOH and placed in the culture hood. The medium was decanted into a waste beaker and 6mL of trypsin was added to the flask and was gently rocked back and forth before being placed back into the incubator for 5 minutes to aid trypsinization. During this time, 18mL of the appropriate cell line culture medium was added to a new 75cm³ flask(s). After 5 minutes, cells were removed from the incubator and observed under an inverted microscope in order to confirm that the cells were no longer adhered to the bottom of the flask. Once this was confirmed, the flask was returned to the culture hood and 9mL culture medium was added to the flask to stop trypsinization and multiple washings performed to ensure cell collection. Following this, the cell suspension was transferred to a new 15mL tube and centrifuged at 830rpm for 7 minutes. The supernatant was discarded and the remaining cell pellet was re-suspended in 1mL medium using a micropipette tip to achieve single-cell suspension. An additional 4mL medium was then added and thoroughly mixed to dilute the cells.

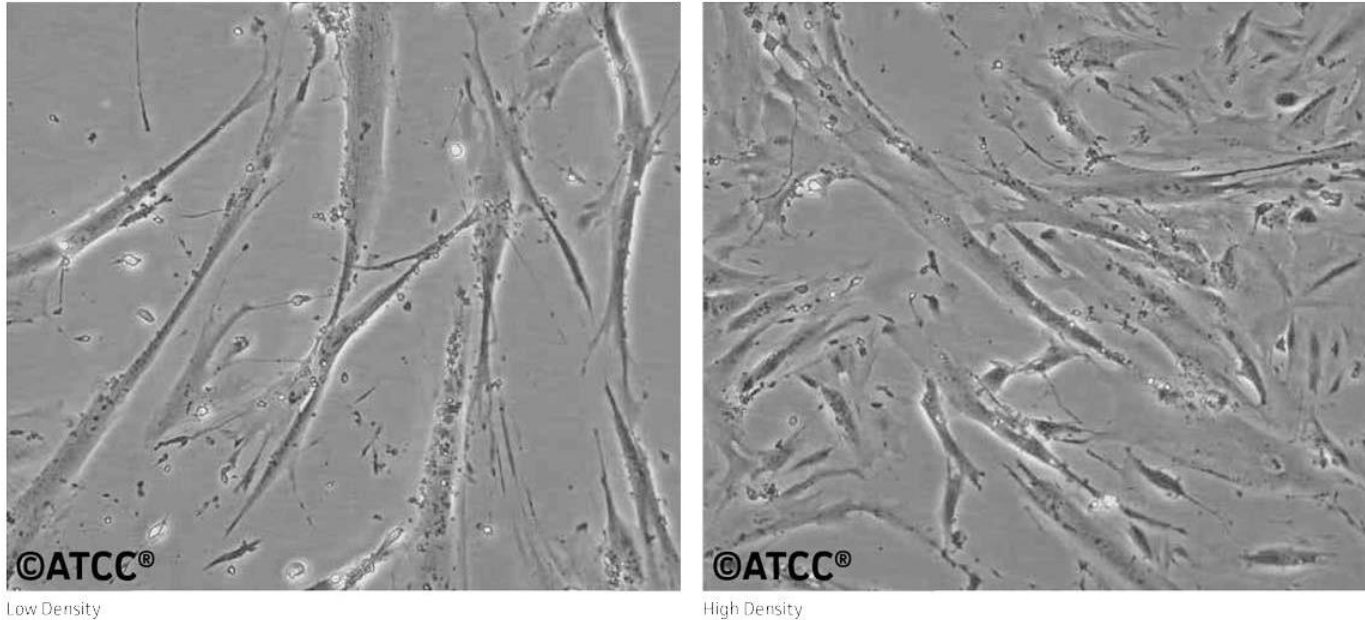
Table 7. Characteristics of Cell Lines

Cell Line	Site of Origin	MSI Status	MAPK Pathway			Subcultivation Ratio [*]	Medium (%FBS)
			KRAS	BRAF	ERBB3		
CCD 841 Normal Colon Epithelium	Colon	-	-	-	-	1:2 – 1:3	EMEM (15)
SW1116 Dukes' A CRC	Colon	Stable	Mutated	WT	WT	1:3 – 1:6	DMEM 1X (10)
SW480 Dukes' B CRC	Colon	Stable	Mutated	WT	WT	1:2 – 1:8	DMEM 1X (10)
HT-29 Dukes' C CRC	Distal Colon	Stable	WT	Mutated	WT	1:3 – 1:8	McCoy's 5A (10)
T84 Dukes' D CRC	Lung Metastasis	Stable	Mutated	WT	WT	1:2 – 1:4	DMEM/F-12 (10)

^{*}Subcultivation refers to the multiple passes or generations of each cell line

Figure 19. Morphology and Growth of Cell Lines CCD 841 CoN – Normal Colon Epithelium

ATCC Number: CRL-1790
Designation: CCD 841 CoN



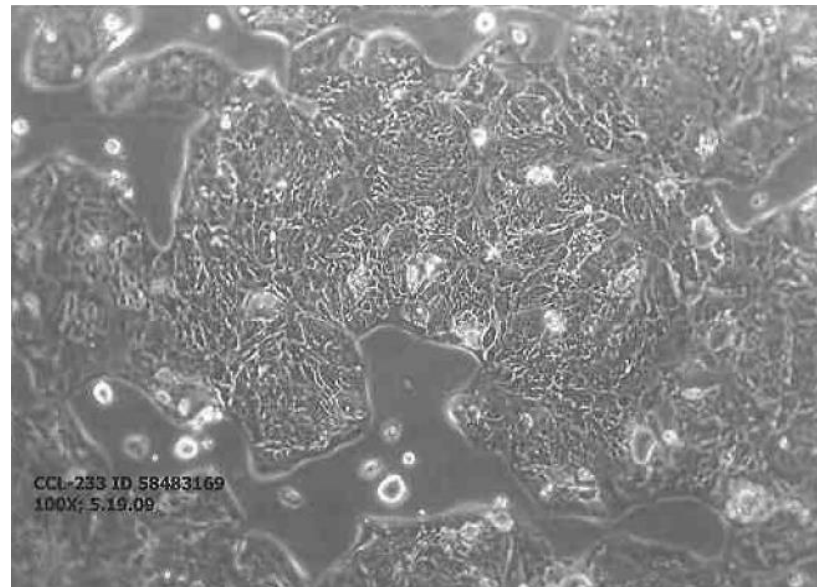
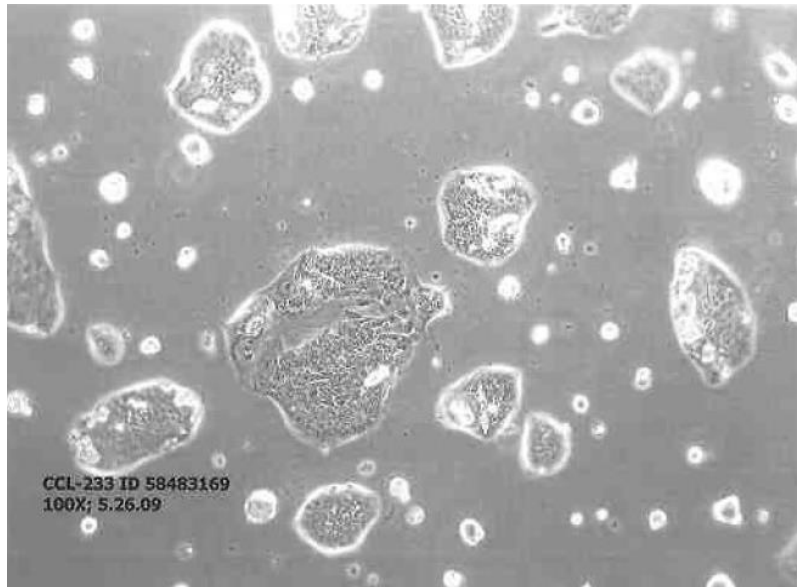
64

The CCD 841 cells are epithelial-like cells that grow as a flattened and disorganized layer in culture. Cells are long and epithelial-like with long thin processes. At low density cells will be relatively sparse and at higher densities cells may begin to overlap one another.

CCD 841 CoN (ATCC® CRL-1790™) is available at: www.atcc.org

Figure 20. Morphology and Growth of Cell Lines SW1116 – Dukes' A CRC

65

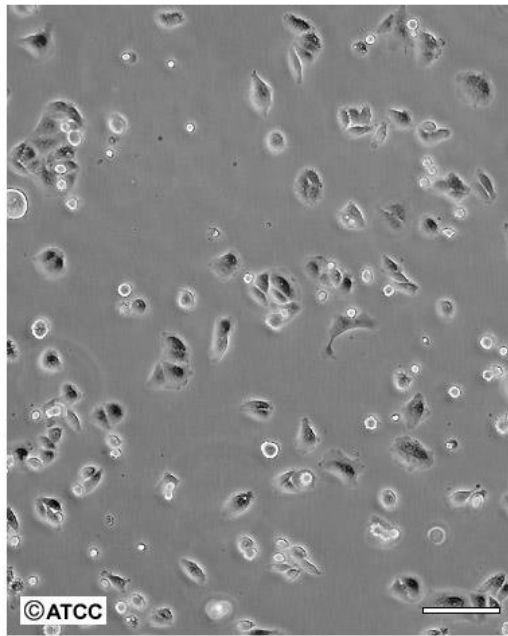


At low density, SW1116 cells can be seen briefly as single, unclumped cells. At higher density, cells appear to grow together in clumps.

SW1116 (ATCC® CCL-233™) is available at: www.atcc.org

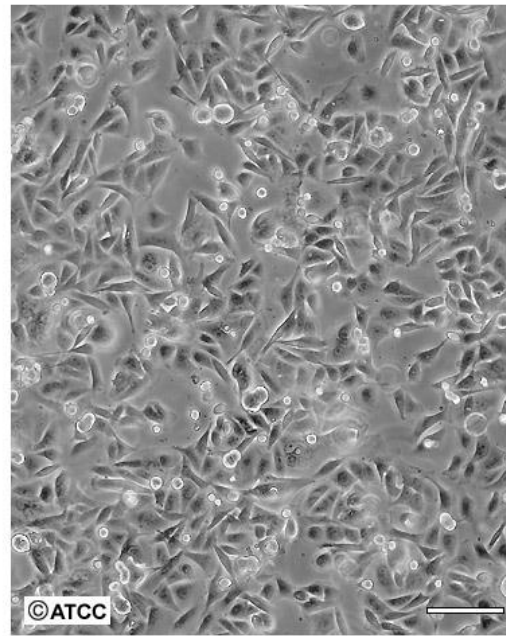
Figure 21. Morphology and Growth of Cell Lines SW480 – Dukes' B CRC

ATCC Number: **CCL-228**
Designation: **SW 480**



Low Density

Scale Bar = 100µm



High Density

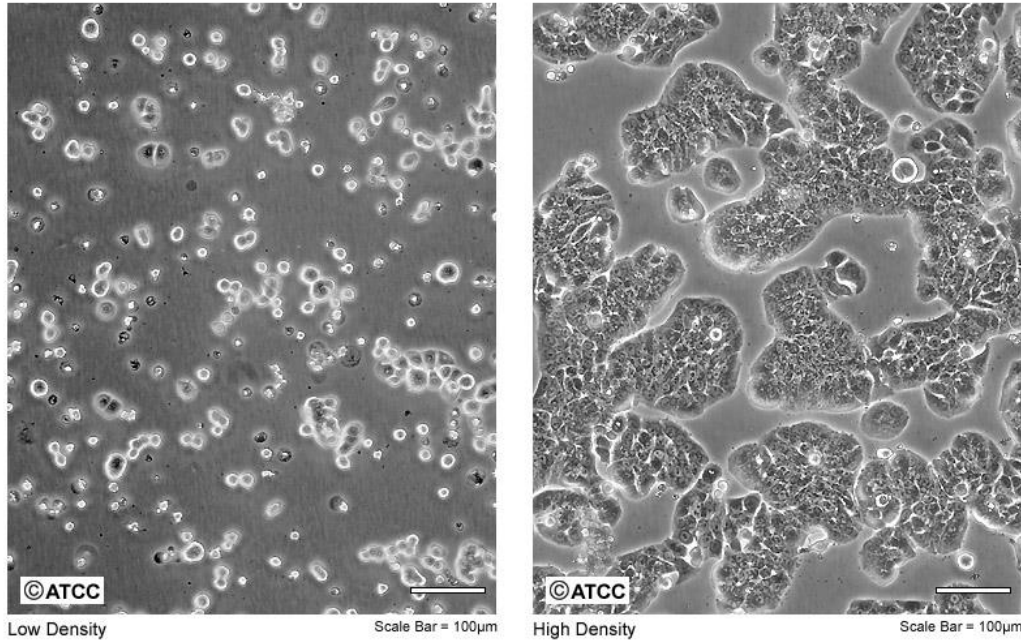
Scale Bar = 100µm

At lower densities, cells will appear more rounded and may appear in small clumps of a few cells. At high densities, the cells will take on a more polygonal shape with defined borders between cells.

SW480 (ATCC[®] CCL-228[™]) is available at: www.atcc.org

Figure 22. Morphology and Growth of Cell Lines HT-29 – Dukes' C CRC

ATCC Number: **HTB-38**
Designation: **HT-29**



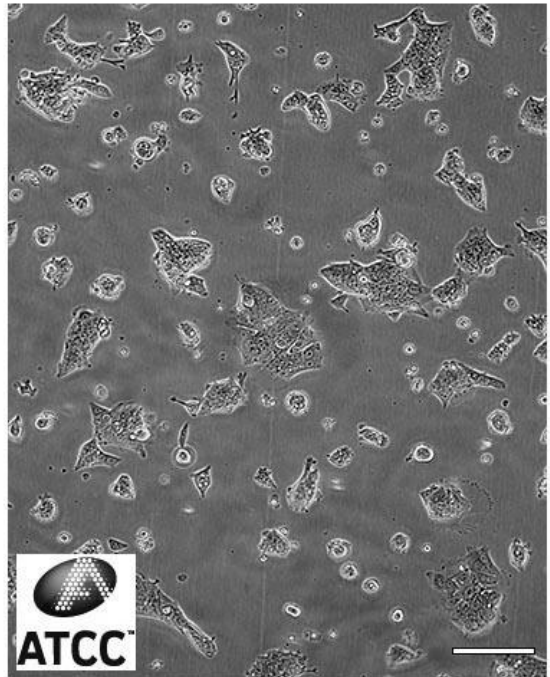
The cells attach in patches as three-dimensional aggregates and as they divide and grow they flatten into an epithelial cell monolayer.

At lower densities, cells will attach as single cells and will be spread apart. Cells appear rounded. At higher densities, many independent "islands" of growing cells come together leading to the large cell mass seen in the higher density picture

HT-29 (ATCC® HTB-38™) is available at: www.atcc.org

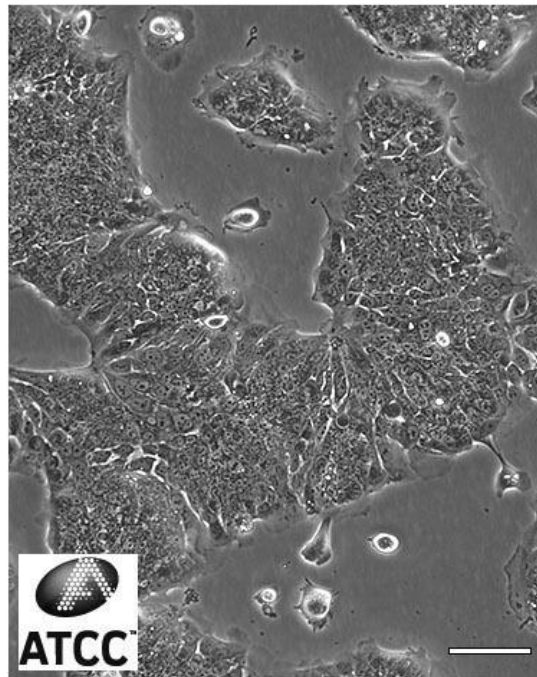
Figure 23. Morphology and Growth of Cell Lines T84 – Dukes' D CRC

ATCC Number: **CCL-248™**
Designation: **T84**



Low Density

Scale Bar = 100µm



High Density

Scale Bar = 100µm

89

Similar to HT-29. At lower densities, small islands of cells will be growing sparsely around the flask. At higher densities, these cell "islands" will join together to form the large cell mass seen in the high density picture

T84 (ATCC® CCL-248™) is available at: www.atcc.org

Cell counting was performed manually and cells stained with trypan blue using a 1:5 dilution by diluting 10 μ L of the cell solution to 40 μ L before sampling a 10 μ L aliquot for counting using a hemocytometer. Total cell count was determined by the following formula:

$$\text{Cell Count} \times \text{Dilution Factor} \times 10^4 \times \text{Volume}$$

Following this, cells were either plated at pre-determined amounts for use in experiments or sub-cultivated to allow for further growth.

3. Freezing of Cells

Cells were split and counted as described above. Cells selected for cryopreservation were at low passage numbers (<10) and viable. Cryo-media was created using a solution of the cells respective media and dimethylsulfoxide (DMSO) so that the final volume contained 5% DMSO. Cells were then added to the solution so that the concentration of cells was 4 x 10⁶ cells/2mL of cryo-media. 2mL of this suspension was labeled and stored in a sterile cryogenic vial (Corning Inc.). These cryogenic vials were then stored in a Cryo 1°C Freezing Container (NALGENE) ethanol bath and placed in a -80°C storage freezer in order to achieve a -1°C/min cooling rate. After 24 hours of cooling in the ethanol bath, these cryogenic vials were transferred to a liquid nitrogen storage container for storage until needed.

c. Harvesting Cells for RNA

Cells were plated at 250,000 cells/2mL in a 6-well plate and allowed to grow to confluence. Once confluent, the medium was carefully removed from each well and discarded. 300 μ L of cell lysis buffer was added to each well. After 5 minutes, the bottom

of each well was scraped using a pipette tip and the resulting product was collected in labelled freeze tubes and stored at -80°C until needed.

d. Harvesting Cells for Protein

Cells were plated at 250,000 cells/2mL in a 6-well plate and allowed to grow to confluence. For every 6-well plate, 1µL of protease and 1µL of phosphatase inhibitors were supplemented to 1mL of radio immune-precipitation assay (RIPA) buffer. The medium was carefully removed from each well and 150µL of RIPA / protease & phosphatase mix was added. The bottom of each well was scraped using the rubber plunger from a syringe, the sample was collected and stored at -80°C in pre-labeled freeze tubes or immediately processed for protein quantification.

e. Total RNA Isolation

Isolation of total RNA from cell lysates was performed using the Ambion® miRVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA). A 1:10 concentration of homogenate was added to each cell sample and incubated on ice for 10 minutes. Equal volumes of Acid-Phenol-Chloroform were added to the lysate. Samples were vortexed and centrifuged for 5 minutes at 10,000g. Careful removal of the aqueous top phase to a new tube was then performed to avoid contamination and maintain purity. The total volume removed was recorded and 1.25 times 100% EtOH was added to the lysate. After mixing, 700µL sample was added to a new spin column. This was centrifuged for 15 seconds at 10,000g and flow-through was discarded. 700µL of Wash Solution 1 was added to each spin column, followed by centrifugation at 10,000g for 10 seconds before the flow-through was discarded. This was followed by the addition of 500µL of Wash

Solution 2 and centrifugation at 10,000g for 10 seconds. The same procedure is then performed for Wash Solution 3. The spin column is removed and the sample placed into a new tube, which is then centrifuged for 1 minute at 10,000g to remove excess liquid. Finally, 40µL of pre-heated elution solution (95°C) was added to the filter in the spin column and centrifuged for 30 seconds at maximum speed. Quantitation of total RNA quantity and quality was then determined using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific®, Middlesex, MA). Samples can be contaminated with other molecules, such as proteins or organic compounds. To assess the nucleic acid quality of our samples we measure the ratio of absorbance at 260nm and at 280nm ($A_{260/280}$). For good quality RNA, the $A_{260/280}$ should be ~1.8-2.2. The total RNA concentration and quality of our samples are shown in Tables 8 & 9.

f. microRNA Screening of Cell Lines

For each cell line, the expression levels of 384 miRNAs were examined using TaqMan® Low Density Array (TLDA) human miRNA card A following the manufacturers protocol (Life Technologies, Carlsbad, CA). Each cell line was screened for expression of dysregulated miRNA four times.

Complementary DNA (cDNA) was generated from total RNA samples using the TaqMan® miRNA Reverse Transcription kit (Life Technologies, Carlsbad, CA). Reverse transcription (RT) total reaction volume for each sample was 75µL, consisting of 45µL master mix and 30µL RNA (4000ng) sample. The RT master mix volume per sample was as follows:

Table 8. Total RNA Concentration and Purity of Normal Colon Epithelial and Dukes' Colorectal Cancer Cell Lysates used for miRNA Cell Line Screening.

Cell Line	RNA Concentration (ng/ μ L)				RNA Purity ($A_{260/280}$)			
	Replicate (no.)				Replicate (no.)			
	1	2	3	4	1	2	3	4
Normal Colon Epithelium CCD 841	137	147	42	34	2.08	2.04	2.02	2.09
Dukes' A CRC (SW1116)	108	451	-	-	2.06	2.18	-	-
Dukes' B CRC (SW480)	247	649	-	-	2.08	2.11	-	-
Dukes' C CRC (HT-29)	496	356	-	-	2.11	2.09	-	-
Dukes' D CRC (T84)	507	497	-	-	2.10	2.10	-	-

Table 9. Total RNA Concentration and Purity following Transfection with miR-200 Family Mimics and Antagomirs.

Cell Line			RNA Concentration (ng/ μ L)					RNA Purity ($A_{260/280}$)				
			Replicate (no.)					Replicate (no.)				
			1	2	3	4	5	1	2	3	4	5
miR-200 family Mimics	Normal Colon Epithelium (CCD 841)	miR-200a	147	176	161	263	126	1.79	1.79	1.75	1.79	1.76
		miR-200b	128	99	263	184	167	1.76	1.71	1.76	1.71	1.71
		miR-200c	132	189	130	256	83	1.80	1.72	1.77	1.76	1.97
		miR-141	185	212	149	90	192	1.76	1.77	1.72	1.99	1.74
		miR-429	105	153	193	132	344	1.88	1.68	1.71	1.69	1.83
		miR-200 All*	181	92	148	175	210	1.73	1.73	1.84	1.70	1.70
		M- [†]	100	80	140	127	94	1.93	1.95	1.80	1.83	1.71
miR-200 family Antagomirs	Dukes' C CRC (HT-29)	miR-200a	156	133	146	134	114	2.05	2.11	2.08	2.11	2.07
		miR-200b	66	141	130	82	121	2.00	2.11	2.02	2.10	2.10
		miR-200c	158	116	138	98	123	2.00	2.07	2.10	2.08	2.10
		miR-141	202	148	148	204	183	2.10	2.04	2.02	2.06	2.06
		miR-429	144	94	190	174	211	2.04	2.03	2.04	2.04	2.09
		miR-200 All*	288	194	198	257	294	2.09	2.08	2.07	2.08	2.09
		A- ^{††}	214	182	192	198	103	2.08	2.07	2.09	2.09	2.05

* Combination of all five miRNA mimics or antagomirs together (125pmol total vs. 25pmol for each individual miRNA)

[†] Mimic Negative Control

^{††} Antagomir Negative Control

Component	Volume (μL) per 75μL Reaction[†]
Megaplex™ Reverse Transcription Human Pool	9.00
A v.2.1 Primers	
100nM dNTPs (with dTTP)	2.25
MultiScribe™ Reverse Transcriptase, 50U/ μL	16.88
10X Reverse Transcription Buffer	9.00
25mM MgCl ₂ Solution	10.20
RNase Inhibitor, 20U/ μL	1.12
Total	48.45

[†] Inclusive of 12.5% excess to compensate for pipetting loss

Once complete, the sample was vortexed, centrifuged to remove air bubbles and run on a pre-set thermal cycler program. Samples were either used immediately or stored at -20°C until further use.

Each TLDA card comprises of 8 fill reservoirs with a fill port on the left and a vent port on the right. For each sample, a master mix of 900 μL was prepared in a 1.5mL microcentrifuge tube, vortexed and centrifuged to eliminate air bubbles. The master mix consisted of the following:

Component	Volume (μL) per TLDA Card[†]
TaqMan® Universal Master Mix II, no	450
UNG	
Nuclease-free water	375
Product from RT reaction	75

†Inclusive of 12.5% excess to compensate for pipetting loss

The TaqMan Array card was removed from its packaging and warmed to room temperature with minimal exposure to sunlight and placed on a lab bench, with the foil side down. Each reservoir was filled with 100 μ L of sample-specific PCR mix made from a single cDNA sample. The micropipette was held at an angled position to dispense the sample and care was taken not to displace the reaction mix outside of the reservoir or introduce air bubbles. After the reservoir had been filled with PCR reaction mix, the card was centrifuged using a Sorvall[®] centrifuge bucket and holder for 1 minute at 1200rpm. Confirmation that filling of the TaqMan card was complete was observed by a consistent reduction in the fluid within each reservoir. The TaqMan Array card was sealed using a TaqMan[®] Array Micro Fluidic Card Sealer, removed and quantitative real-time polymerase chain reaction (qRT-PCR) performed using a ViiA[™] 7 Real-Time PCR System (ThermoFisher Scientific[®], Middlesex, MA).

Expression levels of each individual miRNA were normalized to RNU6 (endogenous internal reference gene), using a cycle threshold (Ct) of 0.2, in order to calculate Δ Ct values for analysis using the comparative Δ Ct method. miRNA expression of each CRC cell line was compared to the miRNA expression of the normal colon epithelium cell line.

g. microRNA Single Assay Validation

For miRNA single assay quantification, total RNA was converted to cDNA using the TaqMan[®] miRNA Reverse Transcription kit and specific TaqMan[®] miRNA primers

for the miR-200 family and RNU6 (Life Technologies, Carlsbad, CA). Specific TaqMan® probes for each miRNA were then used to bind to complementary sequences on target cDNA during qRT-PCR (Life Technologies, Carlsbad, CA). Reverse transcription (RT) total reaction volume for each sample was 15µL, consisting of 7µL master mix, 3µL TaqMan® miRNA primer, and 5µL RNA (2ng/µL) sample. The RT master mix was made using the following:

Component	Volume (µL) per 15µL reaction[†]
100nM dNTPs (with dTTP)	0.17
MultiScribe™ Reverse Transcriptase, 50U/µL	1.13
10X Reverse Transcription Buffer	1.69
RNase Inhibitor, 20U/ µL	0.21
Nuclease-free water	4.68
TaqMan® miRNA primer	3.38
Total	11.25

[†] Inclusive of 12.5% excess to compensate for pipetting loss

Once the master mix for each miRNA was made, the contents were gently mixed and centrifuged to bring the solution to the bottom of the tube, and then placed on ice until the RT reaction was ready to be run. 10µL of each RT master mix was added to wells in a 96-well reaction plate followed by 5µL of RNA sample. The reaction plate was then sealed, vortexed, and centrifuged before running on a pre-set thermal cycler program. Once complete, the cDNA was either used immediately to run qRT-PCR or stored at -20°C until further use.

Nucleic acid quantification was performed using a Step-One Plus qRT-PCR system (Life Technologies, Carlsbad, CA). A 10 μ L qRT-PCR reaction volume was performed and each reaction was performed in duplicate. Similar to the RT reaction, a master mix cocktail for qRT-PCR for each miRNA was made as follows:

Component	Volume (μL) per 10-μL reaction[†]
TaqMan [®] MicroRNA Assay (20X)	0.56
TaqMan [®] Universal Master Mix II, no UNG	5.63
Nuclease-free water	3.57
Product from RT reaction (Minimum 1:15 Dilution)	1.33
Total	11.08

[†] Inclusive of 12.5% excess to compensate for pipetting loss

Once made, the master mix was gently mixed and centrifuged to bring solution to the bottom of the tube. 8.67 μ L PCR master mix was dispensed into each well on a 96-well plate followed by 1.33 μ L of the RT product. Once loaded, the plate was sealed, vortexed and centrifuged to spin down the contents and eliminate air bubbles. The reaction plate was then run on a pre-set thermal cycler program using the Step-One Plus qRT-PCR machine.

The levels of each individual miR-200 family member were normalized to RNU6, using a Ct threshold of 0.1, in order to calculate Δ Ct values for analysis using the comparative Δ Ct method. All reactions were completed in duplicate. Analysis was

performed for expression of individual miR-200 family members for each CRC cell line as compared to that seen in the normal colon epithelium cell line.

h. messengerRNA (mRNA) Quantification

For mRNA quantification, total RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA) with random nucleotide primers. Preparation of a 2X RT master mix per 20 μ L reaction was made as follows:

Component	Volume (μL) per 20-μL reaction
10X RT Buffer	2.00
25X dNTP Mix (100mM)	0.80
10X RT Random Primers	2.00
MultiScribe™ Reverse Transcriptase	1.00
RNase Inhibitor	1.00
Nuclease-free water	3.20
Total	10.00

10 μ L of the 2X RT master mix was pipetted into each well of a 96-well reaction plate followed by 10 μ L of RNA sample (2ng/ μ L) and mixed. The plate was sealed, vortexed and centrifuged to spin down the contents and eliminate air bubbles. The plate was placed on ice until ready to load into and run the thermal cycler on a pre-set program. On completion, the cDNA RT plate was either used immediately to run qRT-PCR or stored at -20°C until further use.

Specific TaqMan® probes for RASSF2 and 18S (endogenous internal reference gene) were used during qRT-PCR and all reactions were completed in duplicate. The total number of PCR reactions was determined and the reaction mix was made as follows:

Component	Volume (µL) per reaction
TaqMan® Fast Advanced Master Mix (2X)	5.00
TaqMan® Gene Expression Assay (20X)	0.50
Nuclease-free water	3.50
Total	9.00

Nine µL PCR reaction mix and 1µL cDNA was added to each well, mixed, and covered with optical adhesive film and sealed. The plate was briefly centrifuged to spin down the contents and eliminate air bubbles. Nucleic acid quantification was performed using a Step-One Plus qRT-PCR system (Life Technologies, Carlsbad, CA). The expression levels of RASSF2 mRNA were normalized to 18S, using a Ct threshold of 0.1, in order to calculate Δ Ct values for analysis using the comparative Δ Ct method. Analysis was performed between the Dukes' C (HT-29) CRC cell line as compared to the normal colon epithelium (CCD 841) cell line.

i. microRNA Transfection

Transfection experiments were conducted in two cell lines; CCD 841 (normal colon epithelium) and HT-29 (Dukes' C CRC). Each cell line was transfected with each individual member of the miR-200 family, all five members of the family combined, and

scrambled oligonucleotides as a negative control, using mirVana™ miRNA mimics (for CCD 841) and antagomirs (for HT-29) (Life Technologies, Carlsbad, CA).

For our experiments, the concentration of the cell suspension was determined and cells plated into individual wells depending on the type of experiment being performed (Table 10). Once plated, cells were allowed a 24-hour acclimation period to adhere to the plate prior to transfection. After 24 hours when cells were 60-80% confluent in each well they were transfected using Lipofectamine® RNAiMAX transfection reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Firstly, miRNA (10µM) was diluted in Opti-MEM® Medium, followed by dilution of Lipofectamine® RNAiMAX Reagent in Opti-MEM® Medium. The diluted miRNA and Lipofectamine® were then combined in a 1:1 ratio and incubated at room temperature for five minutes. The miRNA-lipid complex was then added to each well. Incubation of the cells at 37°C was performed for 24 hours after transfection prior to cell harvesting and further analysis. Cell samples were stored in labelled tubes and stored at -80 °C until further use.

Table 10. Transfection Protocol Procedure Details.

Experiment	Plate Format	Cell Concentration	Final Transfection miRNA Per Well (pmol)	Final Lipofectamine® RNAiMAX Per Well (μL)	miRNA-Lipid Complex Per Well (μL)
miRNA mRNA qRT-PCR	6-well	250,000/2mL	25	7.5	250
RASSF2 and K-Ras Protein Quantification	6-well	250,000/2mL	25	7.5	250
Cell Proliferation	12-well	0.5 – 1 x 10 ⁵ /mL	5	1.5	50
ERK1/2 Total/Phosphorylated ELISA Assay	6-well	250,000/2mL	25	7.5	250

j. Protein Quantification

Cell samples were removed from storage and thawed over ice. Two 1.5mL freeze tubes per sample were labelled with the cell line, date and miRNA clearly visible. The freeze tubes underwent sonication with two pulses per sample and were returned to ice. The sonicator was wiped with kimwipes between each sample and the process was repeated. Once complete, the samples were transferred into a 1.5mL tube and then centrifuged at 10,000rpm for ten minutes in order to pellet the debris. The upper layer of pure protein was transferred into a new 1.5mL labelled tube.

1. Preparation of the protein standards

Protein concentration was determined using the bicinchoninic acid (BCA) assay and performed in a 96-well plate. Protein standards were prepared in a serial 2-fold dilution down the plate. To begin, 20 μ L of double distilled water was pipetted into the wells of rows B-H in the first two columns on the plate. In the first two columns of row A, 40 μ L of BCA standard was added. Transferal of 20 μ L of BCA standard from well A to B was performed and mixed well. A further 20 μ L from mixed well B was transferred to well C. This process was repeated until row G. The remaining row H contained only 20 μ L of double distilled water. The resulting total volume in each well was 20 μ L.

2. Preparation of the protein samples (1:20 dilution)

Depending on the number of samples to be quantitated, 38 μ L of double distilled water was added to wells in the odd numbered columns from the third column onwards. Two microliters of protein sample was then added to each corresponding well and mixed thoroughly to make a total volume of 40 μ L. From each mixed water/protein sample,

20 μ L was transferred to the adjacent well. To read the amount of protein in each sample, equal amounts of Pierce[™] BCA Protein Assay Reagent A and B (Thermo Scientific, Rockford, IL) were combined before 160 μ L was added to every well used, covered and incubated at 37°C for 30 minutes. The 96-well plate was then read using a SPECTRAmax[®] PLUS 384 microplate spectrophotometer at a wavelength of 540nm.

k. Western Blot

Western blots (n=5 for each protein) were performed for target proteins in the MAPK pathway (Table 11). Cell samples were prepared to contain a standardized amount of 40 μ g/ μ L of protein. Bolt[®] (4X) lithium dodecyl sulfate sample buffer was added to each sample, vortexed and heated for 10 minutes at 90°C. A 10-well Bolt[™] 8% Bis-Tris Plus Gel was placed in a Bolt[®] Mini Gel Tank which was then filled half-way with NuPAGE[®] MOPS SDS running buffer. Wells were loaded with 8 μ L molecular marker standards and 30 μ L protein sample. The remainder of the tank was then filled with running buffer. Gels were run at 165mV for 45 minutes in order to identify the proteins of interest. Once complete, the gel was transferred over 10 minutes onto a nitrocellulose membrane using the iBlot[®] Gel Transfer Device. The nitrocellulose membrane was then cut between the protein of interest and the endogenous internal reference, β -actin (MW 42kDa). The membrane was then blocked for β -actin in 5% milk or 5% Bovine Serum Albumin (BSA) in Tris-buffered saline and Tween 20 (TBS-T) for the protein of interest for 1 hour at room temperature on an orbital shaker. During the hour, primary antibodies for target proteins were made according to the manufacturer's instructions. Primary antibodies were poured over the membranes and incubated

Table 11. MAPK Pathway Proteins of Interest with Molecular Weight and Primary and Secondary Antibody Dilutions

Protein	Observed Molecular Weight (kDa)	Primary Antibody Dilution	Secondary Antibody Dilution
RASSF2	37	1:1000	1:1000
K-Ras	21	1:250	1:1000
β -Actin (Internal Reference)	42	1:10000	1:1000

overnight at room temperature while being mixed. After three 5 minute washings with TBS-T, each membrane was submersed with horseradish-peroxidase-conjugated secondary antibody and mixed at room temperature for 1 hour. The membrane was again washed three times for 5 minutes each with TBS-T, incubated in equal parts of Enhanced Chemiluminescence (ECL) Reagents A and B for 5 minutes in the dark in order to detect the target protein, and then developed and imaged using a ChemiDoc MP imager (BioRad, Hercules, CA).

1. Cell Proliferation

For cell proliferation studies, cells were grown in flasks until ready for use. Cells were split as previously described (pg.62) and cell concentration determined. HT-29 cells and CCD841 were plated in duplicate at a concentration of 100,000 cells/mL and 50,000 cells/mL in individual wells in 12-well plates, respectively. Cells were left for 24 hours to adhere to the plate before being serum starved. For this, the medium from each well was removed and replaced using medium with a 0.5% FBS concentration. After 2 hours, this was removed and replaced with the normal growth medium and cells were then transfected with the respective antagomirs and mimics as previously described (pg.79-80). Transfection was stopped after 24 hours, cells were trypsinized and once the cells were confirmed no longer adherent to the bottom of the plate, the trypsinization process was stopped. A 10 μ L sample from each well was inserted into a dual chamber counting slide (Bio-Rad, Hercules, CA) and cell number was measured each day for 5 days using an automated cell counter (TC20[™] Bio-Rad, Hercules, CA).

m. Enzyme-Linked Immunosorbent Assay (ELISA)

Total and phosphorylated ERK1/2 was measured using the InstantOne™ ELISA 96 Well (Affymetrix, Santa Clara, CA). Cell sample lysates were prepared from grown cells that had been plated and transfected in 6-well plates as previously described (pg.79-80). Sample preparation of adherent cultured cells was prepared by removing media from the cells, and 300μL of pre-prepared cell lysis mix (1X) was added to each well. The plate was shaken at room temperature for 5 minutes. Each well was scraped with a pipette tip and the samples were collected in labeled tubes and stored at -80°C until further use. Protein concentration was determined using the BCA assay previously described to ensure protein concentration was in the range of at least 0.1-0.5mg/mL in order to be detected.

When ready for use, cell lysates were thawed over ice and the desired number of InstantOne™ microplate strips needed for the experiment, including the positive control lysate and negative control, were determined. Unused microplate well strips were returned to the storage pouch and sealed until next use. Fifty μL of the positive and negative controls, and cell sample lysates to be tested were added to each of the assay wells. In addition, 50μL of prepared antibody cocktail mix was added to each well; the plate sealed and then incubated for 1 hour at room temperature on a microplate shaker at 300rpm. Following incubation, the wells were washed three times with 200μL wash buffer (1X) /well and on completion any remaining wash solution was removed by inverting the plate on a paper towel. To this, 100μL of a detection reagent was added to each well and incubated for 30 minutes at room temperature with shaking at 300rpm. After 30 minutes, the reaction was stopped by adding 100μL stop solution to each well.

The plate was read by measuring the absorbance of the samples using the SPECTRAMax[®] PLUS 384 microplate spectrophotometer at a wavelength of 450nm.

n. Statistical methods and analytic techniques

1. Where possible, for each cell line and transfected miRNA, samples were prepared in replicates of five, unless stated. Cell culture samples were grown from new freezes and transfected at the same passage to maintain a true sample size of five. All reactions were run in duplicate and the averages of the duplicates used for analysis. Once obtained, all data was analyzed and graphs were prepared in GraphPad Prism v6.01 (GraphPad Software, Inc. La Jolla, CA). Data was tested for normality, and where confirmed, comparison of two data sets was conducted using an unpaired two-tailed Student's *t* test. Statistical significance was defined as $p < 0.05$ and data presented as mean \pm standard deviation.

A *t* test was chosen as the most appropriate method for statistical analysis as we were performing comparisons between two groups for each experiment. If three or more groups (i.e. multiple cell lines) were being compared, analysis using ANOVA would have been performed. Sample size was justified at $n=5$, based on the preliminary cell line screening and validation of single miRNA expression. At a power of 80%, and adjusted $\alpha=0.05$, if we were to detect a difference in mean miRNA expression between Dukes' C CRC and normal colon epithelial cells of 1000 fold, a sample size of $n=2$ would be sufficient to detect a significant difference in miRNA expression between cell lines. We chose to perform experiments with $n=5$, to permit detection of smaller differences in

mean miRNA expression and other variables such as amount of protein, mRNA, and proliferation, and to keep all experimental replicates consistent.

2. qRT-PCR

Raw fluorescence data (Ct values) generated by the Step-One Plus qRT-PCR instrument (Life Technologies, Carlsbad, CA) were exported for analysis using the comparative $\Delta\Delta\text{Ct}$ method. The data was visualized on an amplification curve, and the threshold bar was set at 0.1. The value for the threshold bar was determined as it was consistently above the background noise, was within the exponential phase and was below the plateau and linear phases of the amplification curve. Where the threshold bar cut the amplification curve a Ct value was derived from the x-axis. All undetermined values were replaced with a Ct value of 40. The Ct value of each miRNA and mRNA was normalized to an endogenous control (RNU6 for miRNA and 18S for mRNA) generating a ΔCt . The $\Delta\Delta\text{Ct}$ was calculated by the following;

$$\Delta\text{Ct} = \text{Ct}_{(\text{target case})} - \text{Ct}_{(\text{reference case})}$$

$$\Delta\text{Ct} = \text{Ct}_{(\text{target control})} - \text{Ct}_{(\text{reference control})}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct}_{(\text{case})} - \Delta\text{Ct}_{(\text{control})}$$

The resulting normalized gene expression level was calculated by $2^{(-\Delta\Delta\text{Ct})}$, representing the fold change between samples.

3. Western Blot

The chemiluminescent blots were imaged first with the ChemiDoc MP imager (BioRad, Hercules, CA). The Band Analysis tools of ImageLab software version 5.2.1

(BioRad, Hercules, CA) were used to select and determine the background-subtracted density of the bands. Normalization was performed by dividing the relative density units of the protein of interest by the relative density units of the endogenous control, β -actin. The ratios obtained from this were analyzed as described above by averaging the ratios of each replicate for each sample and comparing the sample of interest against the negative control.

4. ELISA Assay

Each sample replicate was run in duplicate and analyzed using the spectrophotometer at 450nm. Results were adjusted against the negative control (blank) and the absorbance units for each sample were averaged. A ratio of phosphorylated to total ERK 1/2 was calculated for each sample and a comparison of the ratios between each transfected miRNA sample and its corresponding negative control was performed.

5. Cell Proliferation

Total cell numbers for each sample were obtained and averages of the duplicates were taken. For each of the five days, a comparison between total cell numbers of each transfected miRNA sample compared to the negative control was performed. Statistical analysis was conducted as described above.

CHAPTER VII

COLORECTAL CANCER CELL LINE EXPRESSION OF RASSF2 mRNA AND PROTEIN

a) Introduction

We have previously identified increased expression of all members of the miR-200 family in colorectal cancer compared to normal colon epithelium cell lines (pg. 36). The members of the miR-200 family are highly enriched in epithelial tissues and they have been linked to several cancers including CRC.(146) Using the results of our screening data, pathway analysis software identified all members of the miR-200 family to have a validated regulatory role upstream of Ras proteins in MAPK canonical pathway (pg. 45). Further analysis of the miR-200 family confirmed validated target interaction with Ras associated domain-containing protein 2 (RASSF2) (pg. 47). RASSF2 is one of six proteins in the RASSF family that in humans is encoded by the RASSF2 gene. RASSF2 is a negative regulator of Ras and binds directly to K-Ras within the Ras effector domain in a GTP-dependent process.(138) RASSF2 has previously been shown to promote apoptosis, cause cell cycle arrest and is regarded as a novel K-Ras-specific effector and potential tumor suppressor. Therefore, we hypothesize that RASSF2

expression will differ between CRC cell lines and a normal colon epithelial (CCD 841) cell line.

b) Results

1. RASSF2 mRNA Expression in Colorectal Cell Lines

RASSF2 mRNA expression was present in normal colon epithelial cell line samples across all replicates. Conversely, RASSF2 mRNA expression was lower or absent in each Dukes' Stage CRC cell line. Dukes' A (SW1116) and C (HT-29) CRC cell lines had no expression of RASSF2 mRNA, as determined by Ct values ≥ 40 , whereas Dukes' B (SW480) CRC had lower expression and Dukes' D (T84) CRC cell lines had partial expression of RASSF2 mRNA, respectively. A representative qRT-PCR amplification plot of RASSF2 mRNA expression for all colorectal cell lines is shown in figure 24. Average Ct values (\pm standard deviation) representing relative expression of RASSF2 mRNA along with the internal control, 18S, are shown in table 12.

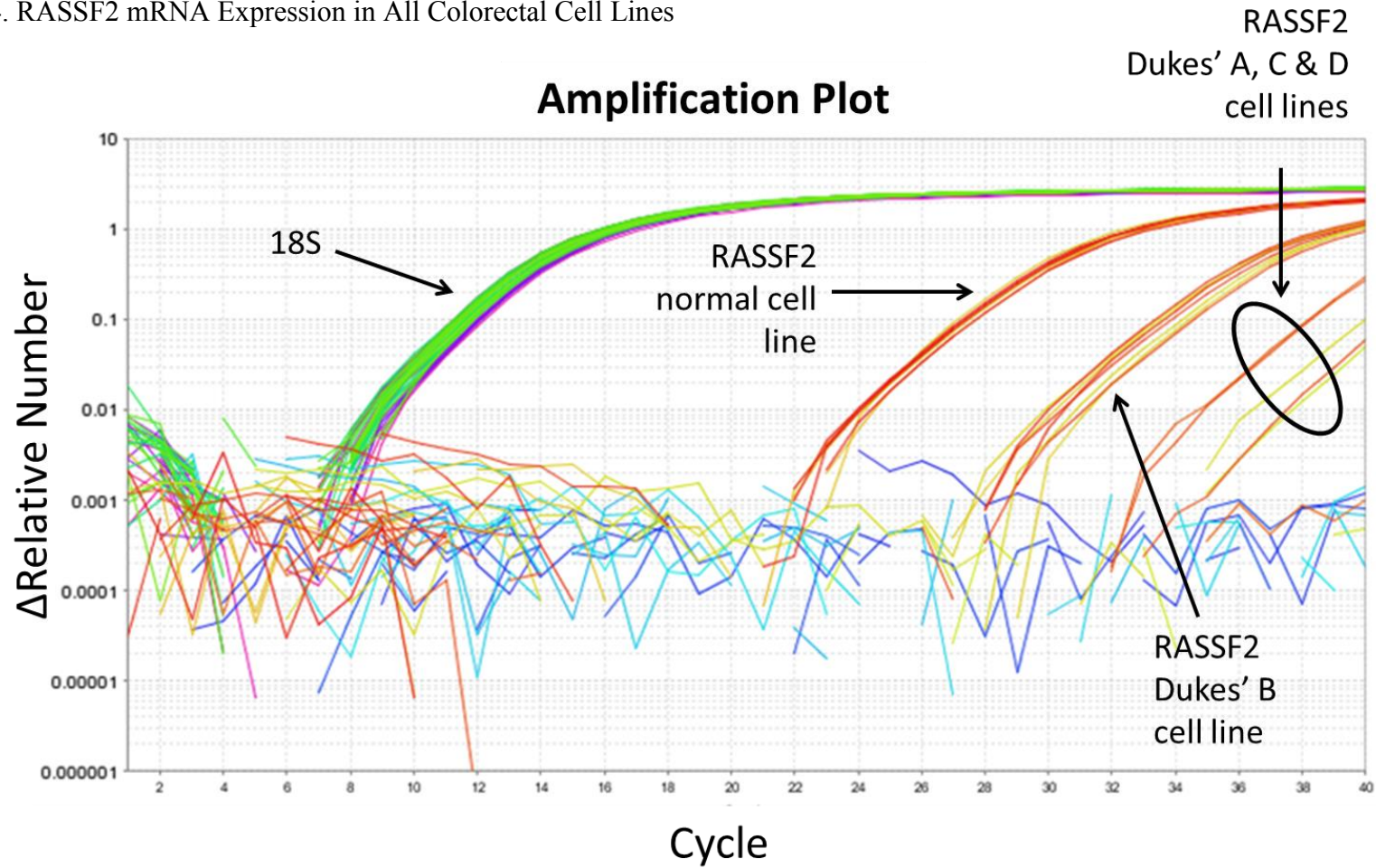
Comparison of expression of RASSF2 mRNA between each Dukes' Stage CRC and the normal colon epithelial cell line showed significant downregulation in all Dukes' Stage CRC cell lines. RASSF2 mRNA was downregulated with a fold regulation between -80 and -6828. Of all the CRC cell lines, Dukes' C (HT-29) stage CRC had the largest downregulation of RASSF2 mRNA -6828 compared to the normal colon epithelial (CCD 841) cell line (Figure 25).

2. RASSF2 Protein Expression in Colorectal Cell Lines

The genes in DNA encode protein molecules which are required to carry out all functions necessary for life. Expressing a gene means manufacturing its corresponding protein. Initially, DNA is transferred to mRNA by a process called transcription. The resulting mRNA is a single-stranded copy of the gene, which then undergoes translation into a protein molecule. We have ascertained downregulation of RASSF2 mRNA in colorectal cancer cell lines compared to normal colon epithelium, the next step in the process to see whether this downregulation translated to the expression of RASSF2 protein.

We observed the presence of RASSF2 protein in all normal colon epithelium (CCD 841) cell line samples, with a RASSF2/ β -actin ratio of 0.014. No RASSF2 protein was detected in Dukes' A (SW1116), C (HT-29) or D (T84) CRC samples, whereas Dukes' B (SW480) CRC had a RASSF2/ β -actin ratio of 0.016. Western blot results for all cell lines are shown in figure 26. There was a significant downregulation of RASSF2 protein in Dukes' A (SW1116), C (HT-29) and D (T84) CRC cell lines compared to the normal colon epithelial (CCD 841) cell line ($p < 0.05$), whereas there was no difference in RASSF2 protein expression observed between the Dukes' B (SW480) CRC cell line and the normal colon epithelium (CCD 841) cell line ($p = 0.99$).

Figure 24. RASSF2 mRNA Expression in All Colorectal Cell Lines



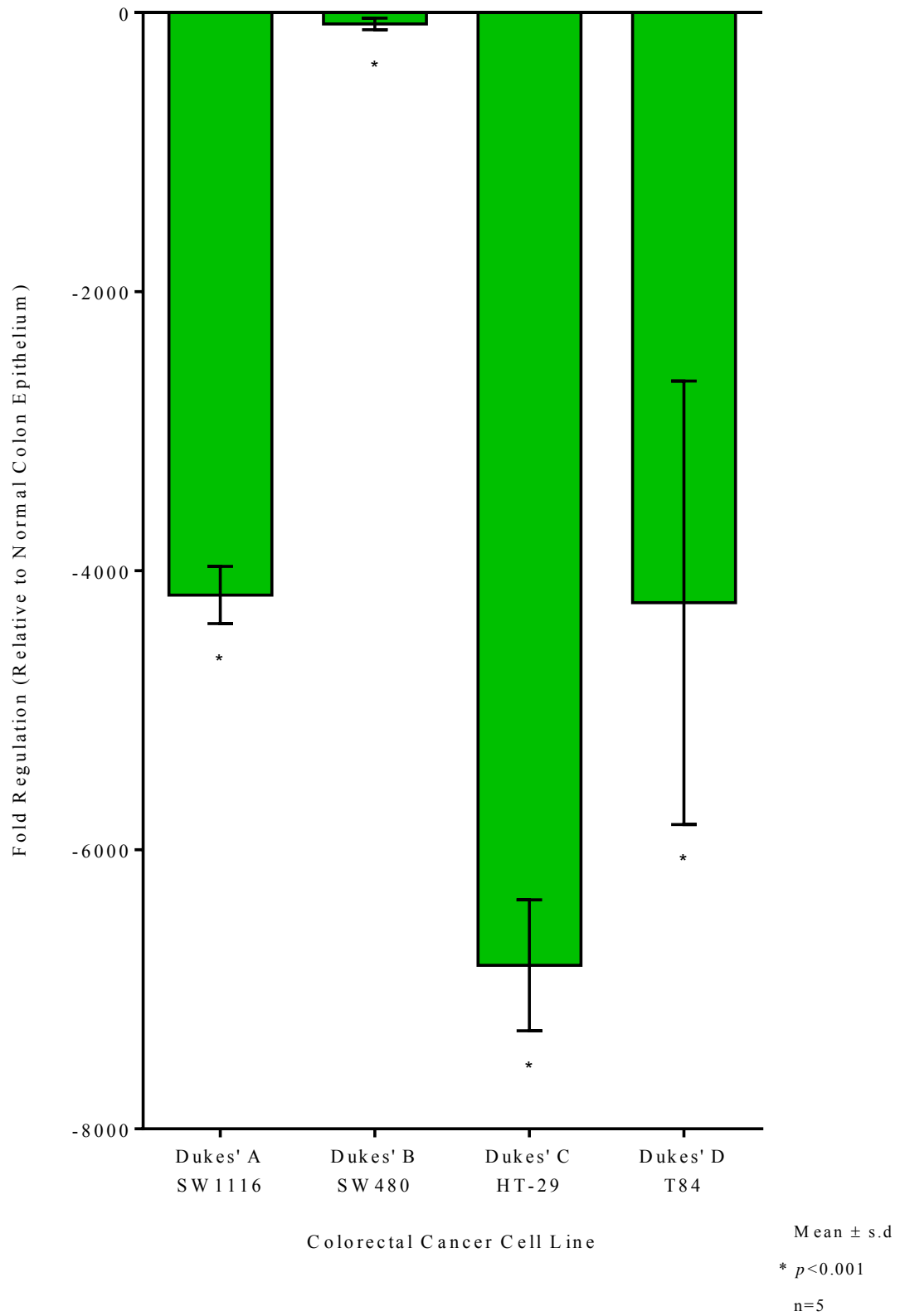
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RASSF2 mRNA expression in Colorectal Cell Lines. Downregulation of RASSF2 mRNA was observed in all CRC cell lines compared to the normal colon epithelium cell line.

Table 12. Average Cycle Threshold and $\Delta\Delta\text{Ct}$ Values, and Fold Regulation for Expression of RASSF2 mRNA in All Colorectal Cell Lines

Colorectal Cell Line	Average Ct Values (+/- sd)		Average $\Delta\Delta\text{Ct}$ Values (+/- sd)	Fold Regulation
	RASSF2	18S		
Dukes' A CRC SW1116	40.0 (+/- 0.0)	12.0 (+/- 0.1)	12.0 (+/- 0.1)	-4174
Dukes' B CRC SW480	33.9 (+/- 0.4)	11.6 (+/- 0.3)	6.3 (+/- 0.6)	-80
Dukes' C CRC HT-29	40.0 (+/- 0.0)	11.3 (+/- 0.1)	12.7 (+/- 0.1)	-6828
Dukes' D CRC T84	39.5 (+/- 0.5)	11.5 (+/- 0.2)	12.0 (+/- 0.5)	-4229
Normal Colon Epithelium CCD 841	27.4 (+/- 0.2)	11.5 (+/- 0.2)	-	-

Figure 25. RASSF2 mRNA Expression in Colorectal Cancer Cell Lines Compared to a Normal Colon Epithelial (CCD 841) Cell Line.



c) Discussion

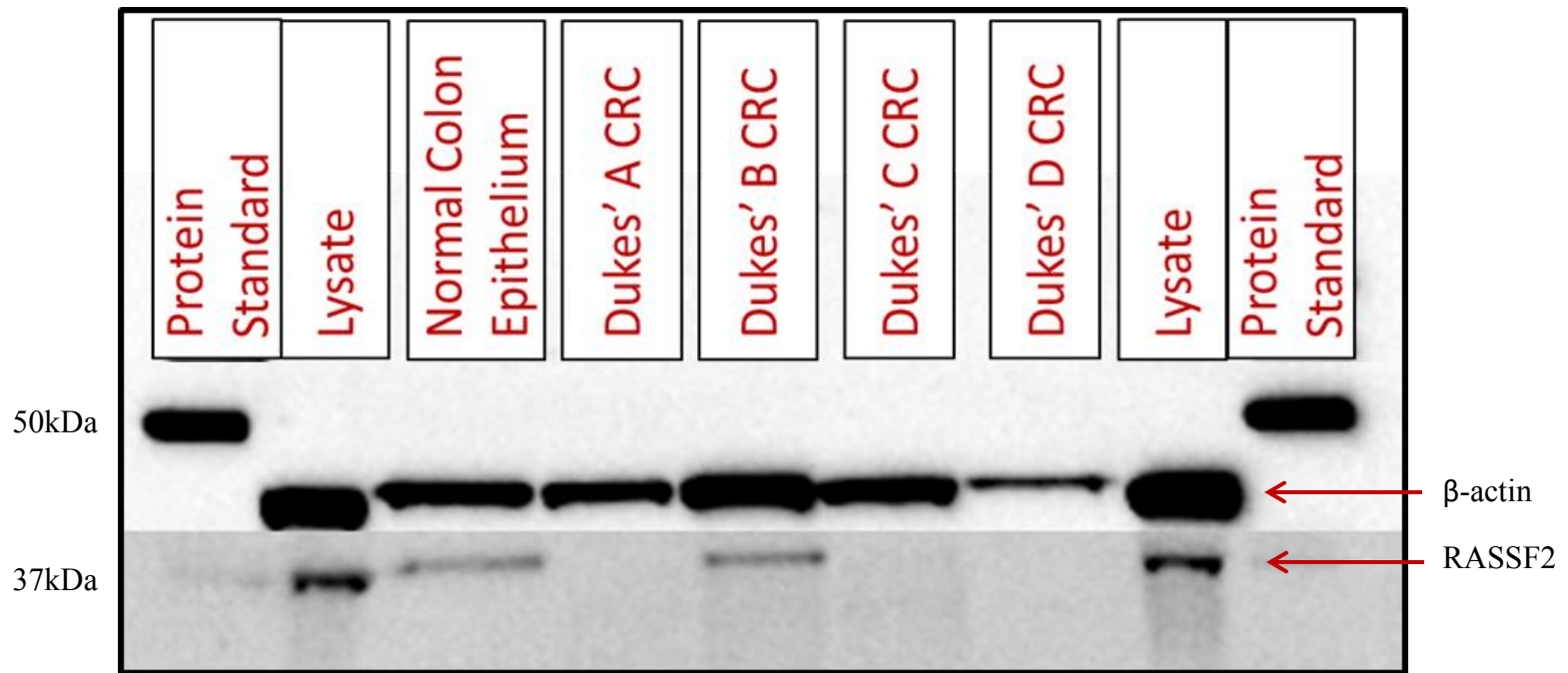
We found that there is a decreased expression of RASSF2 mRNA and protein in Dukes' A (SW1116), C (HT-29) and D (T84) colorectal cancer cell lines compared to the normal colon epithelial (CCD 841) cell line, in keeping with previously reported studies.

Multiple studies have identified RASSF2 to have properties and behavior of a tumor suppressor gene. RASSF2 is a negative effector of Ras protein and it is inactivated by promoter hypermethylation in many cancers. Frequent downregulation of RASSF2 has been detected in colorectal, gastric, lung, ovarian, cervical, and thyroid cancers; to name a few. Our results suggest that RASSF2 is implicated in colorectal carcinogenesis due to the significant difference observed in expression between normal colon epithelial and colorectal cancer cell lines.

Previous studies have identified association with methylation status of RASSF2 and colorectal cancers and adenomas. RASSF2 methylation has frequently been observed in colorectal cancers and adenomas. One study reported RASSF2 methylation to be present in approximately 40% of all CRC and colorectal adenoma samples tested.(142) In addition to this, methylation of RASSF2 was significantly associated with KRAS and or BRAF mutations. Another more recent study showed that RASSF2 methylation was positively correlated with KRAS mutation in MSS, but not MSI, CRC.(140) Interestingly another study observed methylation of RASSF2 to be negatively associated with KRAS mutation in sporadic CRC. In this study, 75% of CRC specimens with RASSF2 methylation had no mutation of KRAS.(141) Methylation of RASSF2 was observed in

Figure 26. Western Blot Showing Expression of RASSF2 Protein in Normal Colon Epithelium (CCD 841) and Dukes' Colorectal Cancer Cell Lines.

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Presence of RASSF2 protein in normal colon epithelium and Dukes' B colorectal cancer cell lines. Significant downregulation of RASSF2 protein in Dukes' A (SW1116), C (HT-29) and D (T84) colorectal cancer cell lines compared to normal colon epithelium ($p < 0.05$)

almost 90% of all colorectal adenoma samples, suggesting that RASSF2 methylation is an early event in CRC. Another series observed concomitant KRAS and BRAF mutations in 73% of colorectal adenoma samples suggesting that the epigenetic changes occur simultaneously and may work synergistically.(147)

The CRC cell lines used in this study have differing KRAS and BRAF mutation status. We specifically chose to investigate MSS sporadic CRC cell lines. Of the four cell lines, only Dukes' C (HT-29) CRC was KRAS wild type with BRAF mutation. The remaining three all had mutations of KRAS. It is possible that RASSF2 expression was present in the Dukes' B (SW480) CRC cell line due to fact that not all CRC will have methylation of RASSF2 as previously described. If RASSF2 is not methylated, then inactivation will not occur and it is likely that CRC develops due to other genetic abnormalities and aberrant signaling events.

Observing absent or downregulation of RASSF2 in colorectal cancer cell lines compared to a normal colon epithelial (CCD 841) cell line provides us with a platform in which to investigate the development of CRC. RASSF2 behaves as a Ras effector and functions as a tumor suppressor, therefore; if we are able to alter the expression of RASSF2 we may be able to change cell behavior by modulating Ras/Raf/MEK/ERK activation.

CHAPTER VIII

GAIN OF FUNCTION OF miR-200 FAMILY IN A NORMAL COLON EPITHELIAL (CCD 841) CELL LINE

a) Introduction

We have seen that miR-200 family levels are decreased and RASSF2 mRNA and protein levels are present in a normal colon epithelial (CCD 841) cell line compared to the Dukes' C (HT-29) CRC cell line. The purpose of the next series of experiments was to confirm the proposed oncogenic action of the miR-200 family members and the inhibitory effect they have on RASSF2 mRNA and subsequent RASSF2 protein expression.

Gain of function experiments with miRNA mimics are used to augment the function of endogenous miRNA for easier detection of a phenotypic change. In the case of the normal colon epithelium (CCD 841) cell line, endogenous levels of miR-200 family members were low. Transfecting with miRNA mimics to supplement miR-200 family function would therefore decrease the expression of the target gene RASSF2, and subsequent presentation of the phenotype.

From our previous knowledge, miRNA-200 family members all target RASSF2. RASSF2 is a negative regulator of K-Ras. Therefore; we hypothesized that increased

expression of miR-200 family members, would cause RASSF2 mRNA and protein levels to decrease in normal colon epithelial cells

b) Results

1. Overexpression of miR-200 family members in the normal colon epithelial (CCD 841) cell line

Transfection of miR-200a, miR-200b, miR-200c, miR-141, and miR-429 mimics into normal colon epithelial cells was successful (Figure 27). Increased expression of each individual miR-200 family member was achieved when compared to the negative control transfection (M-). For all members of the miR-200 family, miRNA mimics achieved from 1084 to 5638 fold increase in the respective miRNAs ($p < 0.001$) (Figure 28).

We also performed an experiment evaluating the additive effect of combining all five miR-200 family members into the same transfected wells, in an attempt to replicate what occurs in the natural environment. Transfection of the five miRNA mimics in combination increased expression of every miR-200 family member, as measured by qRT-PCR, compared to the negative control (Figure 29).

2. Effect of Overexpression of miR-200 Family Members on RASSF2 mRNA

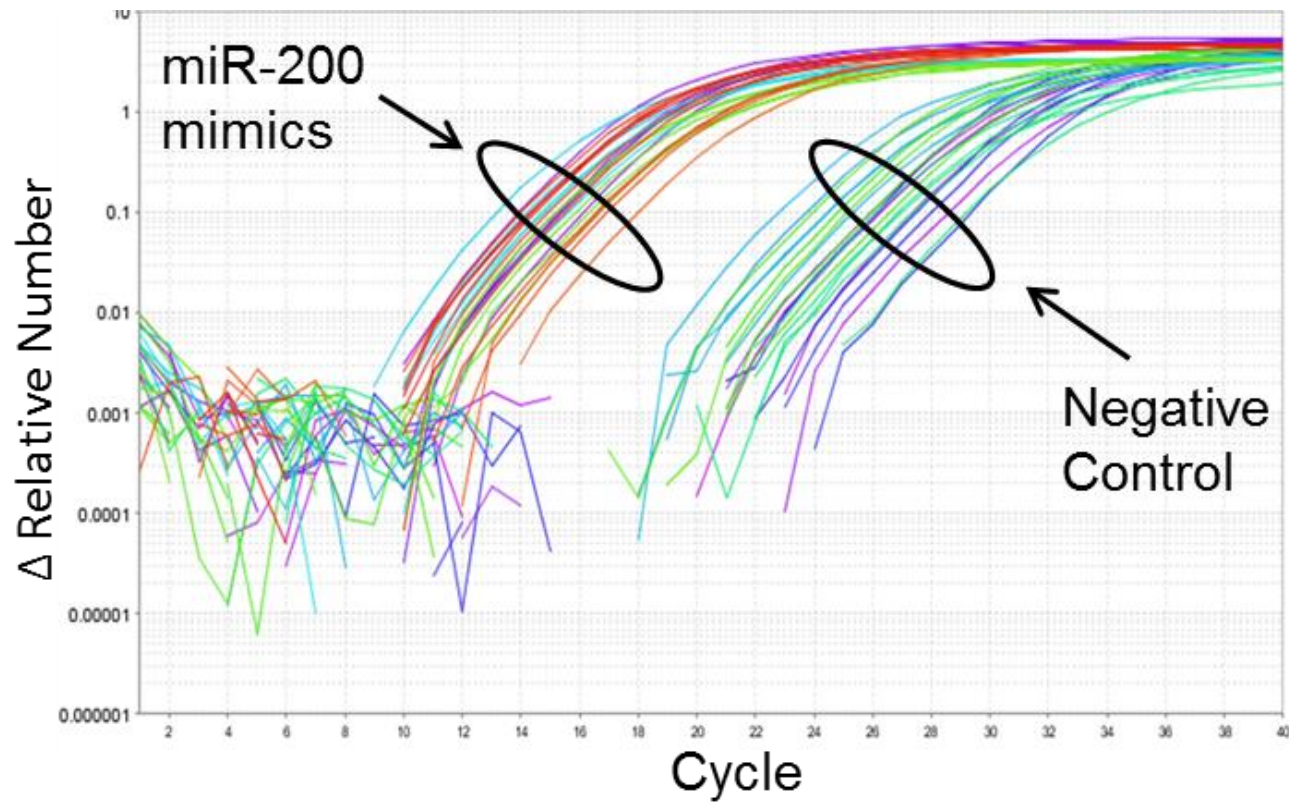
Manipulation of miR-200 expression led to changes in RASSF2 mRNA expression (Figure 30). miR-200a, miR-200b, miR-200c, and the combination of all five miR-200 family members were associated with decreased RASSF2 mRNA expression. miR-200b significantly downregulated RASSF2 mRNA with a fold regulation of -1.74

(p 0.016). There was no change in RASSF2 mRNA expression in the normal colon epithelial (CCD 841) cell line transfected with miR-141 and miR-429 (p 0.5 and 0.6, respectively) (Figure 31).

3. Effect of Overexpression of miR-200 Family Members on RASSF2 Protein

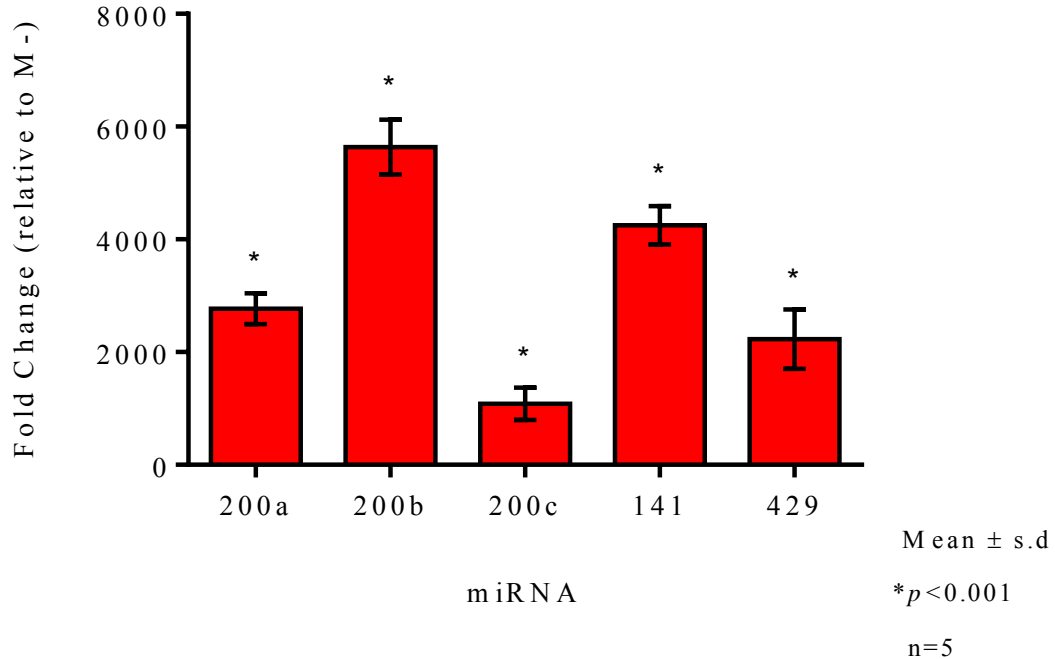
Following transfection with miR-200 family member mimics, we observed a decrease in the relative expression of RASSF2 protein in the normal colon epithelial (CCD 841) cell line. RASSF2 protein levels were significantly lower in cells when transfected with miR-200a, miR-200b, miR-429, and the combination of all five miR-200 family members ($p < 0.05$) (Figures 32 & 33).

Figure 27. miR-200 Family Members Amplification Plot Following Transfection



Amplification plot showing increased expression (denoted by the lower cycle number) of all miR-200 family mimics in the normal colon epithelial (CCD 841) cell line compared to the negative control.

Figure 28. miR-200 Family Expression in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with Individual miR-200 Family Mimics.



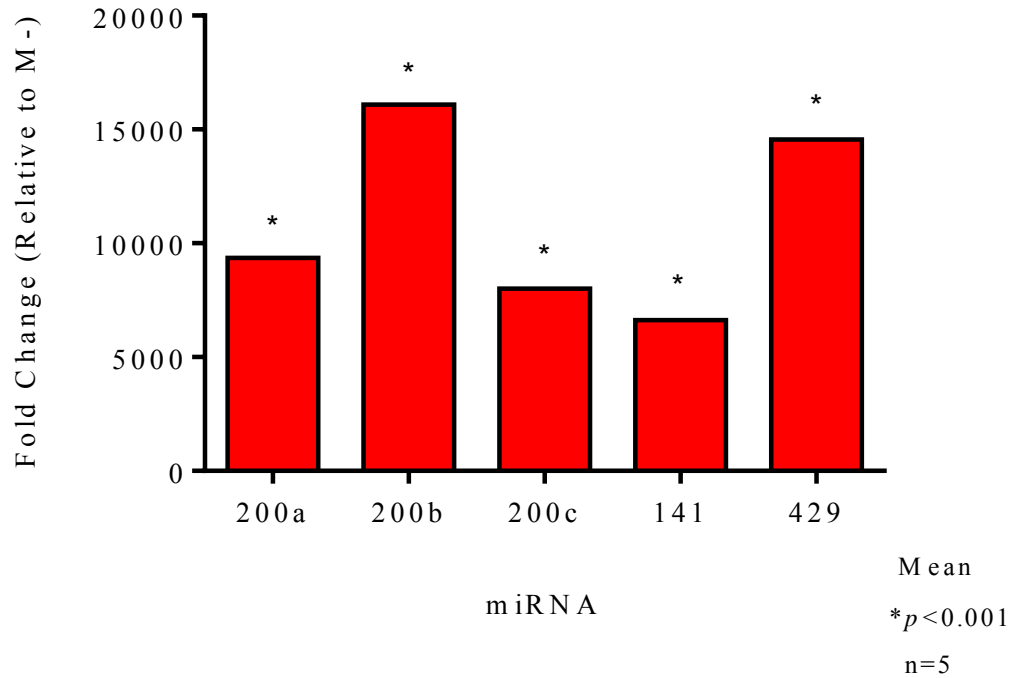
Transfected miRNA	Fold Change (\pm sd)	p -value
miR-200a	2769 (\pm 274)	<0.001
miR-200b	5638 (\pm 485)	<0.001
miR-200c	1084 (\pm 289)	<0.001
miR-141	4252 (\pm 341)	<0.001
miR-429	2230 (\pm 528)	<0.001

Upregulated

Transfection with individual miR-200 family miRNA mimics led to significant over expression of each individual miR-200 family member 24 hours after transfection of a normal colon epithelial (CCD 841) cell line. Data shown are mean \pm standard deviation.

* indicates p <0.001 vs. negative control.

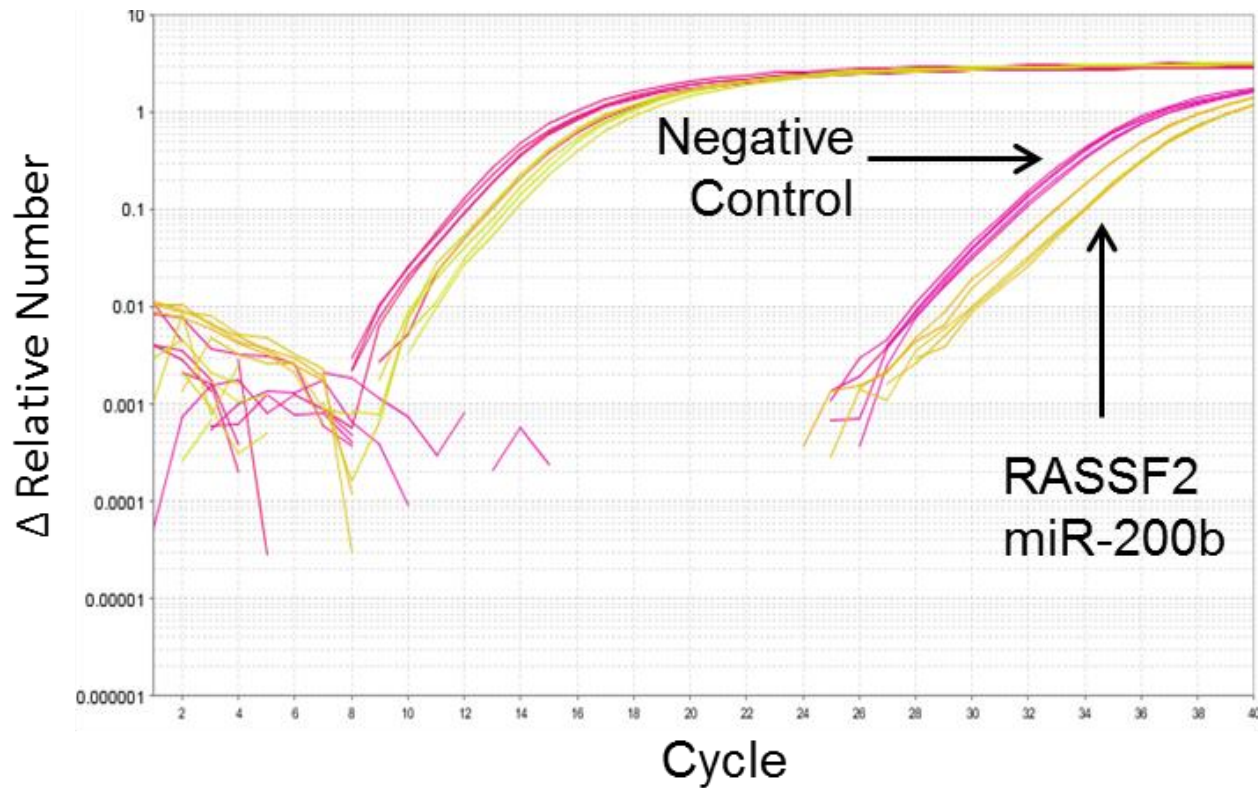
Figure 29. miR-200 Family Expression in a Normal Colon Epithelium (CCD 841) Cell Line Following Transfection with All miR-200 Family Mimics in Combination.



Transfected miRNA	Fold Change	<i>p</i> -value
miR-200a	9353	<0.001
miR-200b	16093	<0.001
miR-200c	8013	<0.001
miR-141	6626	<0.001
miR-429	14551	<0.001

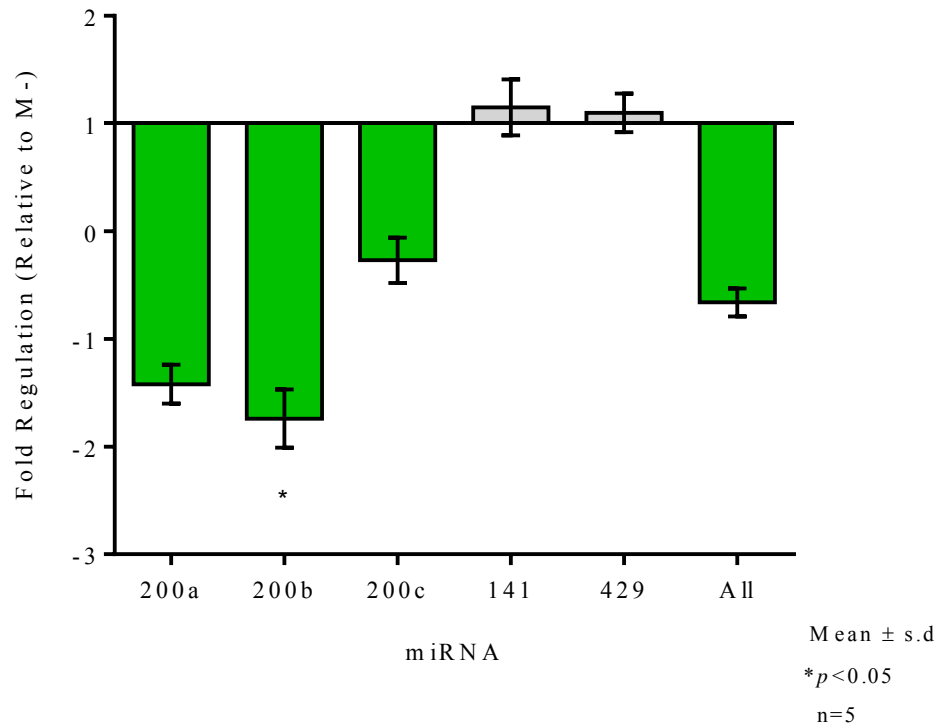
*Transfection with the combination of all miR-200 family miRNA mimics led to a significant increase in expression of miR-200a, miR-200b, miR-200c, and miR-429 after 24 hours. Data shown are mean * indicates $p < 0.001$ vs. negative control (M-).*

Figure 30. RASSF2 mRNA Amplification Plot Following Transfection of Normal Colon Epithelial (CCD 841) Cell Line with miR-200b.



Significant downregulation of RASSF2 mRNA was observed in the normal colon epithelial (CCD 841) cell line transfected with miR-200b after 24 hours. This can be observed by the later expression (higher cycle number) of RASSF2 compared to the negative control.

Figure 31. RASSF2 mRNA Expression in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics.



Transfected miRNA	Fold Regulation (\pm sd)	<i>p</i> -value
miR-200a	-1.42 (\pm 0.18)	0.077
miR-200b	-1.74 (\pm 0.27)	0.016
miR-200c	-0.27 (\pm 0.21)	0.760
miR-141	1.15 (\pm 0.26)	0.500
miR-429	1.10 (\pm 0.18)	0.610
miR-200 Family (All)	-0.66 (\pm 0.13)	0.750

Downregulated

Transfection with miR-200a, miR-200b, miR-200c, and the combination of all five miR-200 family members led to decreased RASSF2 mRNA expression. Data shown are mean \pm standard deviation. * indicates $p < 0.05$ vs. negative control (M-).

c) Discussion

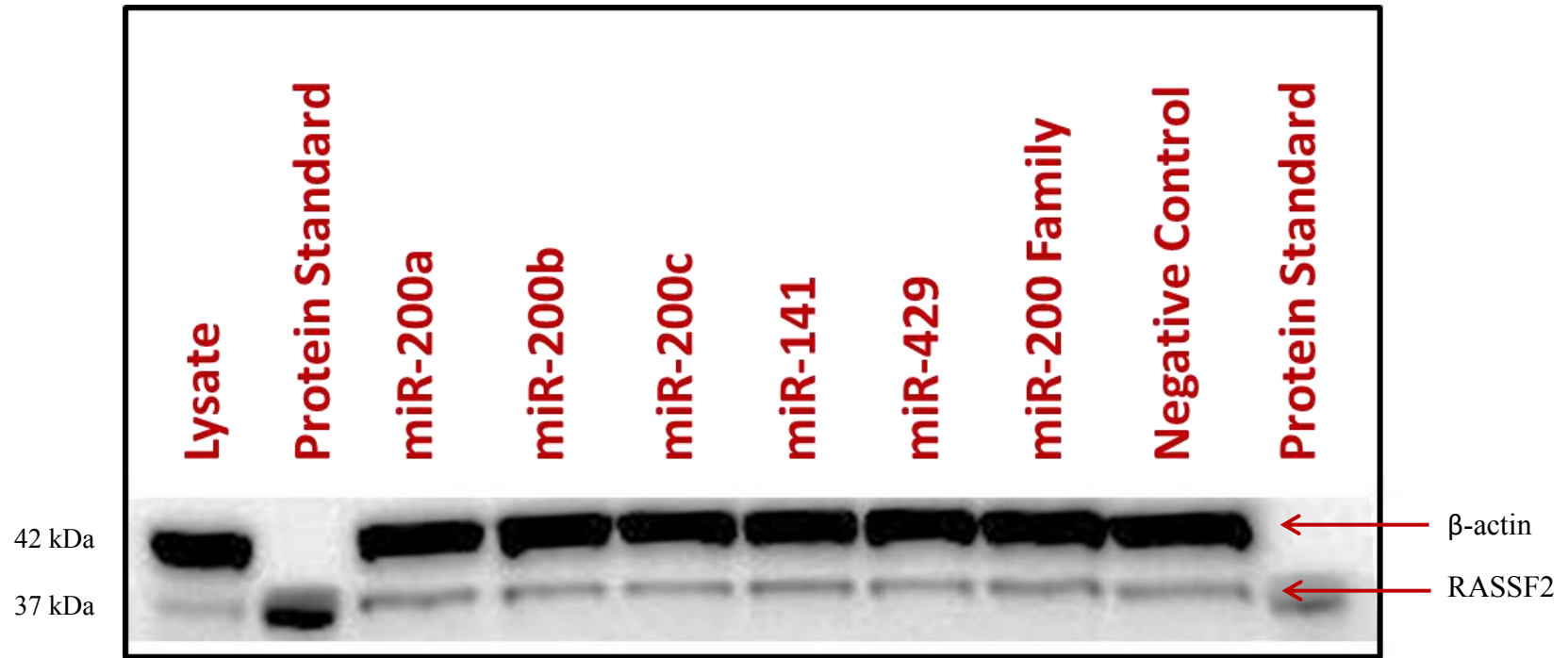
miRNA mimics provide a means to study the function of specific miRNAs in a range of organisms, and to validate their role in regulating target genes. miRNA mimics are small, chemically modified double-stranded RNA molecules that are designed to mimic endogenous mature miRNAs, resulting in artificial down-regulation of target mRNA translation. Our results demonstrate the ability to successfully transfect and increase the expression of the miR-200 family. We have seen an increase in each individual miR-200 family member when transfected into normal colon epithelial cells, which in turn has led to a decreased expression of RASSF2 mRNA and protein levels compared to the negative control. Of importance to note, following transfection with miR-200 family mimics, the largest increase in fold change was observed with miR-200b. This corresponded with the largest downregulation of RASSF2 mRNA and subsequent protein expression, consistent with the idea that the higher the fold change observed, the more likely a response in down-regulation of the target mRNA.

We also were successful in transfection of all five miR-200 family members in combination. By adding each miR-200 family mimic to cultured wells and measuring each individual expression, we observed much higher upregulation of miRNA expression than when transfected alone. This also resulted in the largest down-regulation of RASSF2 protein expression.

It is worth noting the use of miRNA mimics and interpreting results with caution. It has recently been reported that it remains unclear as to whether transient transfection of miRNAs behave similarly to endogenous miRNAs. Transient transfection of miRNA

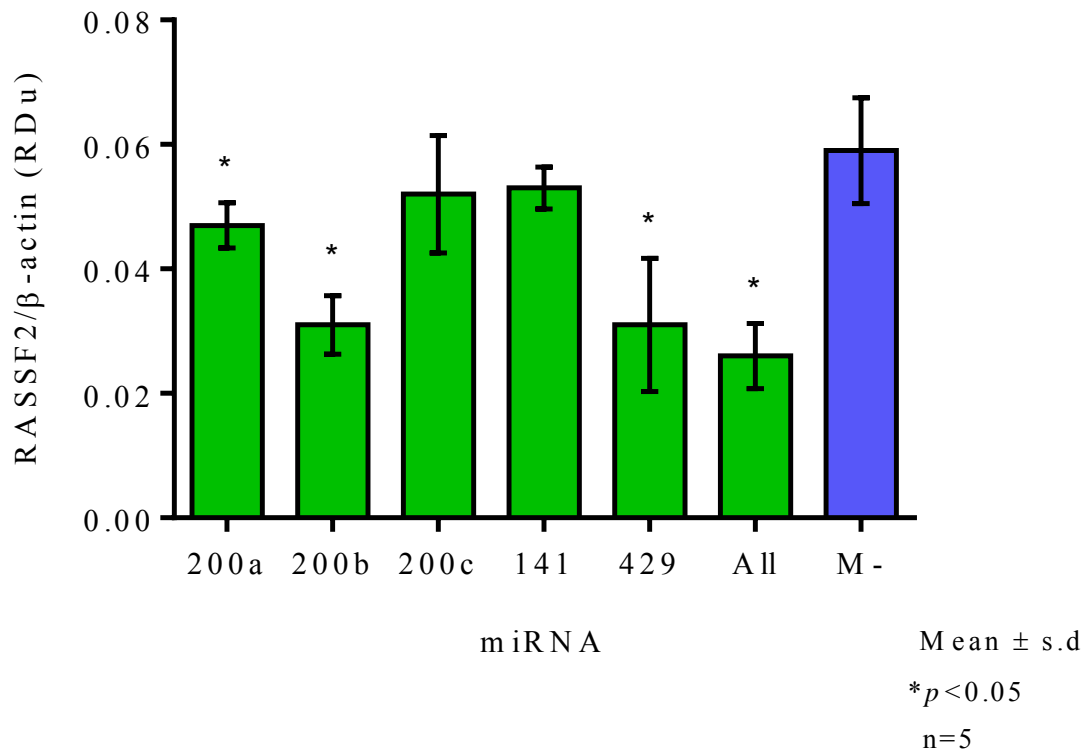
Figure 32. Western Blot of RASSF2 Protein in Normal Colon Epithelial Cell Line when Transfected with miR-200 Family Mimics.

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Transfection of miR-200a, miR-200b, miR-429, and a combination of all five miR-200 family mimics led to a significant decrease in RASSF2 protein level compared to the negative control.

Figure 33. RASSF2 Protein Expression in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics. The Results Show the Ratio of RASSF2 Protein Relative to β -Actin, Expressed as Relative Density Units (RDu), for each miRNA compared to the Negative Control (M-).



Decreased expression of RASSF2 protein was observed in a normal colon epithelial (CCD 841) cell line following transfection with miR-200a, miR-200b, miR-429, and the combination of all miR-200 family members. Data shown are mean \pm standard deviation.

** indicates $p < 0.05$ vs. negative control (M-).*

mimics at high concentrations in HeLa cells caused non-specific alterations in gene expression, while at low concentrations achieved expression levels comparable to other methods, such as lentiviral infection or plasmid transfection, but failed to efficiently suppress target gene expression. (148) We performed transfection at 24 and 48 hours and at 48 hours saw inconsistency within miRNA expression of the negative control. This led us to believe that transfection after 48 hours was inadequate and the results could not be appropriately interpreted, hence a transfection time of 24 hours was implemented. To minimize the risk of over expressing miRNA levels beyond normal pathological levels, we followed the manufacturer's recommendations. For each individual miR-200 family member a 25pmol transfection concentration was used, up to a maximum total of 125pmol when combining all five miR-200 family members together. These concentrations were within the limits of transfection described by Jin *et al.*

In conclusion we have managed to demonstrate significant upregulation of the miR-200 family in normal colon epithelial cells in contrast to the relatively low or absent baseline expression. This has in turn led to a decrease in RASSF2 mRNA expression consistent with the hypothesis that miR-200 family members target RASSF2. By demonstrating reduced RASSF2 protein expression we are now able to examine the effects of increasing the expression of the miR-200 family and its role within activity of the MAPK signaling pathway in colorectal cancer.

CHAPTER IX

LOSS OF FUNCTION OF miR-200 FAMILY IN DUKES' C (HT-29) COLORECTAL CANCER CELL LINE

a) Introduction

Dukes' C (HT-29) CRC cell line was chosen for investigation over other cell lines for three reasons,

- it had consistently higher expression of each individual miR-200 family member on initial screening compared to the other three CRC cell lines. We believed that in order to reduce expression of miRNAs using miRNA antagomirs, a cell line with higher expression of the miRNAs to be investigated would provide the best opportunity to detect downregulation.
- the HT-29 Dukes' C CRC cell line is microsatellite stable, within the MAPK pathway it is KRAS is wild type and is not mutated like the other CRC cell lines we screened.
- it is a metastatic cell line (derived from a cancer with lymph node metastasis) and therefore observations made on it would have direct clinical relevance and applicability with respect to designing newer treatment modalities.

miRNA antagomirs suppress the function of endogenous miRNAs. They prevent miRNAs from binding to its desired mRNA binding site, thus increasing its target gene expression. In previous experiments we have observed miR-200 family members to be highly expressed in the Dukes' C (HT-29) CRC cell line. We have also seen that increasing the expression of miR-200 family members in a normal colon epithelial (CCD 841) cell line reduces the expression of RASSF2 mRNA and RASSF2 protein.

The purpose of these experiments is to determine whether the reverse process is true in a CRC cell line. We therefore; hypothesized that by decreasing expression levels of miR-200 family members we would expect to see increased expression of RASSF2 mRNA and subsequent RASSF2 protein expression.

b) Results

1. Inhibition of miR-200 family members in the Dukes' C (HT-29) CRC cell line

Transfection of miR-200 family antagomirs to reduce expression of each miRNA was confirmed by qRT-PCR. Downregulation was observed in three of these miRNAs, significantly for cells transfected with miR-200b and miR-200c antagomirs with a fold downregulation of -2.8 and -10.6, respectively ($p < 0.01$) (Figure 34). When transfection of all five miR-200 family members in combination was performed, there was significant downregulation of miR-200a, miR-200b, miR-200c, and miR-429 ($p < 0.01$) (Figure 35).

2. Effect of Inhibition of miR-200 Family Members on RASSF2 mRNA

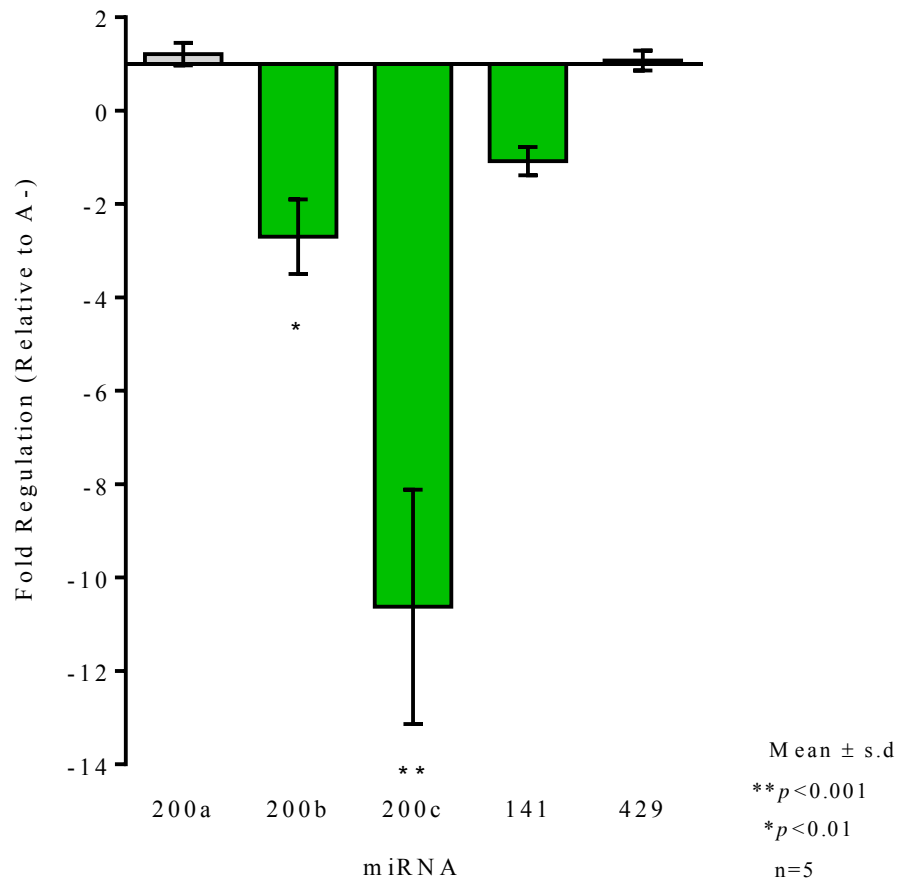
In our preliminary cell line screening, we did not identify RASSF2 mRNA expression in the Dukes' C (HT-29) CRC cell line. By knocking down expression of the

miR-200 family, we observed an increase in RASSF2 mRNA expression in this Dukes' C (HT-29) CRC cell line, significantly for miR-429 ($p < 0.001$) Transfection of all five miR-200 family members in combination, significantly up-regulated RASSF2 mRNA expression 3.7 fold ($p < 0.01$) (Figure 36).

3. Effect of Inhibition of miR-200 Family Members on RASSF2 Protein

In our preliminary cell line studies, we did not detect the presence of RASSF2 protein in the Dukes' C (HT-29) CRC cell line. Transfection with miR-200 family antagonists demonstrated expression of RASSF2 protein in all samples, as detected by Western blot (Figure 37). Significant up-regulation of RASSF2 protein was observed in the Dukes' C (HT-29) CRC cell line transfected with miR-141, miR-429, and all five miR-200 family members in combination, compared to the negative control (A-) ($p < 0.05$) (Figure 38).

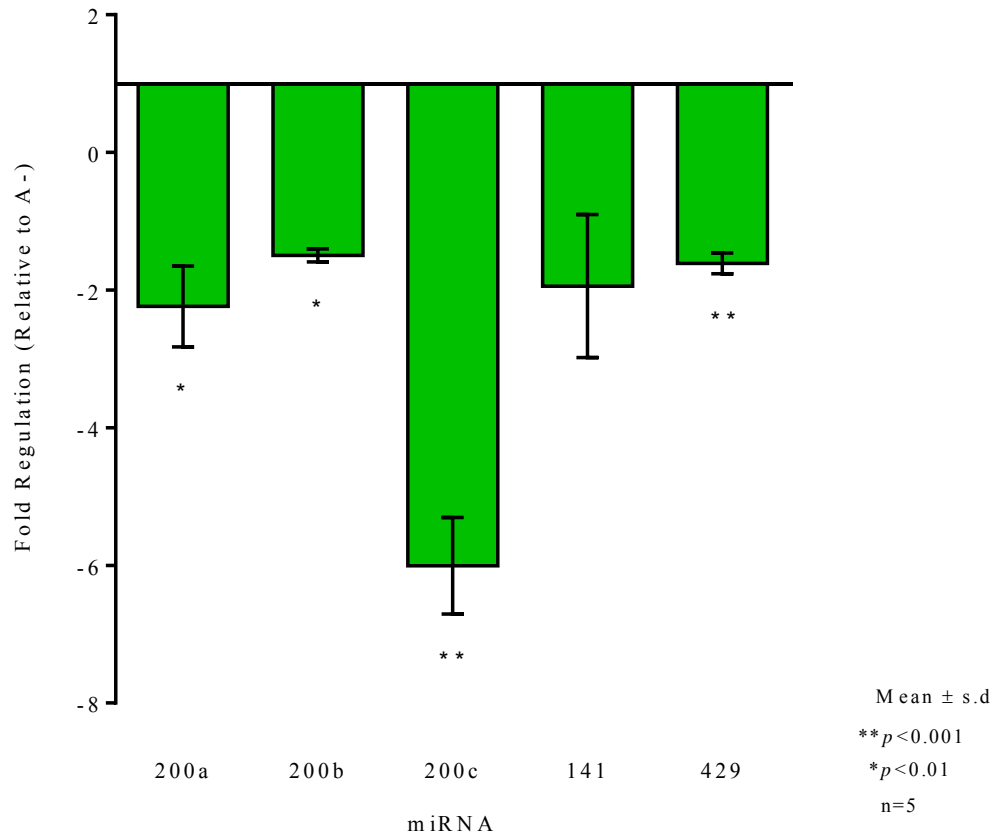
Figure 34. miR-200 Family Expression in a Dukes' C (HT-29) Cell Line Following Transfection with Individual miR-200 Family Antagomirs.



miRNA Antagomirs	Fold Regulation (± sd)	p-value
miR-200a	1.2 (±0.2)	0.149
miR-200b	-2.8 (±1.1)	<0.01
miR-200c	-10.6 (±2.1)	<0.001
miR-141	-1.1 (±0.4)	0.751
miR-429	1.1 (±0.2)	0.594

Transfection with miR-200 family miRNA antagomirs led to a significant decrease in expression of miR-200b and miR-200c after 24 hours. Data shown are mean ± standard deviation. * indicates $p < 0.01$ vs. negative control (A-).

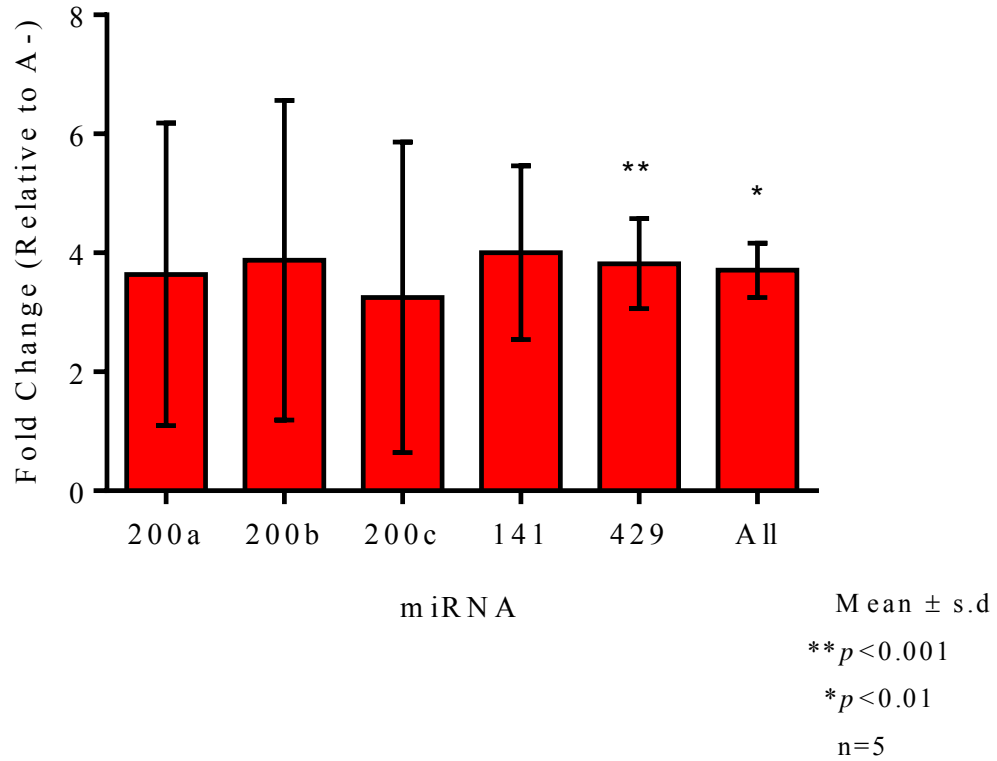
Figure 35. miR-200 Family Expression in a Dukes' C (HT-29) CRC Cell Line Following Transfection with All miR-200 Family Antagomirs in Combination.



miRNA Antagomirs	Fold Regulation (\pm sd)	<i>p</i> -value
miR-200a	-2.16 (\pm0.6)	<0.01
miR-200b	-1.49 (\pm0.1)	<0.01
miR-200c	-5.97 (\pm0.7)	<0.001
miR-141	-1.74 (\pm1.0)	0.07
miR-429	-1.61 (\pm0.2)	<0.001

Transfection with the combination of all miR-200 family miRNA antagomirs led to a significant decrease in expression of miR-200a, miR-200b, miR-200c, and miR-429 after 24 hours. Data shown are mean \pm standard deviation. * indicates $p < 0.01$ vs. negative control (A-).

Figure 36. RASSF2 mRNA Expression in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



Transfected miRNA	Fold Change (\pm sd)	<i>p</i> -value
miR-200a	3.64 (\pm 2.5)	0.12
miR-200b	3.88 (\pm 2.7)	0.08
miR-200c	3.25 (\pm 2.6)	0.15
miR-141	4.00 (\pm 1.5)	0.12
miR-429	3.82 (\pm 0.8)	<0.001
miR-200 Family (All)	3.71 (\pm 0.5)	<0.01

*Transfection with miR-200 family antagomirs led to increased RASSF2 mRNA expression. Data shown are mean \pm standard deviation. * indicates $p < 0.01$, ** $p < 0.001$ vs. negative control (A-).*

c) Discussion

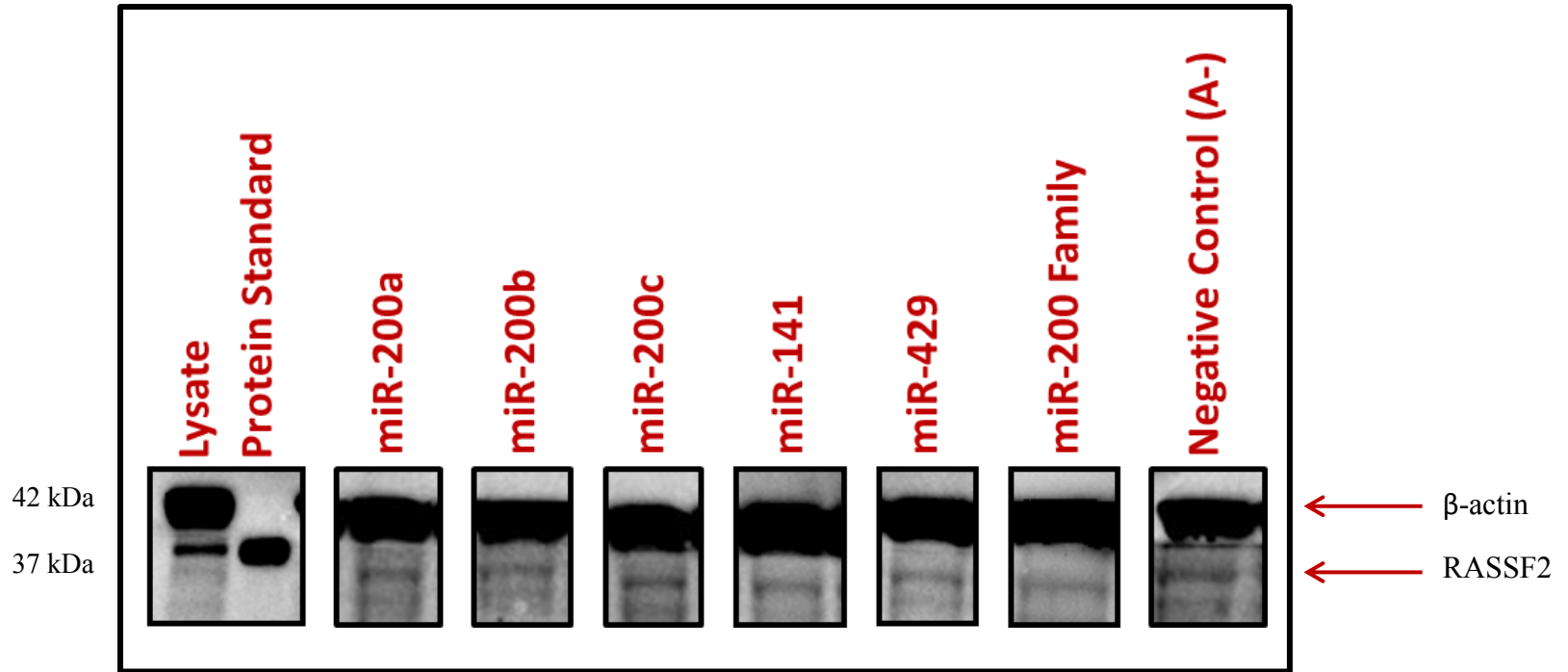
Following transfection of a Dukes' C (HT-29) CRC cell line with miR-200 family antagomirs we observed down-regulation of individual miR-200 family members up to 10 fold. miRNA inhibitors are chemically modified, single-stranded oligonucleotides designed to specifically bind to and inhibit endogenous miRNAs, resulting in artificial up-regulation of target mRNA translation. The binding of the antagomir with the target miRNA leads to the degradation of the miRNA by the same process as described for short interfering RNAs.(149)

We demonstrated significant down-regulation of miR-200b and miR-200c in a Dukes' C (HT-29) CRC cell line and upregulation of RASSF2 mRNA when transfecting cells with each individual miR-200 family antagomir. Although not a significant finding, we believed the more important observation of miR-200 family inhibition would manifest at the protein level.

In untransfected Dukes' C (HT-29) CRC cell lines, there was absent expression of both RASSF2 mRNA and protein. The fact we were able to observe amplification of RASSF2 at any level was encouraging. Again, transfection of all five miR-200 family members in combination produced the most significant RASSF2 mRNA expression.

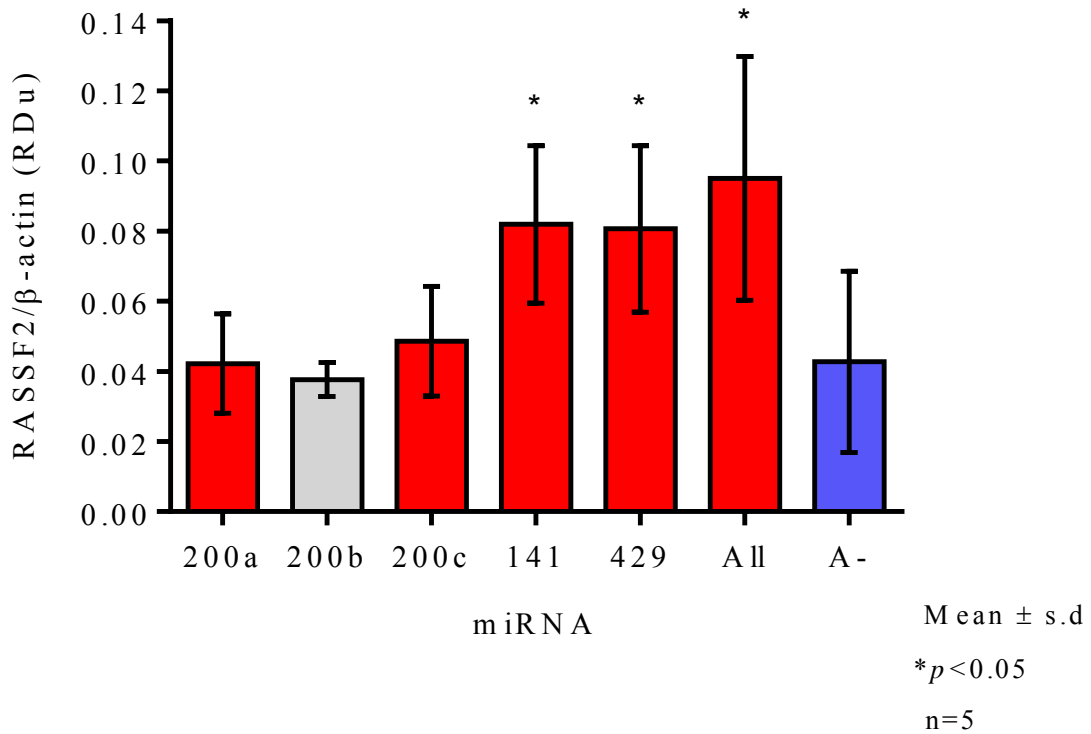
It is possible that transient transfection could affect miRNA silencing and ultimately the results we observe. Anecdotally, inhibition of miRNA using miRNA antagomirs is less successful than miRNA mimics. A number of factors can influence the

Figure 37. Western Blot of RASSF2 Protein in Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



Knockdown of miR-200 family members led to an increase in RASSF2 protein level compared to the negative control (A-).

Figure 38. RASSF2 Protein Expression in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs. The Results Show the Ratio of RASSF2 Protein Relative to β -Actin, Expressed as Relative Density Units (RDu), for each miRNA compared to the Negative Control (A-).



*Increased expression of RASSF2 protein was observed in the Dukes' C (HT-29) CRC cell line following transfection with miR-141, miR-429, and the combination of all miR-200 family members. Data shown are mean \pm standard deviation. * indicates $p < 0.05$ vs. negative control (A-).*

amount to which expression of a target gene is reduced, such as, transfection efficiency, transcription rate of the gene of interest, mRNA and protein stability, and the growth characteristics of the particular cell line used.(150) In our experience, Dukes' C (HT-29) CRC cells are very quick to divide and grow and this may be a factor in the limited success of miRNA antagomir transfection as accelerated growth may outweigh the supply. Lipofectamine RNAiMAX used in our experiments has been developed to increase the transfection efficiency of RNA into in vitro cell cultures. Lipofectamine reagent contains lipid subunits that can form liposomes in an aqueous environment, which entraps the transfection agent, in this case miRNA antagomirs. It is a cationic liposome formulation which joins with negatively charged nucleic acid molecules allowing them to overcome the electrostatic repulsion of the cell membrane.(151, 152) Fusion with the negatively charged plasma membrane occurs allowing the nucleic acid to cross into the cytoplasm. The contents are available to the cell for replication or expression, however; the nucleic acid must reach the cell nucleus to begin transcription. Issues could arise, for example, if the material never reaches the nucleus in the first place, or if it becomes trapped in the reassembling nuclear envelope following mitosis.(150)

Another consideration would be to perform multiple transfections. This would enable newly grown cells to take up the lipid complex and miRNA antagomir. However, transfection-mediated cytotoxicity can be an issue and can mask the true phenotype of the target gene being studied and also affect cell viability. Increasing the frequency of transfection was not considered to be a viable option in our experiments, as we have already demonstrated that a greater than 24 hour transfection was detrimental to cell function and that interpretation of the results at 48 hours was unreliable.

In conclusion, we have demonstrated the ability to down-regulate expression of members of the miR-200 family which were highly expressed in Dukes' C (HT-29) CRC cells. In doing so, we have upregulated expression of RASSF2 mRNA and protein. This suggests that inhibition of miR-200 family members increases RASSF2 expression. This warrants further investigation on the effect of inhibition of the miR-200 family and subsequent RASSF2 expression on KRAS and MAPK pathway activity.

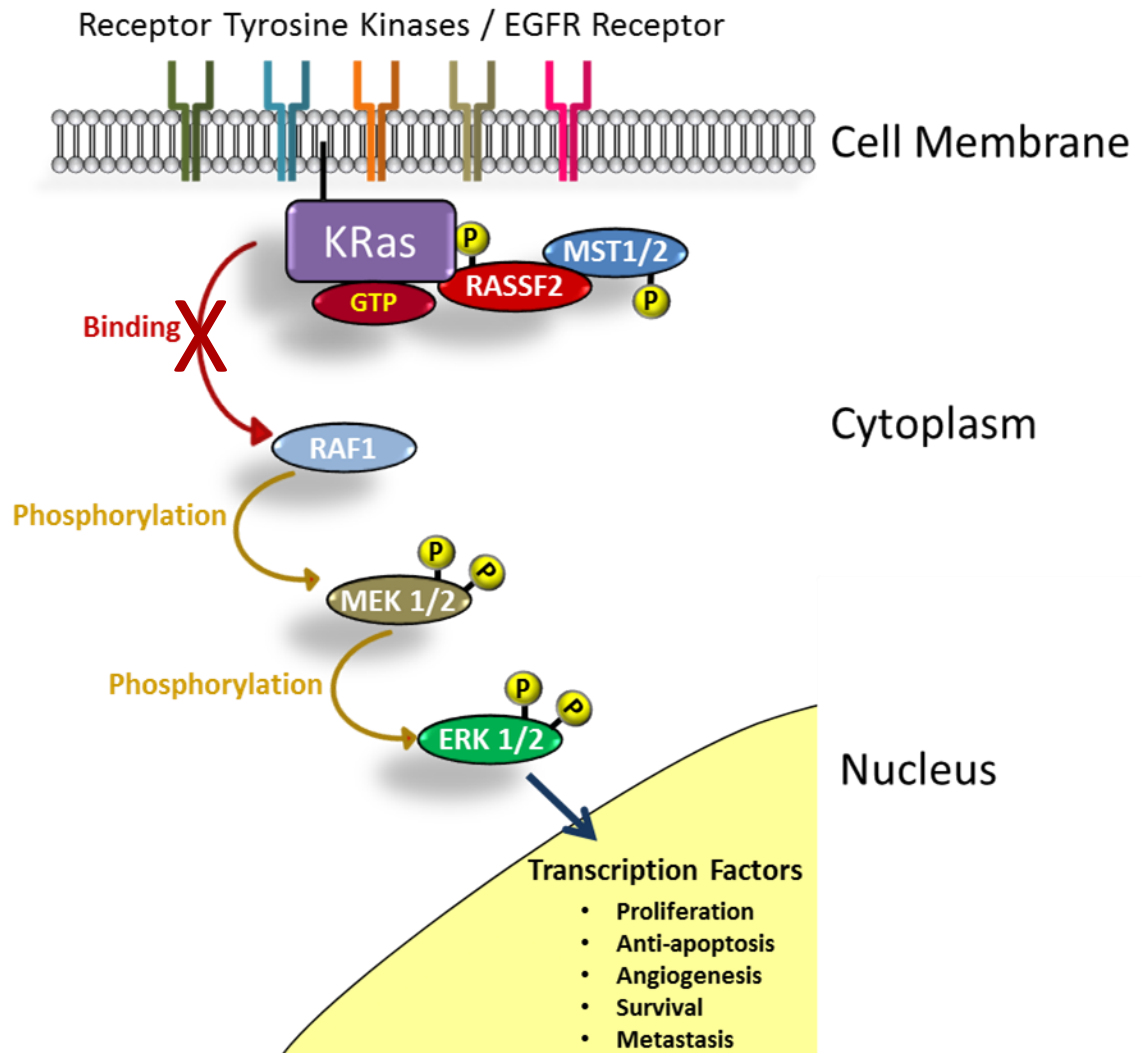
CHAPTER X

MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY ACTIVATION AND CELL PROLIFERATION

a) Introduction

In our previous experiments we demonstrated that successful overexpression of the miR-200 family in a normal colon epithelial (CCD 841) cell line leads to reduced expression of RASSF2 mRNA and RASSF2 protein. Conversely, knockdown of the miR-200 family members in a Dukes' C (HT-29) CRC cell line leads to an increased expression of RASSF2 mRNA and RASSF2 protein. RASSF2 is a known negative regulator of Ras and binds directly to K-Ras, within the MAPK pathway, in a GTP-dependent manner. Once K-Ras is activated, it initiates a phosphorylation cascade of downstream proteins resulting in altered gene expression. miRNAs from the miR-200 family have been shown to promote cell proliferation when transfected into various cell lines and may impact K-Ras expression and downstream pathways.(153) As the miR-200 family target RASSF2, and considering that RASSF2 is a negative regulator of K-Ras, manipulation of miR-200 family expression may lead to changes within the MAPK pathway affecting transcription factors and the resulting downstream functional processes such as cell proliferation (Figure 39).

Figure 39. Association between RASSF2 and K-Ras in the MAPK Pathway.



RASSF2 is a negative regulator of Ras. It binds directly to K-Ras in a GTP-dependent manner via the Ras effector domain. The miR-200 family target RASSF2 and changes in expression could affect transcription factors and the resulting downstream functional processes such as cell proliferation.

We propose that by manipulating the expression of the miR-200 family members in a normal colon epithelial (CCD 841) and a Dukes' C (HT-29) CRC cell line we will affect total K-Ras expression thereby leading to a change in activity within the MAPK pathway, as measured by the phosphorylation of ERK 1/2, a downstream protein within this pathway. We also hypothesize that increased activation of ERK 1/2 in a normal colon epithelial (CCD 841) cell line will result in increased cell proliferation, suggesting oncogenic-like properties, whereas decreased ERK 1/2 activation in a Dukes' C (HT-29) CRC cell line will result in decreased cell proliferation, highlighting the potential use of miR-200 family as a therapeutic target in CRC.

b) Results

Total K-Ras Expression

Following successful transfection of a normal colon epithelial (CCD 841) cell line with miR-200 family members we observed an increase in total K-Ras expression in cells transfected with miR-200a, miR-141 and miR-429 compared to the negative control (M-) ($p < 0.05$) (Figures 40 & 41).

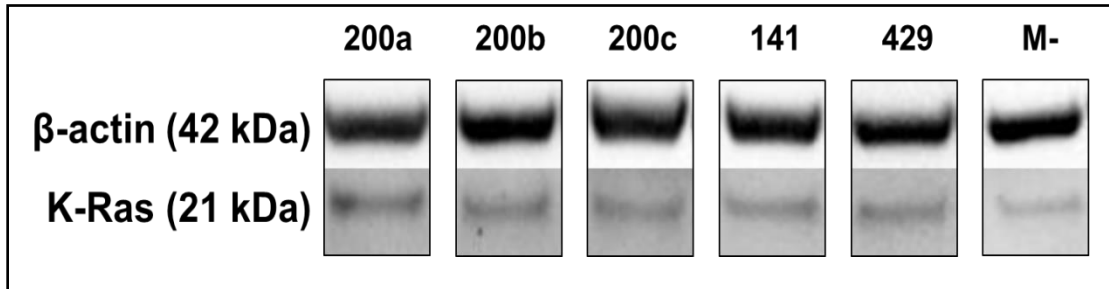
In a Dukes' C (HT-29) CRC cell line total K-Ras was increased compared to the negative control (A-) following transfection with miR-200a and miR-200c ($p < 0.05$) (Figures 42 & 43).

ERK 1/2 Activation

In a normal colon epithelial (CCD 841) cell line, measurement of total and phosphorylated ERK 1/2 was performed following transfection with miR-200 family mimics. We observed an increased ratio of phosphorylated to total ERK 1/2 compared to the negative control (M-). This indicates a significant activation of the MAPK pathway for all miR-200 family mimics ($p < 0.01$) (Figures 44 & 45).

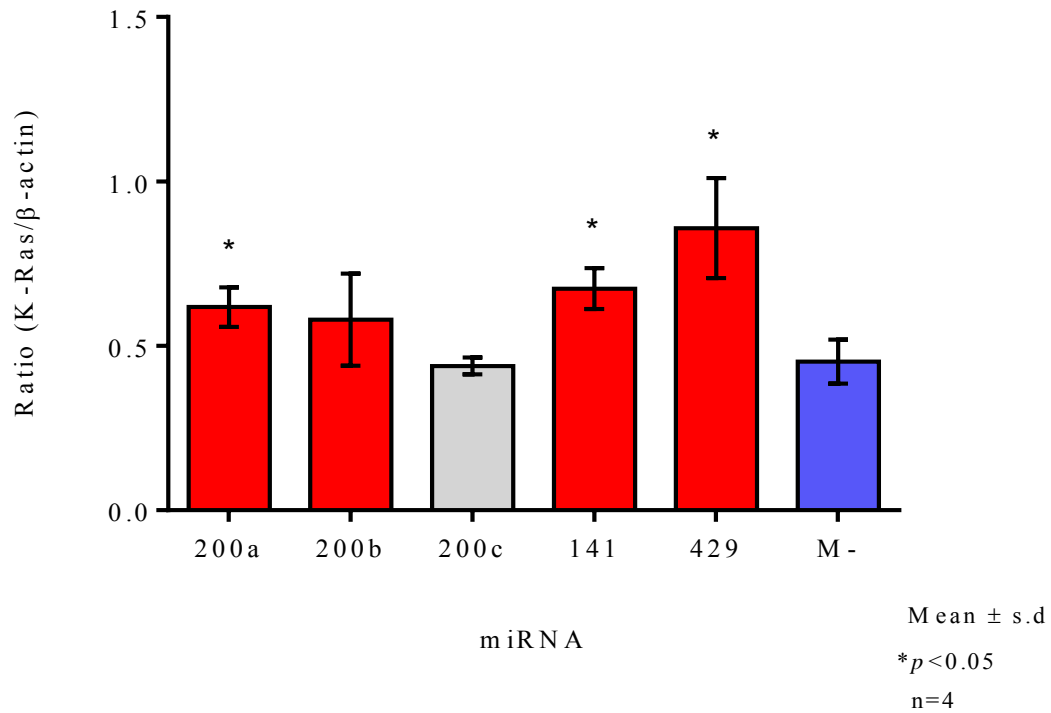
In the Dukes' C (HT-29) CRC cell line, measurement of total and phosphorylated ERK 1/2 following transfection with miR-200 family antagomirs, showed a decreased ratio of phosphorylated to total ERK 1/2 compared to the negative control (A-) indicating a significant decrease in MAPK pathway activation for cells transfected with miR-200a, miR-200b, miR-141, and the combination of all five miR-200 family member antagomirs ($p < 0.05$) (Figures 46 & 47).

Figure 40. Western Blots for Total K-Ras Protein Expression in a Normal Colon Epithelial (CCD 841) Cell Line.



Representative Western blot images showing total K-Ras expression. Bands were detected at 21kDa in all cells transfected with miR-200 family mimics.

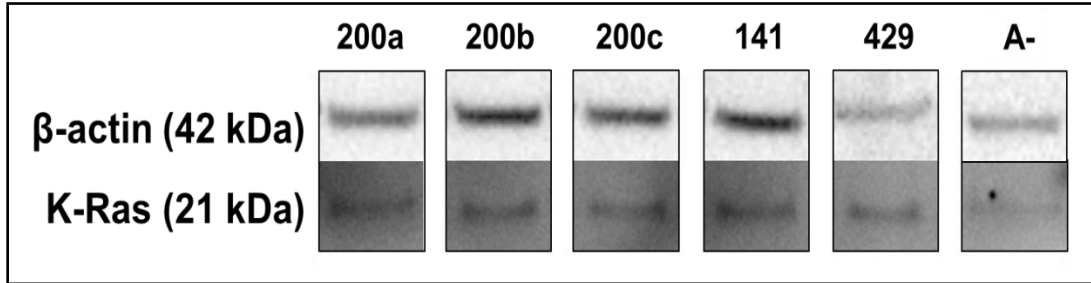
Figure 41. Total K-Ras Protein Expression in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics.



miRNA mimics	Average Ratio (K-Ras/ β -actin)	<i>p</i> -value
miR-200a	0.618	0.026
miR-200b	0.580	0.178
miR-200c	0.439	0.769
miR-141	0.674	0.009
miR-429	0.643	0.029
M-	0.452	

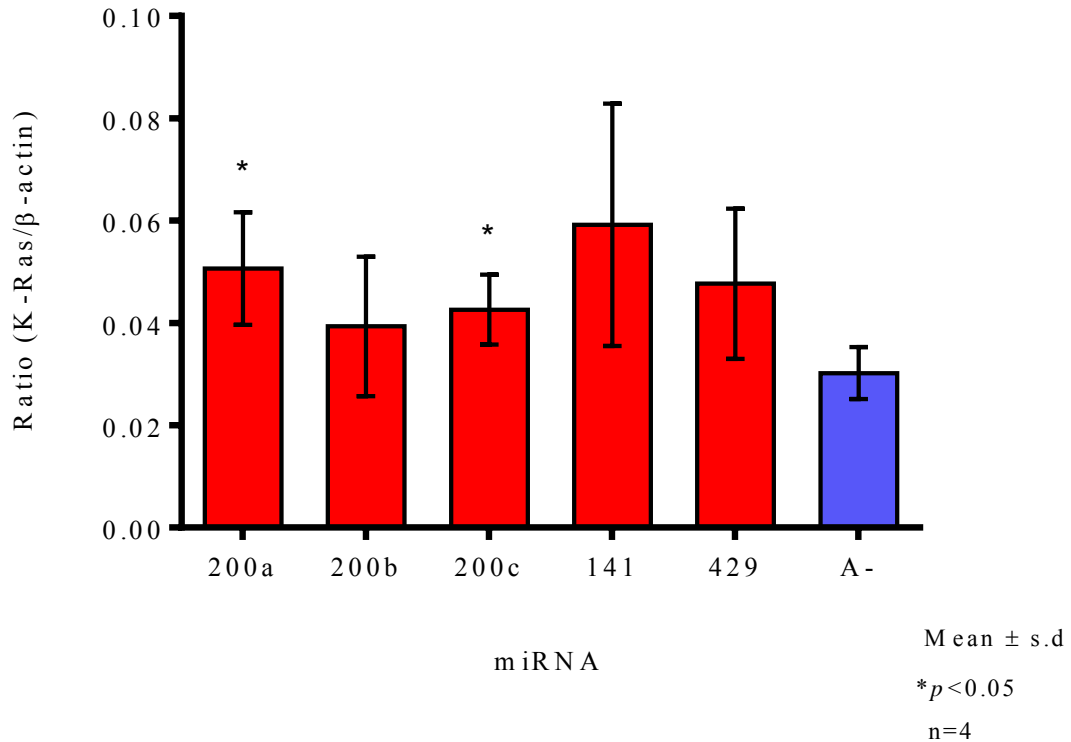
An increase in total K-Ras expression was observed in cells transfected with miR-200a, miR-141 and miR-429 compared to the negative control (M-). Data shown are mean \pm standard deviation. * indicates $p < 0.05$ vs. negative control (M-).

Figure 42. Western Blots for Total K-Ras Protein Expression in a Dukes' C (HT-29) CRC Cell Line.



Representative Western blot images showing total K-Ras expression. Bands were detected at 21kDa in all cells transfected with miR-200 family antagomirs.

Figure 43. Total K-Ras Protein Expression in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



miRNA antagomirs	Average Ratio (K-Ras/ β -actin)	p -value
miR-200a	0.051	0.027
miR-200b	0.039	0.286
miR-200c	0.043	0.040
miR-141	0.059	0.089
miR-429	0.048	0.166
A-	0.030	

An increase in total K-Ras expression was observed in cells transfected with miR-200a, and miR-200c compared to the negative control (A-). Data shown are mean \pm standard deviation. * indicates $p < 0.05$ vs. negative control (A-).

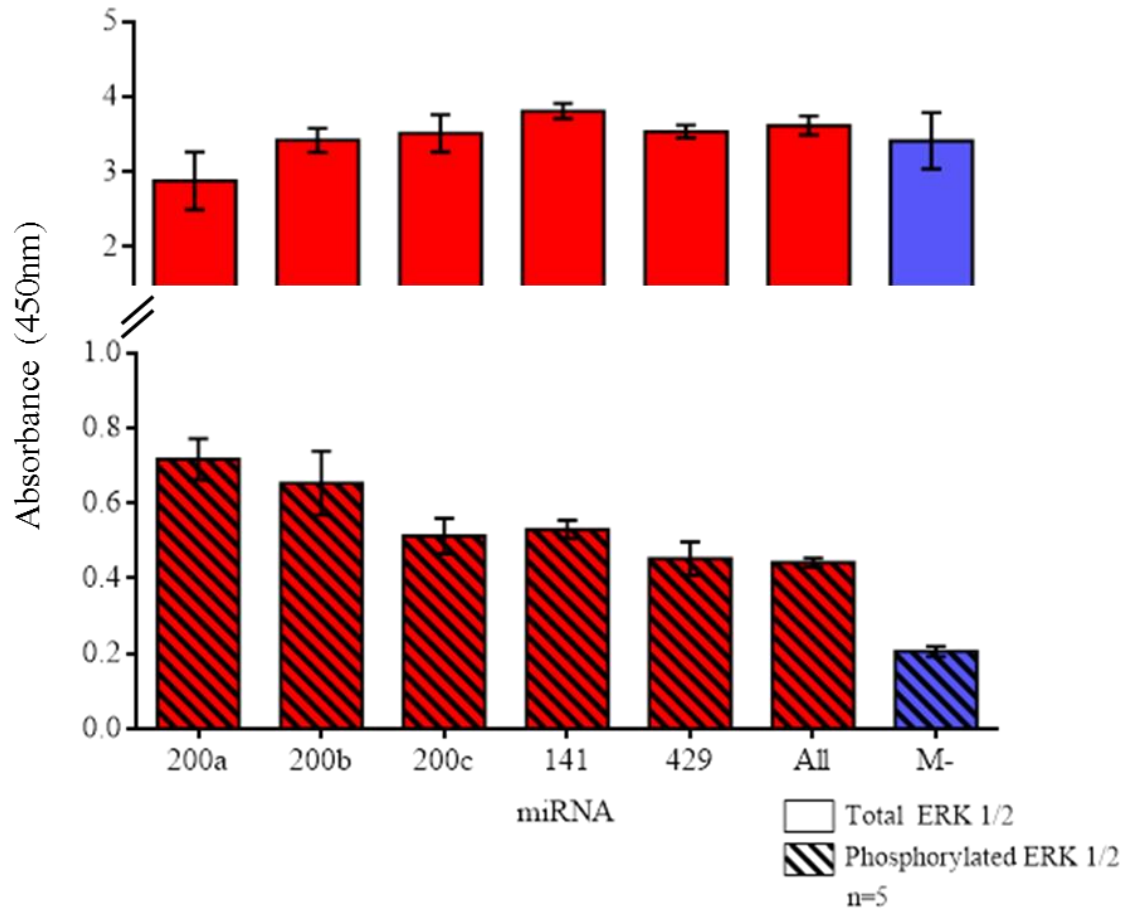
Cell Proliferation

Measuring cell proliferation as the resulting functional outcome of activity within the MAPK pathway was performed for both normal colon epithelial (CCD 841) and Dukes' C (HT-29) CRC cell lines. Following transfection of miR-200 family mimics in the normal colon epithelial (CCD 841) cell line, we observed a significant increase in cell proliferation at day five in cells transfected with miR-200a, miR-200c and miR-141 mimics compared to the negative control ($p < 0.05$) (Figure 48). Cell proliferation was also increased in the normal colon epithelial (CCD 841) cell line following transfection with miR-200a at days 2, 4 and 5 ($p < 0.01$).

No significant increase in cell proliferation was observed for miR-200b, miR-429, and when all five miR-200 family members were transfected into cells in combination. Cell proliferation growth curves for each miR-200 family member are shown in figure 49.

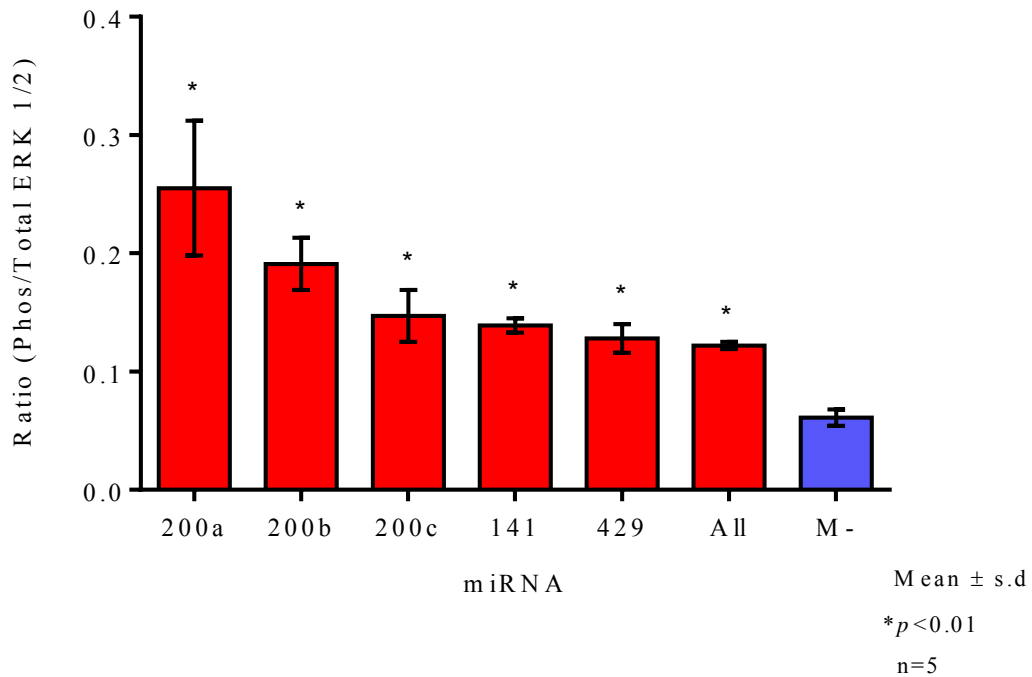
In the Dukes' C (HT-29) CRC cell line, cell proliferation was significantly decreased following transfection for individual ($p < 0.001$) and the combination of all miR-200 family member antagomirs ($p < 0.05$) at day five compared to the negative control (Figure 50). miRNAs-200b, miR-200c, miR-141, miR-429, and the combination of all five miR-200 family members had a significant decrease in cell proliferation at day four ($p < 0.05$). Cell proliferation growth curves for each miR-200 family member are shown in figure 51.

Figure 44. ELISA Measurement of Total and Phosphorylated ERK 1/2 in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics.



Data shown are mean \pm standard deviation.

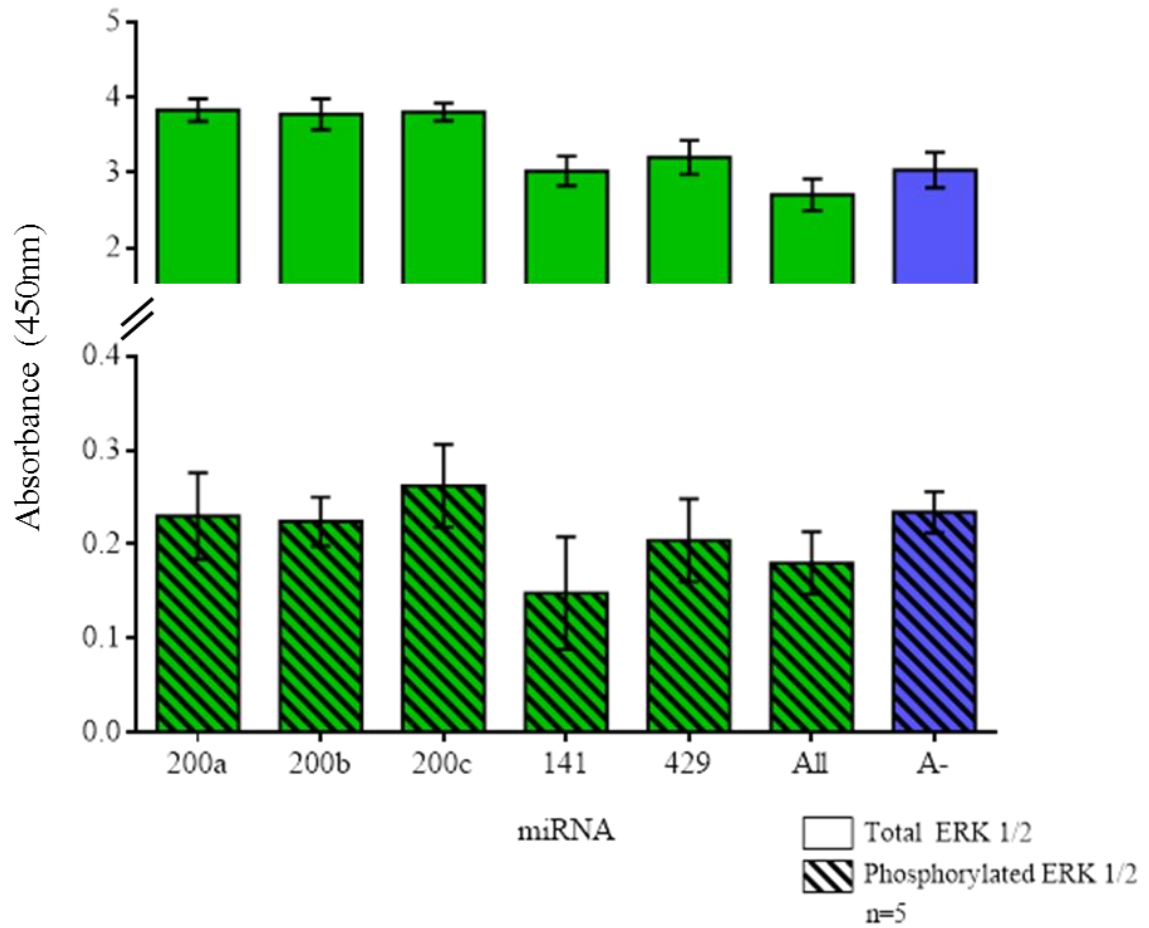
Figure 45. ERK 1/2 Activation in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics.



miRNA	Ratio ERK1/2 (Phos/Total)	<i>p</i> -value
200a	0.249	0.002
200b	0.191	<0.001
200c	0.146	<0.001
141	0.139	<0.001
429	0.128	<0.001
miR-200 Family (All)	0.122	<0.001
M-	0.060	

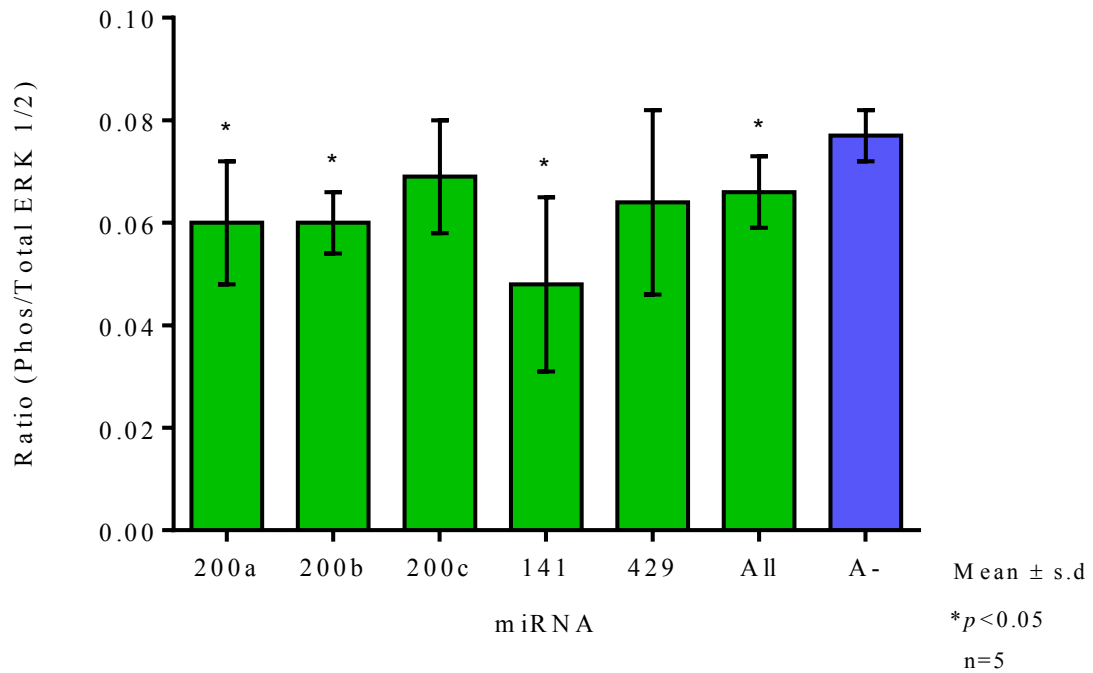
*Increased ratio of phosphorylated to total ERK1/2 compared to negative control (M-) indicates significantly increased activation of the MAPK pathway for all miR-200 family mimics. Data shown are mean \pm standard deviation. * indicates p <0. 01 vs. negative control (M-).*

Figure 46. ELISA Measurement of Total and Phosphorylated ERK 1/2 in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



Data shown are mean \pm standard deviation.

Figure 47. ERK 1/2 Activation in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



miRNA	Ratio ERK1/2 (Phos/Total)	p-value
200a	0.060	0.031
200b	0.060	0.001
200c	0.069	0.166
141	0.048	0.017
429	0.064	0.189
miR-200 Family (All)	0.066	0.024
A-	0.077	

Decreased ratio of phosphorylated to total ERK1/2 compared to negative control (A-) indicates significantly decreased activation of the MAPK pathway for miR-200a, miR-200b, and 141 and the combination of all five miR-200 family member antagomirs. Data shown are mean \pm standard deviation. * indicates $p < 0.05$ vs. negative control (A-).

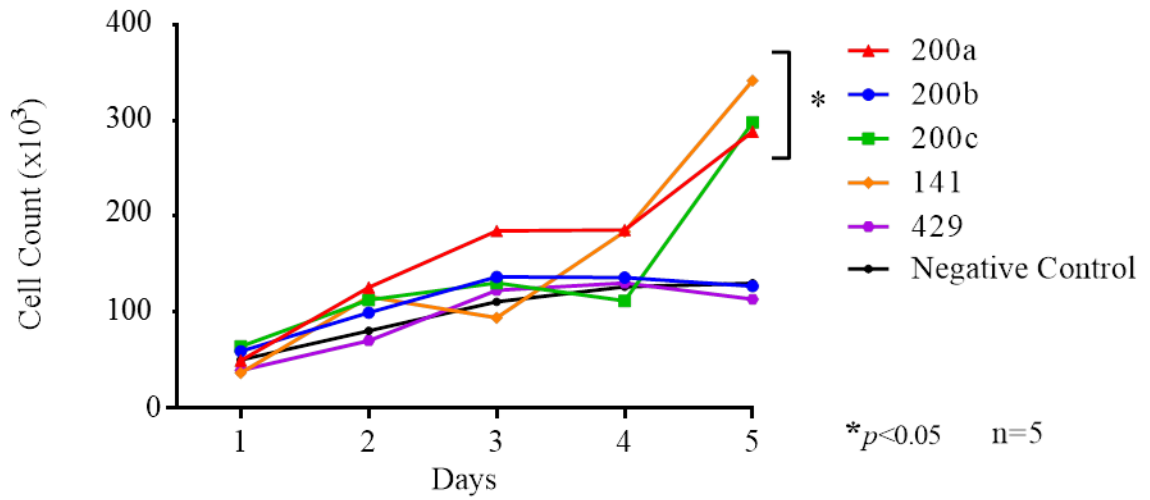
c) Discussion

We have demonstrated that overexpression of miR-200 family members and a reduction in RASSF2 protein expression leads to increased K-Ras expression in normal colon epithelial (CCD 841) cells transfected with miR-200a, miR-200b, miR-141, and miR-429 mimics. In addition, greater expression of miR-200 family members also increases activation of the MAPK signaling pathway as demonstrated by enhanced phosphorylation of ERK 1/2 in relation to total levels (Figure 39). This increase in activity may also explain the observed increase in cell proliferation as a measure of a functional outcome of the increased MAPK pathway activity.

Conversely, loss of function of miR-200a and miR-200c in Dukes' C (HT-29) CRC cells results in increased K-Ras expression, confirming previous findings that miR-200c is inversely correlated with K-Ras expression in certain cancers. (154) Despite the increase in K-Ras expression, we observed a decreased ratio of phosphorylated to total ERK 1/2 associated with loss of function in miR-200a, miR-200b, and miR-141, indicating MAPK pathway inactivation. MAPK pathway inactivation may explain the significant decrease in cell proliferation seen for all members of the miR-200 family.

The ERK MAPK signaling pathway is regarded as one of the most important regulators of cell proliferation.(155) There is growing evidence to suggest that activation within this pathway is involved in the pathogenesis, progression, and oncogenic behavior of colorectal cancer.(52) Disruption of the cell cycle in CRC is common and contributes

Figure 48. Cell Proliferation in a Normal Colon Epithelial (CCD 841) Cell Line Following Transfection with miR-200 Family Mimics.



Cell count as a measurement of proliferation shows statistically significant increase in count by day 5 for miR-200a, miR-200c, and miR-141 mimics.

Figure 49. Cell proliferation growth curves for each miR-200 family member in a Normal Colon Epithelial (CCD 841) Cell Line.

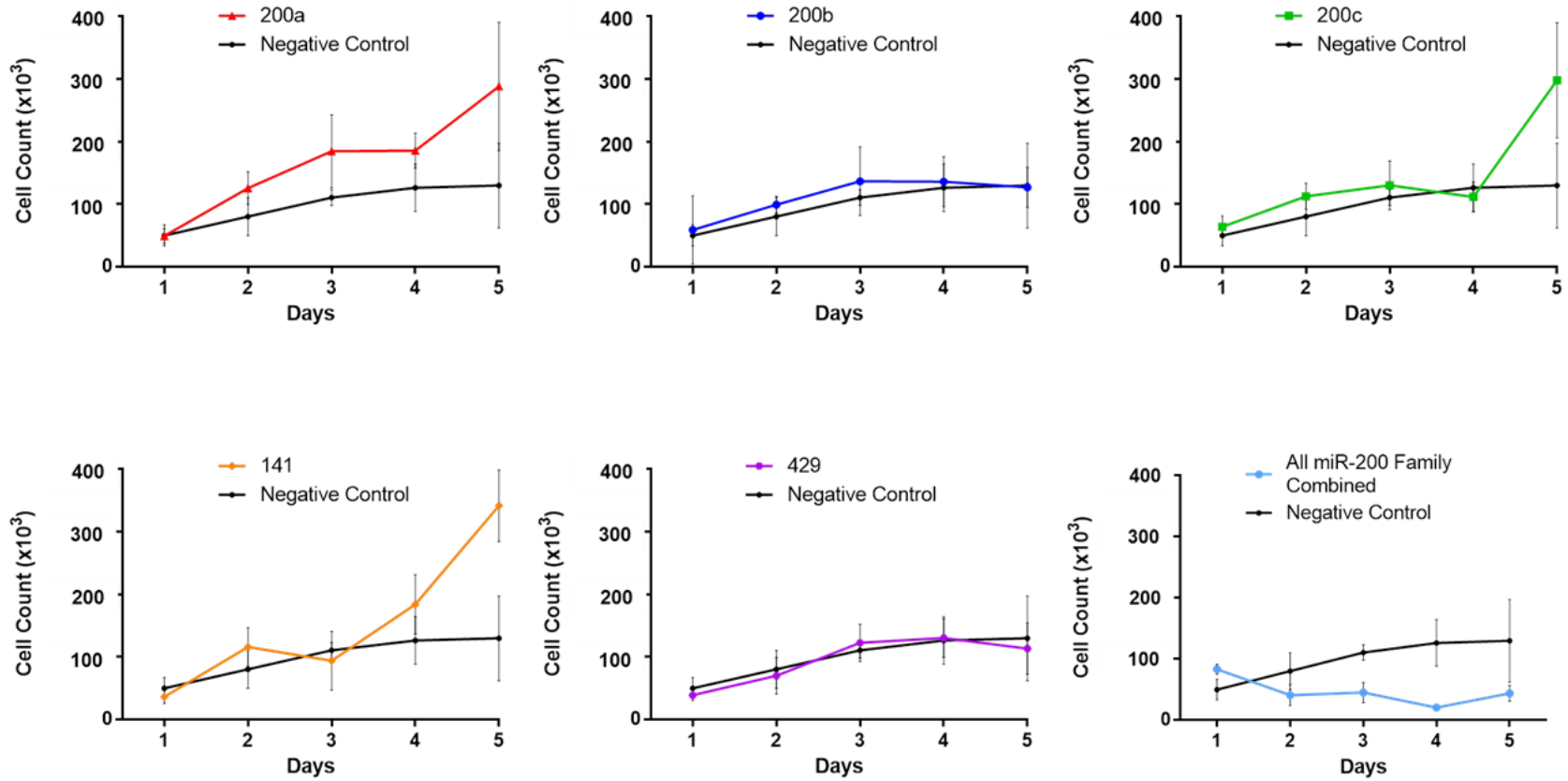
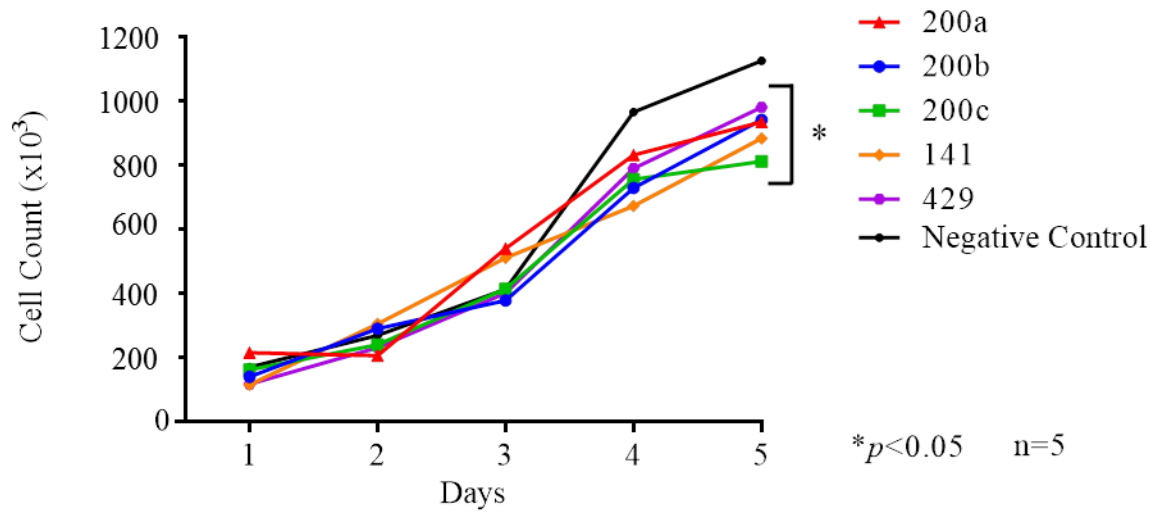
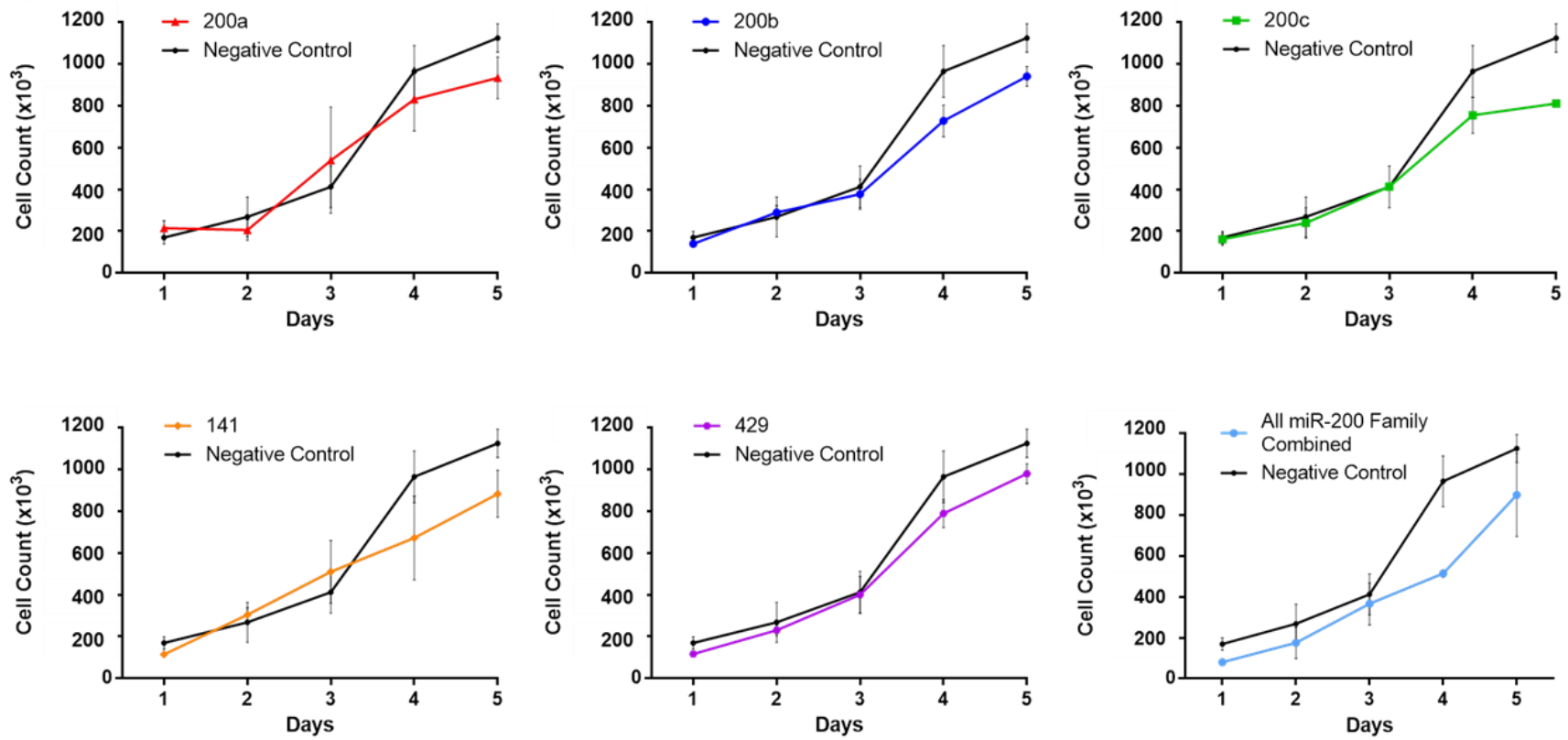


Figure 50. Cell Proliferation in a Dukes' C (HT-29) CRC Cell Line Following Transfection with miR-200 Family Antagomirs.



Cell count as a measurement of proliferation shows statistically significant decrease in count by day 5 for all miR-200 family antagomirs.

Figure 51. Cell proliferation growth curves for each miR-200 family member in a Dukes' C (HT-29) CRC Cell Line.



to tumorigenesis. Changes in ERK signaling may contribute to cell cycle arrest in the G2/M phase.(156) Ras activity is another observation suggestive of MAPK pathway activation. High Ras activity is accompanied by an increased ERK 1/2 activity.(157) We have demonstrated higher K-Ras expression following transfection with miR-200 family mimics and antagomirs. Whilst we might expect to see lower levels of K-Ras in Dukes' C (HT-29) CRC cell lines transfected with miR-200 family antagomirs, hence reducing downstream ERK 1/2 activity, we saw an increase in total K-Ras levels compared to the negative control. Although miRNAs are typically associated with decreased protein expression, a possible explanation could be differential activation of K-Ras expression and repression of translation through separate base-pairing mechanisms. In addition, although we cannot make a direct comparison between the normal colon epithelial (CCD 841) cell lines, transfected with miR-200 family mimics, compared to the Dukes' C (HT-29) CRC cell lines, transfected with miR-200 family antagomirs, it would be worth noting the total K-Ras expression ratios. The ratio of total K-Ras to β -actin in normal colon epithelial (CCD 841) cell lines was approximately ten times higher than the ratio of total K-Ras to β -actin in Dukes' C (HT-29) CRC cell lines (~ 0.6 vs. 0.05). This might suggest to us that we did observe a reduction in overall levels of total K-Ras in Dukes' C (HT-29) CRC cells hence the resulting decrease in ERK 1/2 activation seen.

Association of the miR-200 family and interaction with the MAPK signaling pathway is well documented. The majority of knowledge regarding the miR-200 family is the involvement with epithelial to mesenchymal transition (EMT). The miR-200 family promotes the epithelial state by suppressing epithelial gene transcriptional repressors, Zeb1/Zeb2. In one study, overexpression of miR-200c was associated with enhanced

EGFR and MAPK signaling pathways and lead to suppression of cell invasion in mouse breast cancer cells.(158) There is also evidence to support the association of miR-200 and KRAS. In a mutant K-Ras lung adenocarcinoma mouse model, overexpression of miR-200a, miR-200b, and miR-429 inhibited tumor growth and metastasis.(159) In addition to the miR-200 family association with tumor cell invasion, they have been identified to govern other tumor cell biologic processes, such as proliferation.(160) miR-200c has been shown to modulate KRAS in breast cancer cell lines. KRAS silencing by miR-200c lead to reduced proliferation in KRAS wild-type but not KRAS mutated breast cancer cell lines.(154)

Dysregulation of the miR-200 family has been observed to be both up-regulated and down-regulated in different types of cancer. The majority of functional studies, particularly in breast cancer, concentrate on down-regulation of miR-200, suggesting that miR-200 members prevent tumor progression.(161) Despite this, identification of up-regulation of the miR-200 family in cancers, including colorectal cancer, suggests that miR-200 family members may in fact be promoting progression of certain cancers. In melanoma cell lines, the levels of miR-200 family members were increased compared to normal melanocytes, and expression did not suppress tumor invasion.(160) They identified that up-regulation of miR-200a and miR-200c drives different modes of tumor cell invasion, either “mesenchymal-type” or “amoeboid-like”. Overall, this group questioned previous findings of the role of the miR-200 family in suppressing invasion and metastasis. To support these findings, two other recent studies identified that overexpression of the miR-200 family promoted cell proliferation in culture, and metastasis in vivo.(153, 162)

We believe our findings that a reduction in miR-200 family members leads to a decrease in MAPK pathway activity and a reduction in cell proliferation in Dukes' C (HT-29) CRC cell lines is novel, and suggests that the miR-200 family acts as an oncogene in CRC. This provides an opportunity to develop new and targeted therapies to treat colorectal cancer.

CHAPTER XI

CONCLUDING REMARKS AND DIRECTION OF FUTURE STUDIES

The identification of high expression of the miR-200 family in CRC cell lines compared to a normal colon epithelial (CCD 841) cell line allowed us to investigate the role of this family in the progression of CRC. Previous research with the miR-200 family has focused on EMT of cancer cells where dysregulation is considered to exhibit tumor suppressive behavior. However; in various cancers increased levels of some individual members of the miR-200 family contradicts the tumor suppressive role of this miRNA family. The purpose of this project was to investigate the role of the miR-200 family in CRC cell lines in order to gain a better understanding regarding the role that this family plays in colorectal cancer.

Here, we have identified an association between the miR-200 family and the RASSF2 tumor suppressor, and have investigated their impact on activity within the MAPK signaling pathway. Overexpression of the miR-200 family in a normal colon epithelial (CCD841) cell line reduces the expression of RASSF2 protein. RASSF2 binds to K-Ras. Loss of RASSF2 protein expression increases K-Ras and subsequent activity with the MAPK signaling pathway, as measured by the downstream pathway protein ERK 1/2. The resulting outcome is increased cell proliferation, a marker of oncogenesis.

Conversely, when expression of the miR-200 family in a Dukes' C (HT-29) CRC cell line is reduced, RASSF2 protein expression increases and subsequent MAPK signaling pathway activity decreases. Measurement of cell proliferation confirmed the reduced growth of these cells. A summary of our results are outlined on page 147.

We have demonstrated the ability of the miR-200 family to transform normal epithelial cells into cells with more cancer-like behavior, and to transform CRC cells to cells with a more normal, less malignant behavior. These findings suggest the potential of the miR-200 family to be developed as a therapeutic target in CRC. Current standard therapies used in the treatment of CRC include the chemotherapy regime of FOLFOX (oxaliplatin, 5-FU and leucovorin). This is based on the MOSAIC trial which showed that in patients with Stage II or III colon cancer, those treated with FOLFOX therapy versus 5-FU and leucovorin had a significantly higher 3-year disease free survival.(163) More recently, newer agents have been developed to specifically target the gene and protein changes in cells that cause cancer. Epidermal growth factor receptor (EGFR) protein is often highly expressed on the surface of cancer cells and is recognized as an important player in CRC initiation and progression. Anti-EGFR drugs have been developed to target this protein and reduce cell growth, however; in patients who have a KRAS mutated CRC, the treatment is ineffective.(164) Similarly, MEK inhibitors have been developed to target MAPKK enzymes, MEK 1/2, in the MAPK pathway which is often overactive in some cancers. MEK inhibitors are effective in the treatment of KRAS/BRAF mutated, but not wild-type CRC.(165)

There are several limitations to this work. Firstly, the use of colorectal cell lines in research is a cost effective, easy to use method, and provides a pure population of cells

that are consistent and reproducible, to name but a few advantages. The popularity for the use of cell lines in research is growing, based upon the number of cell lines available for use and numerous publications. However; care must be taken when interpreting the results as serial passage of cell lines can cause phenotypic changes within these cells and these cells may not always accurately replicate characteristics of the primary cells. Another problem to note is that of contamination with other cell lines and infection with mycoplasma. Secondly, transient transfection is most commonly used to investigate the short-term impact of alterations in gene and protein expression. During transient transfection, the nucleic acid sequence is not integrated into the cell genome and the effect on the target gene expression is temporary. Our transfection time was limited to 24 hours to allow for this. Lastly, we investigated the change in miR-200 family expression in a KRAS wild-type sporadic CRC cell line. KRAS mutations are present in up to 50% of cases of CRC and occur as point mutations at codons 12 and 13. RASSF2 binds to K-Ras in the effector binding area on the protein, separate from the area where the mutations occur, therefore, for the purposes of this study, the KRAS mutation status should not affect our results. However; for completeness it would be worthwhile to investigate the role of the miR-200 family in a KRAS mutated CRC cell line for comparison.

Based on sample size calculations from the screening and validation of dysregulated miRNAs in colorectal cancer cell lines compared to the normal colon epithelial cell line (CCD841) a sample size of $n=2$ was required to achieve a power of 80%. As previously described we chose to perform all experiments with five experimental replicates to allow for the detection of smaller differences in other

variables. When measuring RASSF2 mRNA levels, we observed a trend in upregulation or downregulation, depending on the cell line and miRNA transfection in our replicates, but this did not reach statistical significance for all the individual miR-200 family members. It is possible that by increasing the sample size, e.g. n=10, for experiments where differences in expression were found to be more subtle, we would narrow the standard deviation and variability, and observe significant differences in cells transfected with either miR-200 family mimics or antagomirs.

Future considerations include clinical correlation with CRC patient tissue samples. For example, the use of a tissue microarray to permit simultaneous analysis of miR-200 family expression and RASSF2 mRNA and protein levels and correlate the findings with clinical factors would be of interest. In addition, the effects of the miR-200 family on CRC could be measured by using a mouse model. An established miR-200c/miR-141 knockout mouse would allow for investigation into the oncogenic features of the miR-200 family and also allow for testing of miR-200 therapies, such as miRNA inhibition therapy using miRNA antagonists e.g. siRNA, as a potential treatment in CRC.

From our colorectal cell line screening we found significant dysregulation of many miRNAs. While we chose to study the upregulated miR-200 family it is important to consider the significantly downregulated miRNAs. Comparison of the downregulated miRNAs in our colorectal cancer cell lines revealed five miRNAs to be common to all, miR-199a, miR-409, miR-485, miR-541 and miR-654. Each of these miRNAs has been shown to be involved in cancer pathogenesis.(166-170) Of interest, miR-409 expression was significantly downregulated in colorectal cancer tissues and negatively regulated

CRC metastatic capacities, including suppressing cancer cell migration, invasion and metastasis.(167) miR-199a and miR-541 have previously been regarded as tumor suppressors and miR-199a has been identified to regulate the tumor suppressor MAP3K. An area of interest for future study would involve investigation of these downregulated miRNAs and their associated pathways. It would be interesting to evaluate any targets common to both the miR-200 family and the downregulated miRNAs with the aim to enhance miRNA therapy in colorectal cancer in addition to the newer targeted drug therapies.

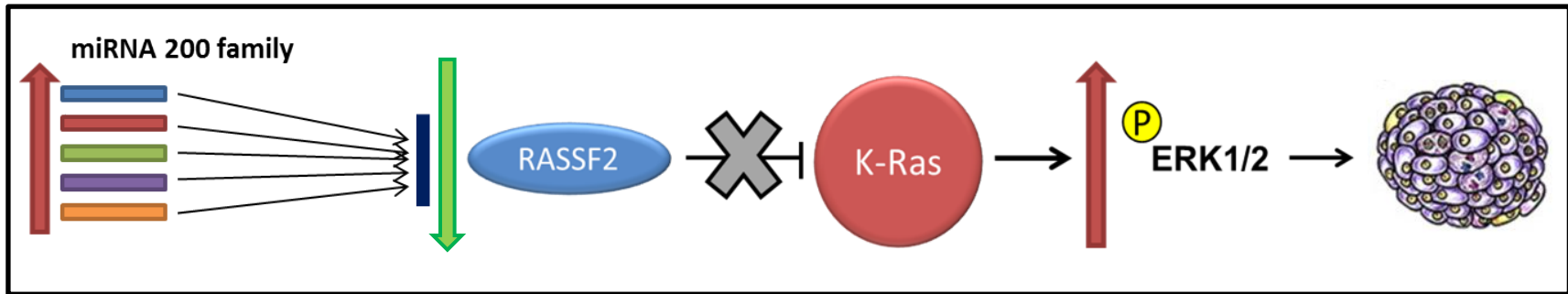
The miR-200 family has previously been studied in regards to its regulation of EMT. EMT is the process where an epithelial cell loses apical/basolateral polarity, severs intercellular adhesive junctions, degrades basement membrane components and becomes a migratory mesenchymal cell i.e. enables cells from the primary cancer to travel to regional nodes or metastatic sites. The miR-200 family plays an important role in regulating EMT inhibiting the initial step of metastasis by maintaining the epithelial phenotype through direct targeting of transcriptional repressors of E-cadherin, ZEB1 and ZEB2. Members of the miR-200 family have therefore been believed to play an essential role in tumor suppression.(161) One of the main pathways involved in this process is the transforming growth factor beta (TGF- β) signaling pathway. TGF- β signaling pathway is involved in many cellular processes and suppresses tumor formation by inhibiting cell growth and promoting apoptosis. In advanced cancers it has been shown to promote tumor formation by its ability to induce EMT.(171) TGF- β also induces other signaling pathways and activates the ERK MAPK pathway by allowing the binding of the Grb2-Sos1 complex which activates Ras. The interaction of the miR-200 family in both of

these signaling pathways is interesting as they play differing roles within each. This would certainly be an area of interest to study further and improve our understanding of the cellular processes regulating cancer development.

A recently identified pathway regulated by the miR-200 family is the PI3K/AKT pathway. This pathway is important in regulating the cell cycle and is directly related to cell proliferation and apoptosis. Overactivation of this pathway is commonly observed in cancer. miR-200 activates the PI3K/AKT pathway by targeting FOG2 acting as a PI3K antagonist. miR-200 was observed to activate AKT through a novel mechanism involving inactivation of S6K, thus promoting tumor growth.(172)

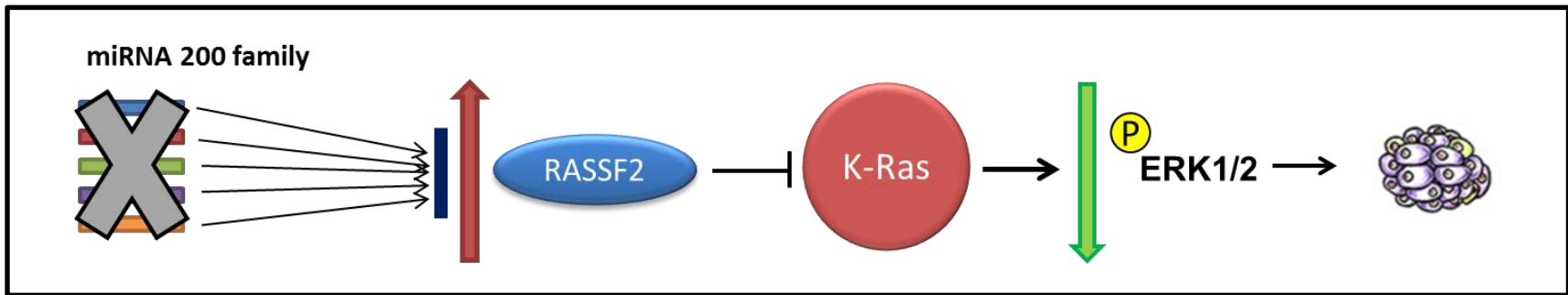
In conclusion we have identified a novel finding regarding the role of the miR-200 family on the tumor suppressor RASSF2 and subsequent activity within the MAPK signaling pathway in CRC. We have demonstrated the transition of a normal colon epithelial (CCD 841) cell line towards a “cancer-like” colorectal cell by increasing the expression of members of the miR-200 family. Conversely, reduction of miR-200 family expression in a Dukes’ C (HT-29) CRC cell lines reverses this process and the CRC cells begin to show less “cancer-like” features. This improved understanding of the oncogenic effects of the miR-200 family may result in a targeted therapy in colorectal cancer.

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miR-200 Family and Dukes' C (HT-29) Colorectal Cancer



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APPENDIX: ABBREVIATIONS

A-	miRNA Antagomir Negative Control
AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B (PKB)
APC	Adenomatous Polyposis Coli
AUC	Area Under the Curve
BCA	Bicinchoninic Acid Assay
BRAF	v-Raf Murine Sarcoma Viral Oncogene Homolog B
BSA	Bovine Serum Albumin
CA 19-9	Carbohydrate Antigen 19-9, tumor marker
CA11-19	Colorectal Cancer Tumor marker
CAA	Colorectal Advanced Adenoma
CCD 841	Human Normal Colon Epithelial Cell Line
CEA	Carcinoembryonic Antigen, tumor marker

CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CRC	Colorectal Cancer
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial-Mesenchymal Transition
ERK 1/2	Extracellular Signal-Related Kinase pathway; part of the MAPK
EtOH	Ethanol
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum

FOBT	Fecal Occult Blood Test
FOG2	Friend of GATA 2
FOLFOX	Chemotherapy Regimen, FOL – Folinic Acid (Leucovorin), F – Fluorouracil (5-FU), OX - Oxaliplatin
GDP	Guanosine Diphosphate
Grb2-SOS1	Growth Factor Receptor-Bound Protein 2 – Son of Sevenless homolog 1
GTP	Guanosine Triphosphate
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
IPA	Ingenuity Pathway Analysis
JNK	c-Jun N-terminal Kinase pathway; part of the MAPK pathway
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
HT-29	Human Dukes' C Colorectal Adenocarcinoma Cell Line
M-	miRNA Mimic Negative Control
MAPK	Mitogen-Activated Protein Kinase
MAPKK / MAP2K	Mitogen-Activated Protein Kinase Kinase

MAPKKK / MAP3K	Mitogen-Activated Protein Kinase Kinase Kinase
MAPK14	Mitogen-Activated Protein Kinase 14, also called p38- α
MEK 1/2	Mitogen-Activated Protein Kinase Kinase; phosphorylates mitogen-Activated protein kinase
MET	Mesenchymal-Epithelial Transition
miRNA	microRNA: short, single stranded RNA
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MOSAIC	Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer Trial
mRNA	messengerRNA: longer, single stranded RNA; serves as a template for protein translation
MSI	Microsatellite Instability
MSS	Microsatellite Stable
NRAS	Neuroblastoma Rat Sarcoma Viral Oncogene Homolog
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PKC	Protein Kinase C

qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RASSF2	Ras Association Domain-Containing Protein 2
RIPA	Radioimmunoprecipitation Assay Buffer
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
S6K	Ribosomal Protein (S6) Kinase
SNAIL	Zinc Finger Protein SNAI1
siRNA	Short Interfering RNA; synthetic RNA duplex designed to specifically target a particular mRNA for degradation
SW1116	Human Dukes' A Colorectal Adenocarcinoma Cell Line
SW480	Human Dukes' B Colorectal Adenocarcinoma Cell Line
T84	Human Dukes' D Colorectal Adenocarcinoma Cell Line
TBS-T	Tris-Buffered Saline and Polysorbate 20
TGF- β	Transforming Growth Factor Beta
TNM	Tumor, Node, Metastasis Classification of Malignant Tumors
TP53	Tumor Protein p53

TS	Thymidylate Synthase
Wnt	Wingless-Related Integration Site; Signal transduction pathway
ZEB 1/2	Zinc Finger E-box Binding Homeobox 1/2
5-FU	5-Fluorouracil

OTHER PUBLICATIONS DURING TIME OF PhD STUDIES

1. **Carter J**, Roberts H, Pan J, et al.,
A Highly Predictive Model for Diagnosis of Colorectal Neoplasms Using Plasma MicroRNA: Improving Specificity and Sensitivity.
Ann Surg. 2016 Oct;264(4):575-84. doi: 10.1097/SLA.0000000000001873.
2. **Carter J**, Pan J, Rai S, Galandiuk S
ROC-ing along: Evaluation and Interpretation of Receiver Operating Characteristic Curves.
Surgery. 2016 Jun;159(6):1638-45. doi: 10.1016/j.surg.2015.12.029.
3. Nelson B, **Carter J**, Eichenberger M, Netz U, Galandiuk S.
Genetic polymorphisms in 5-FU related enzymes predict pathologic response after neoadjuvant chemoradiation for rectal cancer.
Surgery. 2016 Nov;160(5):1326-1332. doi: 10.1016/j.surg.2016.05.017.
4. Galbraith N, Walker S, **Carter J**, Polk HC
Past, present and future of augmentation of monocyte function in the surgical patient.
Surg Infect (Larchmt). 2016 Oct;17(5):563-9. doi: 10.1089/sur.2016.014.
5. Bennett J, **Carter J**, Foy C, Rodd C, Longman R.
Unsupervised use of laparoscopic box trainers in a cohort of UK core surgical trainees.
Journal of Surgical Simulation, 2016. Accepted In Press.

UNDER REVIEW

1. **Carter J**, Galbraith N, Yang D, Burton J, Walker S, Galandiuk S.
Blood-Based MicroRNA as Biomarkers for the Diagnosis of Colorectal Cancer: A Systematic Review and Meta-Analysis.
Submitted to: British Journal of Cancer.
2. Galbraith N, **Carter J**, Netz U, Yang D, Fry D, McCafferty M, Galandiuk S.
Laparoscopic Lavage in the Management of Perforated Diverticulitis: A Contemporary Meta-analysis.
Submitted to: JAMA Surgery
3. Netz U, **Carter J**, Eichenberger M, Dryfen G, Pan J, Rai S, Galandiuk S.
Genetic Polymorphisms Predict Anti-TNF Treatment Response in Crohn's Disease.
Submitted to: The American Journal of Gastroenterology

CURRICULUM VITAE

JANE V. CARTER M.B. Ch.B. MRCS

Date of Birth: 20th September 1984

Place of Birth: Northallerton, North Yorkshire, United Kingdom

Home Address : 1639 Jaeger Avenue
Louisville, Kentucky 40205

Office Address : Price Institute of Surgical Research
511 South Floyd Street
Louisville, Kentucky 40202

Telephone: Home (502) 202-6864
Office (502) 852-5552

Email Address: jane.carter@louisville.edu

GMC Number 6156497

Medical Defence Medical Defence Union
444731H

EDUCATIONAL HISTORY

Postgraduate Certificate Med Ed	University of Bristol Faculty of Medicine & Dentistry	2013
Fellow of the Higher Education Academy	University of Bristol Faculty of Medicine & Dentistry	2013
MRCS (Eng)	Royal College of Surgeons of England	2010
Medical School	University of Bristol	2002-2007
School	Polam Hall Independent School for Girls, Darlington	1995-2002

QUALIFICATIONS

PG Certificate Med Ed		2013
Fellow of the Higher Education Academy		2013
MRCS (Eng)		2010
MB ChB		2007

AWARDS AND PRIZES

Price Institute Surgical Research Fellowship	University of Louisville School of Medicine, Kentucky, USA	2014 -
Honorary Clinical Surgical Tutor	Severn School of Surgery	2011-2013
Honorary Clinical Tutor	University of Bristol	2011-2012
Charles Hastings Audit Award	Worcestershire Royal Hospital Shortlisted	2008

EMPLOYMENT HISTORY

Current Post

RESEARCH

PhD Candidate Oct 14 - current
Department of Physiology
University of Louisville School of Medicine, Kentucky, USA

Price Institute Surgical Research Fellowship. Oct 14 – current
Price Institute, Department of Surgery,
University of Louisville School of Medicine, Kentucky, USA

Previous posts

Higher Surgical Training
Severn Postgraduate Medical Education School of Surgery 2012-
2014

Clinical Teaching Fellow
Gloucestershire Academy, University of Bristol 2011-
2012

Core Surgical Training
Severn Postgraduate Medical Education School of Surgery 2010-
2011

West Midlands Postgraduate School of Surgery 2009-
2010

Foundation Programme Training
West Midlands South Foundation Programme 2007-
2009

PROFESSIONAL COURSES & MEETINGS

American Surgical Association , Chicago	2016
Academic Surgical Congress , Jacksonville	2016
Noncoding RNAs and Cancer: Mechanisms to Medicine , AACR, Boston	2015
European Society for Surgical Research , Liverpool	2015
Academic Surgical Congress , Las Vegas	2015
Surgical Investigators' Course , ASC, Las Vegas	2015
The 4th Bristol Trauma Course , Bristol	2014
Americas Hernia Society 16th Annual Hernia Repair Congress , Las Vegas	2014
South West Surgical Training Network Masterclass: Challenges in the Surgical Management of IBD , Bristol	2013
Annual Scientific Meeting, The Association for the Study of Medical Education , Brighton	2012
Introduction to Medical Education (Invited speaker) , Taunton	2014 2013 2012
Immediate Life Support (Instructor) , Gloucester	2012
BASO ~ The Association for Cancer Surgery Scientific Conference , London	2011
ABC 1 First Consensus Conference , ESO, Lisbon	2011
Simulation Tutor Training , Bath	2011
OSLER Examiner Training , Bristol	2011
Training the Trainers , Bristol	2011
Americas Hernia Society 14th Annual Hernia Repair Congress , San Francisco	2011
CCrISP , Cheltenham	2010

Basic Surgical Skills, Worcester	2009
ATLS, Birmingham	2008
Advance Life Support, Redditch	2007
Immediate Life Support, Bristol	2007
Surgical Skills Course, Bristol	2007

PUBLICATIONS

Books and Book Chapters

1. Polk HC, **Carter J**, Netz U
Chapter Malignant Melanoma. Fischer's Mastery of Surgery 7th Edition. Fischer JE, Bland KI, Callery MP, eds. Lippincott Williams & Wilkins. Philadelphia. **Anticipated 2017**
2. **Carter J**, Vestey S
Chapter 14 Breast Disease. Succeeding in your Medical School Finals: Instant revision notes. BPP Learning Media. December 2012

Original Articles

1. Bennett J, **Carter J**, Foy C, Rodd C, Longman R.
Unsupervised use of laparoscopic box trainers in a cohort of UK core surgical trainees. Journal of Surgical Simulation, 2016. Accepted In Press.
2. **Carter J**, Roberts H, Pan J, et al.,
A Highly Predictive Model for Diagnosis of Colorectal Neoplasms Using Plasma MicroRNA: Improving Specificity and Sensitivity.
Ann Surg. 2016 Oct;264(4):575-84. doi: 10.1097/SLA.0000000000001873.
3. Nelson B, **Carter J**, Eichenberger M, Netz U, Galandiuk S.
Genetic polymorphisms in 5-FU related enzymes predict pathologic response after neoadjuvant chemoradiation for rectal cancer.
Surgery. 2016 Nov;160(5):1326-1332. doi: 10.1016/j.surg.2016.05.017.
4. **Carter J**, Pan J, Rai S, Galandiuk S
ROC-ing along: Evaluation and Interpretation of Receiver Operating Characteristic Curves.
Surgery. 2016 Jun;159(6):1638-45. doi: 10.1016/j.surg.2015.12.029.
5. Galbraith N, Walker S, **Carter J**, Polk HC
Past, present and future of augmentation of monocyte function in the surgical patient.
Surg Infect (Larchmt). 2016 Oct;17(5):563-9. doi: 10.1089/sur.2016.014.
6. Ward S, **Carter JV**, Robertson CS
Herniography influences the management of patients with suspected occult hernia and patient factors can predict outcome.
Hernia. 2011 Oct;15(5):547-51. doi: 10.1007/s10029-011-0825-x.

Published Abstracts

1. **Carter J**, Jenkins M, Coombs N
Pre-operative groin discomfort can predict persistent post-operative groin pain despite a successful laparoscopic inguinal hernia repair
Hernia March 2014; 18 (Supp 1), S60
2. **Carter J**, Hannaway N, Bland H, Fletcher P, Rodd C
Are students closing the book on learning?
Book of abstracts The Association for the Study of Medical Education (ASME) 2012
3. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Beyond the de facto curriculum – making post exams learning fun
Book of abstracts ASME 2012
4. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Pastoral care in tutorials – Do students get it?
Book of abstracts ASME 2012
5. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Video Assisted Learning – Is it always worth it? A randomised control trial
Book of abstracts ASME 2012
6. Hannaway N, **Carter J**, Bland H, Fletcher P, Rodd C
How do medical students view their learning opportunities?
Book of abstracts ASME 2012
7. Hannaway N, **Carter J**, Bland H, Rodd C, Fletcher P
Do financial concerns in medical students lead to perceived challenges in learning?
Book of abstracts ASME 2012
8. Hannaway N, Davies P, Chant H, Bland H, **Carter J**
Introducing the Observed Structured Student Interactive Clinical Learning Environment (OSSICLE) – an interactive structured induction programme for medical students.
Book of abstracts ASME 2012
9. Bland H, Hannaway N, **Carter J**, Fletcher P, Rodd C
Reading anatomy before an imaging tutorial – is there evidence that it aids interpretation of chest radiographs?
Book of abstracts ASME 2012
10. Bland H, Hannaway N, **Carter J**, Fletcher P, Rodd C
Does patient gender affect student learning in the clinical setting?
Book of abstracts ASME 2012

11. Bland H, Nageswaran H, Hannaway N, **Carter J**, Fletcher P, Rodd C
Teaching and learning professionalism – are teachers and learners in harmony?
Book of abstracts ASME 2012
12. **Carter J**, Hunt R, Yin J, Burd E, El-Abbar M, Thorne A, Gill J, Price R
NICE Breast Reconstruction Guidelines. Are we good enough?
EJSO 37 (2011): 993
13. Yin J, **Carter J**, Hunt R, Burd E, El-Abbar M, Price R, Gill J
One UK Breast Unit's experience of GP 2 week wait referrals to breast care clinic – Are they appropriate?
The Breast 20 (2011), S40
14. Hunt R, Ainsworth R, **Carter J**, Burd E, Price R, Gill J
Patient satisfaction after breast conserving surgery in a District General Hospital: A case for liposculpture
EJSO 37 (5): S24-25
15. Ward S, **Carter J**, Robertson C
Herniography influences the management of patients with suspected occult hernia and patient factors can predict outcome
Hernia 2011; 15 (Supp 1), S78
16. Makawaro T, Phillips J, Gee E, **Carter J**, Eaton J, Makar A
A study to evaluate the accuracy of magnetic resonance imaging in the staging of prostate cancer
International Journal of Surgery 8 (7); 576

PRESENTATIONS

International

1. **Carter J**, Rice J, Roberts H, Eichenberger MR, Pan J, Kanaan Z, Rai S, Galandiuk S
A Highly Predictive Model for Diagnosis of Colorectal Neoplasms Using Plasma MicroRNA: Improving Specificity and Sensitivity.
American Surgical Association, Chicago, Illinois, USA
2. Nelson B, **Carter J**, Eichenberger MR, Netz U, Galandiuk S
Genetic polymorphisms in 5-FU related enzymes predict complete pathologic response following neoadjuvant chemoradiation for rectal cancer.
Academic Surgical Congress, Jacksonville, Florida, USA
3. Roberts H, McClain M, Rice J, **Carter J**, Burton J, Galandiuk S
Can Cancer Cell Lines Clarify Molecular Mechanisms of Hereditary Non-Polyposis Colorectal Cancer?
Academic Surgical Congress, Jacksonville, Florida, USA
4. **Carter J**, Rice J, McClain M, Farmer R, Galandiuk S
Plasma microRNA to monitor treatment response in patients with colorectal neoplasia
European Society for Surgical Research Annual Congress, Liverpool, UK
5. Bennett J, **Carter J**, Davies H, Higgs S, Rodd C, Longman R
Pilot study of unsupervised use of laparoscopic box trainers in a cohort of core surgical trainees
Association of Surgeons of Great Britain and Ireland
6. **Carter J**, Hannaway N, Bland H, Fletcher P, Rodd C
Are students closing the book on learning?
Association for the Study of Medical Education, Brighton, UK
7. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Beyond the de facto curriculum – making post exams learning fun
Association for the Study of Medical Education, Brighton, UK
8. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Pastoral care in tutorials – Do students get it?
Association for the Study of Medical Education, Brighton, UK
9. Hannaway N, **Carter J**, Bland H, Fletcher P, Rodd C
How do medical students view their learning opportunities?
Association for the Study of Medical Education, Brighton, UK

10. Hannaway N, **Carter J**, Bland H, Rodd C, Fletcher P
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11. Bland H, Hannaway N, **Carter J**, Fletcher P, Rodd C
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Does patient gender affect student learning in the clinical setting?
Association for the Study of Medical Education, Brighton, UK
13. Bland H, Nageswaran H, Hannaway N, **Carter J**, Fletcher P, Rodd C
Teaching and learning professionalism – are teachers and learners in harmony?
Association for the Study of Medical Education, Brighton, UK

Regional/ Local

1. Roberts H, McClain M, Rice J, **Carter J**, Burton J, Galandiuk S
Can Cancer Cell Lines Clarify Molecular Mechanisms of Hereditary Non-Polyposis Colorectal Cancer?
Kentucky Chapter American College of Surgeons, Louisville, KY. 1st Prize Winner.
2. Nelson B, **Carter J**, Eichenberger MR, Netz U, Galandiuk S
Genetic polymorphisms in 5-FU related enzymes predict complete pathologic response following neoadjuvant chemoradiation for rectal cancer.
Kentucky Chapter American College of Surgeons, Louisville, KY
3. Ward S, **Carter J**, Robertson C
The use of herniography in the management of patients with suspected occult herniae and how patient factors can predict outcome.
West Midlands Surgical Society, Birmingham, UK

POSTERS

International

1. **Carter J**, Pan J, Rai S, Galandiuk S
ROC-ing along: Evaluation and Interpretation of Receiver Operating Characteristic Curves.
Academic Surgical Congress, Jacksonville, Florida, USA (Manuscript published)

2. **Carter J**, States V, Eichenberger MR, Pan J, Rai S, Galandiuk S
Longitudinal Changes in Plasma miRNA in Patients with Benign and Malignant Colorectal Neoplasia.
Noncoding RNAs and Cancer: Mechanisms to Medicine, AACR, Boston , Massachusetts, USA
3. **Carter J**, Jenkins M, Coombs N
Pre-operative groin discomfort can predict persistent post-operative pain despite a successful laparoscopic inguinal hernia repair.
Americas Hernia Society 16th Annual Hernia Repair Congress, Las Vegas, Nevada, USA
4. **Carter J**, Bland H, Hannaway N, Fletcher P, Rodd C
Video Assisted Learning – Is it always worth it? A randomised control trial
Association for the Study of Medical Education, Brighton, UK
5. Hannaway N, Davies P, Chant H, Bland H, **Carter J**
Introducing the Observed Structured Student Interactive Clinical Learning Environment (OSSICLE) – an Interactive Structured Induction Programme for Medical Students
Association for the Study of Medical Education, Brighton, UK
6. Yin J, **Carter J**, Hunt R, Burd E, El-Abbar M, Price R, Gill J
One UK Breast Unit’s experience of GP 2 week wait referrals to breast care clinic – Are they appropriate?
ABC1 First Consensus Conference, European School of Oncology, Lisbon, Portugal
7. **Carter J**, Ward S, Robertson C
The use of herniography in the management of patients with suspected occult hernia and how patient factors can predict outcome
Americas Hernia Society 14th Annual Hernia Repair Congress, San Francisco, California, USA
(Manuscript published)

National

1. **Carter J**, Hunt R, Yin J, Burd E, El-Abbar M, Thorne A, Gill J, Price R
NICE Breast Reconstruction Guidelines. Are we good enough?
BASO ~ The Association for Cancer Surgery Scientific Conference, London, UK
2. Hunt R, Ainsworth R, **Carter J**, Burd E, Price R, Gill J
Patient satisfaction after breast conserving surgery in a District General Hospital: A case for liposculpture
ABS & AGM, Manchester, UK
3. Makarawo T, Phillips J, Gee E, **Carter J**, Eaton J, Makar A
A study to evaluate the accuracy of magnetic resonance imaging in the staging of prostate cancer
ASiT, Hull, UK

Regional/ Local

1. Makarawo T, Phillips J, Gee E, **Carter J**, Eaton J, Makar A
A study to evaluate the accuracy of magnetic resonance imaging in the staging of prostate cancer
South Eastern Uro-Oncology Meeting, Cardiff, UK

RESEARCH EXPERIENCE

As part of my interest in teaching I have initially been involved with research into medical education, both at an undergraduate and postgraduate level. As part of my year as a clinical teaching fellow I was involved in several research projects, 4 of them as principle researcher. Completion of these projects has led to presentations at an international conference. Following this, I have been involved as joint lead in a postgraduate surgical education research project. My interest and experience in research has led me to be involved with a large multicentre national audit as a collaborator.

The principles behind research fascinate me, and as a result I wish to pursue a career in academic general surgery. My rotations to date have led me to have an interest in colorectal disease, in particular colorectal cancer. With my long term interest in general surgery I have also been awarded the Price Institute Surgical Research Fellowship, University of Louisville, Kentucky USA. I commenced this post in October 2014 where I am studying towards a PhD on the role of the miRNA-200 family on the tumor suppressor RASSF2 in the development of colorectal cancer.

AUDITS

J Carter, N Galbraith, S Galandiuk 2015

Very Reliable Ostomy Output Monitoring (VROOM) Training

The purpose of the proposed quality improvement study is to decrease hospital readmission rates due to dehydration and obstruction following creation of either permanent or temporary ileostomies in patients with inflammatory bowel disease and rectal cancer.

J Carter, S Williams, J Cook 2013

Intraoperative molecular analysis (OSNA) of total tumour load in sentinel lymph node biopsy predicting axillary status in breast cancer patients. A retrospective comparison in a UK centre.

J Carter, S Cawthorn 2013

iBRA - A national audit of the practice and outcomes of immediate implant-based breast reconstruction.

J Carter , S Narang, S Khadouri S, N Smart <i>SPARCS National Sepsis Audit</i>	2013
J Carter <i>Time to flexible sigmoidoscopy</i>	2014
J Carter , R Hunt, J Yin, E Burd, M El-Abbar, R Price, J Gill <i>NICE Breast Reconstruction Guidelines. Are we good enough?</i>	2011
J Yin, J Carter , R Hunt E Burd, M El-Abbar, R Price, J Gill <i>One UK breast unit's experience of GP 2 week wait referrals to breast care clinic – Are they appropriate?</i>	2011
R Hunt, R Ainsworth, J Carter , E Burd, R Price, J Gill <i>Patient satisfaction after breast conserving surgery in a District General Hospital: A case for liposculpture</i>	2010
T Makarawo, J Phillips, E Gee, J Carter , J Eaton, A Makar <i>A study to evaluate the accuracy of magnetic resonance imaging in the staging of prostate cancer</i>	2009
J Carter <i>Prescribing in the prevention of osteoporotic fractures</i>	2009
J Carter , J Patel <i>Documentation of blood tests and chest x-rays by doctors clerking medical patients</i>	2008
J Carter , C Robinson <i>Radiation protection in the catheterisation laboratory</i>	2007

POSITIONS OF RESPONSIBILITY / MANAGEMENT

I enjoy taking on managerial responsibilities and projects requiring organisation and leadership qualities. I am effective at motivating people and achieving set goals. I have taken on roles, both in my clinical work and outside of medicine, which show my enthusiasm to develop these skills. I realise that management is an integral part of hospital practice and have so far had the following experience in management.

The Society for Surgery of the Alimentary Tract	Candidate Member The SSAT is committed to advancing the science and practice of surgery in the treatment of digestive disease.	2016
Ad hoc journal reviewer for Diseases of the Colon and Rectum	Reviewer of manuscripts submitted to the world's leading peer-reviewed journal in colorectal surgery (impact factor 3.739). My specialist interest being molecular biology and biomarker detection in colorectal disease.	Aug 2016 - Current
Restructuring of Emergency Surgery Service	Junior representative on restructuring of the emergency surgery service in North Bristol NHS Trust following relocation to Southmead Hospital - Supported by Miss A Pullyblank	August 2014
Commissioning of theatres	Junior lead for commissioning of breast/plastics theatres in North Bristol NHS Trust. Liaison amongst multidisciplinary team members and voice of Consultant body	May-Jun 2014
Clinical Governance Group Surgical Division member	Junior Doctor representative and attendance at monthly CGG meetings	2013-2014

Rota coordinator Breast Surgery	Organisation of the breast surgery rota for junior medical staff at Great Western Hospital	Apr-Oct 2013
Medical School Course Planning	During my year as a Clinical Teaching Fellow I was instrumental in curriculum planning and development, course design and delivery of teaching	2011- 2012
Organiser of “Widening Participation Summer School Programme”	Organiser for 20 6 th form students from local comprehensive schools interested in applying for medicine to attend a week long summer school. University of Bristol, Gloucestershire Royal Hospital	June 2012
SIMman training	Lead organiser for recruitment and provision of simulation training using SIMman for both undergraduate medical and nursing students. Gloucestershire Royal Hospital	Jan-Mar 2012
MBChB Surgical Skills programme	Lead organiser of final year MBChB Surgical Skills Programme. Gloucestershire Royal Hospital	Sep11- Mar 2012
Honorary Clinical Surgical Tutor	Organiser and faculty member of Core Surgical Training Days, Severn Deanery	2011- Current
Trauma Simulation	Organiser of multi-disciplinary trauma simulation training programme. Gloucestershire Royal Hospital	2011- 2012
Honorary Medical Officer	Medical Officer at Point-to-Point Horse Trials. Responsible for jockey and crowd safety. Gelligaer Farmers’ Hunt, Lower Machen	2009

Mess President	Worcestershire Royal Hospital Mess President	2007- 2008
Ski club captain	University of Bristol Medics ski club captain and organiser of ski trip for 50 students	2006-2007
Captain of Sports Teams	Captained lacrosse, netball and hockey 1 st teams at school and university. Player of the year award.	2000-2002 2007
School House Captain	House captain of 100 students	2000-2002

TEACHING

I have taken an interest in teaching throughout my postgraduate career. My experience so far, as well as informal student teaching includes:

QUALIFICATIONS

Postgraduate Certificate in Medical Education 2013

Teaching and learning for Health Professionals. University of Bristol

Fellow of the Higher Education Academy 2013

Training the Trainers 2011

Royal College of Surgeons of England

Formal (Full-Time) Teaching Roles

Clinical Teaching Delivery of medicine and surgery curriculum to 3rd 2011-2012

Fellow

**University of Bristol,
Gloucestershire Royal
Hospital**

and 5th year medical students from the University of Bristol. Involvement with evaluation and restructuring of the undergraduate surgical education. Provision of clinical teaching, small group teaching, lecturing, feedback sessions, pastoral support and examination setting and marking. In addition to medicine and surgery, I was also responsible for delivery of professional behaviour standards, ethics and communication and equality and diversity teaching. I was also involved with course development and design for core surgical skills.

During this post, I managed to maximize the opportunities for my own clinical learning within the department by attending regular theatre lists and clinics.

Other Teaching Roles

University of Louisville, School of Medicine	Oral examiner of Year 3 Medical Students Surgical Rotation	2014-Current
Core Surgical Trainees Teaching, Severn and Peninsula Deaneries	Faculty for core surgical skills days. Organisation and delivery of teaching to surgical trainees.	2011-2014
Introduction to Medical Education, Course Faculty	Invited as speaker and faculty to deliver course on introduction to medical education to surgical trainees.	2012-2014
Honorary Clinical Surgical Tutor	Awarded for delivering teaching to core surgical trainees	2011-2013
Severn School of Surgery		
Examiner for Final MBChB OSCE & OSCE University of Bristol	Regular examiner of long case and OSCE exams for final year medical students	2011-2013
Examiner, Marker & Invigilator	Regular examiner, marker and invigilator for 3 rd year medical student examinations	2011-2012
MBChB OSCE & Data OSCE		
University of Bristol		
University Medical Admissions Interviewer	Invited to interview 6th form and postgraduate students for medical school places	2011-2012
University of Bristol		

Intermediate Life Support	Course facilitator for final year medical students	2012
Course Faculty		
Foundation Year 2 Interviews Faculty	Invited to conduct interview practice for foundation year 2 trainees applying for specialty training	2012
Honorary Clinical Tutor University of Bristol	Awarded for delivering teaching to University of Bristol Medical Students	2011-2012
Student Selected Component (SSC) Marker University of Bristol	Marker of Year 4 MBChB students SSC's. Required to mark 20 3,000 word reports to count towards overall grade	2011
Student Mentor Musgrove Park Hospital University of Bristol	Medical Student Mentorship Programme	2010-2011

OUTSIDE INTERESTS AND ACHIEVEMENTS

Tennis:	USTA League 18 & Over, Louisville, KY	2015-
	Women's State Champions (Lexington, KY)	2016
	Mixed Doubles State Champions (Murray, KY)	2016
	Women's Doubles Combo State Champions (Lexington, KY)	2016
Badminton:	Durham County Elite Squad	98-02
Lacrosse:	Polam Hall School for Girls 1st XII Captain	00-02
Netball:	Polam Hall School for Girls 1st VII Captain	00-02
	Player of the Year	01-02
Hockey:	University of Bristol 2nd X1	02-04
	Polam Hall School for Girls 1 st XI Vice-Captain	00-02
Football:	University of Bristol Medical School 1 st XI Vice-Captain	04-07
	Player of the Year	06-07

Sport has always played a major part in my life. I currently take time to maintain my health by playing tennis. I have recently represented Kentucky at the Southern Sectional tennis championships in Mobile, AL, playing against the champions of eight other southern states for the opportunity to advance to the National finals.

I am a keen traveler and as such I have visited many countries ranging from developed to under-developed and have experienced many different cultures and religions.