MODULATION OF INTESTINAL MICRORNAS BY A CHEMOPROTECTIVE DIET

A Dissertation

by

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Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2012

Major Subject: Genetics

ABSTRACT

We have hypothesized that dietary modulation of intestinal miRNA expression may contribute to the chemoprotective effects of nutritional bioactives (fish oil and pectin). Using a rat colon carcinogen model, we determined miRNAs-let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were modulated by fish oil + pectin. We also demonstrated that BACE1 and PTEN are targets of miR-107 and miR-21, respectively.

To further elucidate the biological effects of diet and carcinogen on miRNAs, we integrated global miRNAs, total and polysomal gene expression datasets obtained from the above mentioned study and used four computational approaches. We demonstrated that polysomal profiling is tightly related to microRNA changes when compared with total mRNA profiling. In addition, diet and carcinogen exposure modulated a number of microRNAs and complementary gene expression analyses showed that oncogenic PTK2B, PDE4B, and TCF4 were suppressed by the chemoprotective diet at both the mRNA and protein levels.

To determine the function of select diet and colon carcinogen modulated miRNAs and to validate their targets, we carried out a series of loss and gain of function experiments along with luciferase reporter assays. We verified that PDE4B and TCF4 are direct targets of miR-26b and miR-203, respectively. PTK2B was determined to be an indirect target of miR-19b. In addition, microRNA physiological function was assessed by examining effects on apoptosis and cell proliferation.

To better understand how the colonic stem cell population responds to environmental factors such as diet and carcinogen, we investigated the chemoprotective effects of dietary agents on miRNAs in colonic stem cells obtained from Lgr5-EGFP-IRES-creER^{T2} knock in mice injected with AOM. We demonstrated that based on relative expression of miR-125a-5p, miR-190b and miR-191 in stem cells vs. daughter cells and differentiated cells, these miRNAs may be stem cell specific miRNAs. We also identified miR-21 to be significantly reduced in stem cells compared to differentiated cells and selectively modulated by these dietary agents in stem cells.

In summary, our results indicate for the first time that fish oil plus pectin protect against colon tumorigenesis in part by modulating a subset of miRNAs and their target genes (mRNAs) implicated in the regulation of the colon stem cell niche and tumor development.

DEDICATION

To my parents and my fiance

ACKNOWLEDGEMENTS

Above all, I am deeply grateful to Dr. Robert S. Chapkin for supporting and guiding my doctoral studies and the work presented here. His insights have been fundamental for all of this research and invaluable at every step for all my work. He has always been patient and supportive, and has shown complete faith in my abilities for which I am very grateful. At any time, the doors of his office were open for discussions on any question or problem that I had and these discussions were extremely important and informative and have facilitated my understanding of concepts and principles. He has also reviewed, commented and edited all the sections of this dissertation at very short notices. It has been a great honor to have him as a mentor and an advisor during my doctoral studies at Texas A&M University.

In the various projects of my doctoral dissertation, I have benefitted greatly from the guidance and insights of my committee members- Dr. Mary Bryk, Dr. Rajesh Miranda, Dr. Joanne R. Lupton. I would like to thank them for generously devoting time and thought into my projects and for providing me with innovative ideas. I am also grateful to them for providing me with expertise in their respective fields in ways and language that I could understand and apply to my work seamlessly. All of them, including Dr. Chapkin, have played a very important role in helping me find a post-doctoral position.

I am also grateful to the senior members of the Chapkin lab – Dr. Laurie Davidson, Dr. Yang-yi Fan, Evelyn Callaway and Jennifer Goldsby for guiding and helping with me many projects, and for making this lab a fun and pleasant place to work.

The animals studies would not have been possible without the help of Evelyn and Jennifer. In addition to being extremely resourceful, Dr. Davidson patiently taught me several laboratory techniques and was a mother-in-lab to me. She was always there to boost my morale. I would also like to thank current and former post-docs and students in the Chapkin Lab – Dr. Jennifer Monk, Dr. Scott Schwartz , Dr. Roger Zoh, Jason Knight, Karen Triff, Rajeshwari Yog, Harmony Turk, Satya Kolar and Qian Jia for helping me and co-operating with me in various research and academic activities. I would like to thank our collaborators for all the projects, especially Dr. Ivan Ivanov and Dr. Beiyan Zhou.

My friends at Texas A&M University made this journey very enjoyable. I have lots of great memories of exploring College Station and Bryan, movies, restaurants, and hanging out with Rajeshwari Yog and many others. They always had words of confidence and encouragement for me and facilitated my work by providing me with food, tea and coffee so that I could concentrate on writing.

The love, affection, confidence, patience and emotional support of my family was indispensible. I would like to express my gratitude and love for my parents, Mr. Shailesh A. Shah and Mrs. Varsha S. Shah, who have always emphasized the value of higher education and given me emotional and financial support through all my career decisions. They have taught me to believe in myself and my abilities while listening to my anxiety and allaying my fears. The proximity of my sister Manisha Goel and her husband Dr. Piyush Goel was very reassuring and has filled the void created by being away from my parents. They have been here for me whenever I needed them. I owe a

special note of gratitude to my fiancé Amit Joshi, who has been a source of stability and strength and has provided me with constructive criticism and moral support as I wrote this thesis.

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction of miRNAs

MicroRNAs (miRNAs), a diverse class of highly conserved small non-coding RNAs (~22 nucleotides long), have been shown to play a critical role in several basic biological processes such as cellular differentiation, apoptosis, cell proliferation and stem cell development, and consequently are also believed to affect complex biological events such as carcinogenesis and immune modulation (1, 2). miRNAs regulate protein expression by acting through perfect or imperfect complementation to 3' untranslated regions (UTRs) of their "target" mRNAs, which results in repression of target gene expression post transcriptionally (2, 3). Currently, more than 800 human and mouse miRNAs have been identified (4). miRNA studies over the last decade have shown their dysregulation in almost all human malignancies, either acting as oncogenes or tumor suppressors.

1.2 Biogenesis of miRNAs

The genomic regions from where the miRNAs are transcribed are largely intergenic regions, but small proportions are also found within exonic or intronic regions. "Mirtrons" is the word coined for those miRNAs that are transcribed from the intronic regions of the genome (5). miRNAs are transcribed from the genome by RNA polymerase II (RNA pol II) from the promoter as long hair-pin shaped primary transcripts (pri-miRNAs) that are polyadenylated at the 3' end and capped at 5' end

(^{7}MGpppG) (Cai et al, 2004). Pri-miRNA is then cropped to pre-miRNA (~70 nucleotide long) by the enzymatic activity of cellular RNAse III-type protein endocnuclease, Drosha, together with DGCR8/Pasha protein (DiGeroge syndrome critical region gene) known as microprocessor complex (6). This pre-miRNA, which has a 2-nt 3' overhang, is recognized by Ran-GTP-dependent transporter exportin 5 and exported from the nucleus to the cytoplasm (7, 8). In the cytoplasm, the pre-miRNA is then further cleaved by RNAse III enzyme Dicer which is associated with TRBP (TAR RNA-binding protein) and Argonuate (AGO1-4) to generate a double stranded (ds) miRNA:miRNA* duplex. This ds duplex is then loaded onto the miRNA associated RNA-induced silencing (RISC) complex and with the help of AGO proteins is delivered to the target mRNA. The guiding miRNA strand is then unwound by a helicase and is referred to as "mature" miRNA. This mature miRNA can then hybridize with the 3' UTR of its "target mRNA" either with imperfect complementarity or with a higher degree of complementarity. If bound with imperfect complementarity, it leads to translational repression, whereas if it binds with high complementarity, it leads to cleavage of target-mRNA (9) **(Figure 1).** Recent studies have demonstrated that miRNAs may also bind to 5' UTR or even the open reading frame (10, 11). Moreover, it has been recently shown that exosomes may contain both mRNA and miRNAs and mediate the exchange of genetic material with other cells (12).

There is also evidence to suggest that there are alternative pathways for non-canonical mirtron biogenesis, which are Drosha independent pathways (13). Mirtrons

can be spliced by spliceosomes and then debranched into pre-mRNA hairpin structures. These hairpin structures are then acted upon by Dicer and thus bypass the microprocessor complex. They are then exported from the nucleus to the cytoplasm, which is mediated by Exportin-5, where it is processed as mentioned above (14) **(Figure 1)**. Alternative mechanisms of generating miRNAs also involve snoRNA, shRNA and tRNA derived pathways (15, 16). Also, it is suggested that miRNAs can be processed by Dicer independent pathways i.e. AGO dependent pathway and tRNaseZ-dependent pathway (17-20) **(Figure 1)**. With respect to intercellular microRNA transport, vesicles of endocytic origin can contain mRNA and miRNAs (12). These exosomes are shuttled between cells to transport miRNAs. Overall, these findings suggest that much remains to be determined regarding the biogenesis of miRNAs.

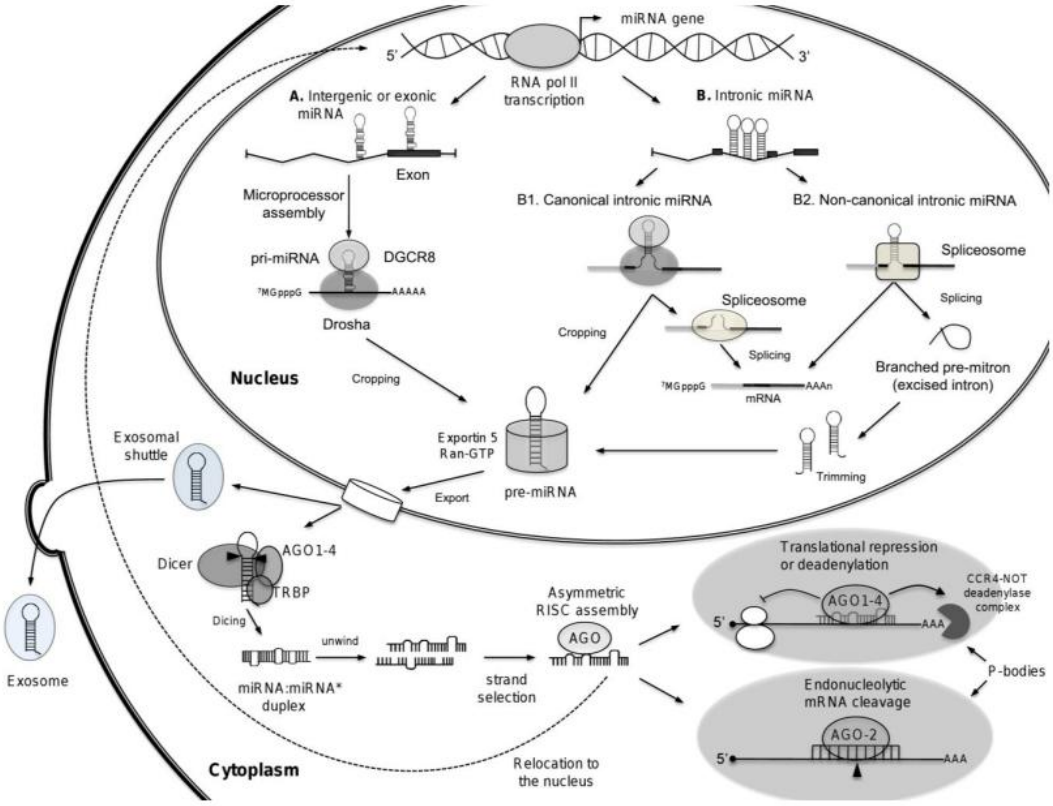

Figure 1. Biogenesis of miRNAs. The processing of miRNAs is mediated by Dicer and Drosha endonucleases. In the first step, the microprocessor complex (Drosha and DGCR8) mediates the nuclear processing of primary-miRNAs (pri-miRNA) into stem-loop precursors of ~70 nucleotides (pre-miRNA). The nuclear export of the precursors is subsequently mediated by exportin-5 in a Ran-GTP dependent manner. In the second step, the pre-miRNA is cleaved in the cytoplasm by Dicer into ~22 nucleotides mature miRNA, which incorporates as single-stranded RNA into the RNA-induced silencing complex (RISC). This complex directs respective miRNAs to their "target mRNAs", which leads either to translational repression or degradation of the target transcripts. Processing of mirtrons by alternative pathways is depicted. miRNAs-dependent repression of gene expression occurs in P-bodies which are also depicted. miRNAs are relocalized to the nucleus, where they may regulate transcription or splicing of transcripts (dotted line). Exosomal shuttle RNA is also represented as budding vesicles containing miRNAs. Image adapted from (21).

1.3 Colorectal cancer

Colorectal cancer ranks as the third leading cause of death among adults. Every year in the United States, more than 150,000 cases of colorectal cancer are diagnosed and around 57,000 patients die of the disease (22). Several molecular events drive the initiation, promotion and progression of colon cancer. These molecular events are influenced by several environmental factors, germ-line mutations in genes and transcriptional factors, which dictate individual cancer susceptibility and causes accumulation of somatic changes in the colorectal epithelium. Also, genomic instability, specifically, chromosomal instability, DNA repair defects and aberrant DNA methylation are involved in colon cancer (23-29). Changes in chromosomal copy number, germ-line and somatic inactivation of mismatch-repair genes and tumor suppressor genes such as APC, p53, SMAD4, MLH1, TGF-β, ABX and MYH lead to initiation, promotion and progression of colon cancer (30-33). Also, the modest depletion of cytosine methylation and aberrant methylation in certain promoter-associated CpG islands contribute to the epigenetic silencing of gene expression (28). Moreover, mutational inactivation of key tumor suppressor genes have been demonstrated to affect key signaling pathways, which contribute to tumor formation **(Figure 2)**.

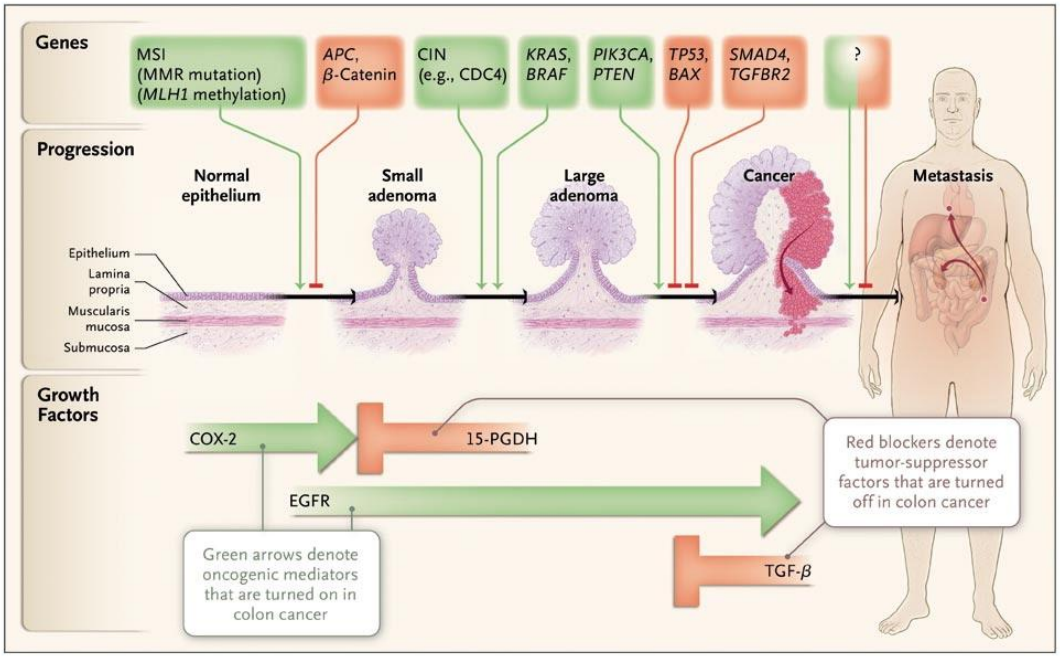

Figure 2 Genes and growth factor pathways that drive the progression of colorectal cancer. In the progression of colon cancer, genetic alterations target the genes that are identified at the top of the diagram. The microsatellite instability (MSI) pathway is initiated by mismatch-repair (MMR) gene mutation or by aberrant *MLH1* methylation and is further associated with downstream mutations in *TGFBR2* and *BAX*. Aberrant *MLH1* methylation and *BRAF* mutation are each associated with the serrated adenoma pathway. The question mark indicates that genetic or epigenetic changes specific to metastatic progression have not been identified. Key growth factor pathways that are altered during colon neoplasia are shown at the bottom of the diagram. CIN denotes chromosomal instability, EGFR epidermal growth factor receptor, 15-PGDH 15-prostaglandin dehydrogenase, and TGF-β transforming growth factor β. Image adapted from (34).

According to the literature, there are three major steps involved in progression and promotion of colon cancer. The first event is associated with APC, which is a well-

known tumor suppressor gene involved in suppressing β-catenin activity in normal colon epithelium and is commonly mutated in colorectal cancer either due to germ line or somatic mutations (35, 36). This leads to constitutive activation of Wnt signaling, which leads to promotion of colorectal cancer. The next step involves TP53. Inactivation of TP53 gene, a well-known tumor suppressor gene has been demonstrated to be one of the main causes of progression of colon cancer, i.e., transition of large adenomas into invasive carcinomas (37). The last step involves TGF-β-tumor suppressor pathway. There is somatic mutational inactivation of TGF-β signaling, which coincides with the transition from adenomas to carcinoma (38). Apart from mutation inactivation of tumor suppressor genes, activation of oncogenic pathways such as MAPK signaling, PI3K signaling and GTPase activity also contributes to development of colon cancer (37, 39-42).

1.4 Effect of polyunsaturated fatty acids (PUFA) in colon cancer

Clinical, experimental and epidemiological evidence demonstrate the significant role of PUFA in colon cancer (43, 44). A plethora of studies have shown that consumption of a diet rich in omega- 3 fatty acids (n-3 PUFA) enriched in fish oil may reduce the risk of colon cancer compared to omega-6 fatty acids (*n*-6 PUFA) (45-48). Fish oil contains significantly higher amounts of eicosapetaenoic acid (EPA, $20{:}5^{\Delta 5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) compared to corn oil which has higher amounts of linoleic acid (LA, $18{:}2^{\Delta 9,12}$), an important source of n-6 in the western diet (49). Recent investigations have demonstrated that highly saturated fatty acids present in fish oil have effects on apoptosis, signal transduction, reactive oxygen species (ROS)

production, transcriptional and translational regulation (50-53). Another dietary agent, fermentable fiber is also considered to be one of the most important nutritional factors considered to be protective against colon cancer (54-56). Butyrate, a short chain fatty acid produced in the intestinal lumen due to bacterial anaerobic fermentation has also been demonstrated to have chemoprotective effects (57). Moreover, we have demonstrated that dietary fish oil and fermentable fiber work synergistically to protect against colon carcinogenesis, primarily by enhancing apoptosis (52, 58-60). Despite all the scientific evidence indicating the protective effect of fish oil and fermentable fiber, there is lack of information regarding the molecular mechanisms by which this combination exerts its protective effect against colon tumorigenesis.

1.5 miRNAs and cancer

It was reported for the first time in 2002 by Calin *et al.*, that a region on chromosome 13q14 frequently deleted in chronic lymphocytic leukemia (CLL), was transcribed into miR-15 and miR-16 (61). Subsequently, miRNAs were shown to be differentially expressed in cancer cells, in which distinct and unique miRNA expression profiles were documented (62). High throughput miRNA profiling studies have determined the implications of aberrant expression of miRNAs in organ development, e.g., colon, (63-67), liver (68, 69), lung (63, 70, 71), breast (72-74), prostate (75-77), pancreas (65, 78-80). Moreover, miRNAs are shown to be correlated to the tumor location and mutation status of several tumor suppressor genes /oncogenes and cancer disease stages. There is also evidence suggesting that miRNA profiles are able to predict disease outcome and response to therapy (81, 82). Hence, these small molecules have been the focus of

scientific attention to determine their potential as clinical biomarkers, with diagnostic, and predictive potential.

1.5.1 Mechanisms affecting miRNA expression

Several factors have been reported to regulate miRNA expression in cancers. One of the key factors is chromosomal abnormality. Genomic regions where miR-15a and miR-16-1 were mapped and are reported to be frequently deleted in cancer (61). Single nucleotide polymorphisms have also been reported to regulate miRNA expression in several types of cancer (83). Apart from structural genetic alterations, miRNA expression can also be deregulated by some key epigenetic events like hypomethylation, which has been demonstrated to increase expression of miRNAs in cancer (84-86) . Moreover, miRNAs have been shown to regulate several components of the epigenetic machinery creating a feedback loop (87-90).

Another mechanism by which miRNA expression is altered is due to defects in miRNA biogenesis machinery (91). Overexpression or loss of Dicer and/or Drosha is either positively or negatively correlated with outcome in colorectal cancer (92, 93) and several other types of cancer (94-96). Alteration in the activity of transcription factors, such as p53, MYC and ZEB1 has been shown to affect miRNA expression (97-99).

1.5.2 Role of select miRNAs in cancer

High throughput miRNA profiling studies have detected aberrant expression of miRNAs in development of colon (63, 65-67, 100), liver (68) (69, 101), lung (70, 71, 102), breast (72-74), prostate (75-77), and pancreatic cancer (65, 78-80). Moreover, miRNAs are also correlated with tumor location, mutation status of several tumor

suppressor genes /oncogenes and cancer disease stages. For example, miR-31 expression was found to be significantly higher in Stage IV tumors as compared to stage II tumors, while miR-21 was positively correlated with colorectal cancer metastasis (64).

Several miRNAs such as miR-21 have been shown to be aberrantly expressed in almost all types of cancer. Two key "tumor suppressors miRNAs, miR-143 and miR-145, are dysregulated in several cancers (64, 66, 67, 103-108). Functional studies identified several key targets such as ERK5, KRAS, MAPK7, DNMT3A, TGFBRE, APC, IRS1, STAT1, YES1 and FLI1 (107, 109, 110). Moreover, overexpressing these miRNAs leads to inhibition of cell growth by increasing apoptosis and decreasing cell proliferation (111, 112).

A diverse array of cellular activities has been shown to be modulated by the let-7 family. For example, it has been demonstrated that members of the let-7 family act as a tumor suppressoror oncogene based on the tissue type and histological grade of cancer as compared to normal tissue (109, 113-120). Some of the well-defined targets of the let-7 family are RAS, HMGA2, Blimp-1 and eIF4F (118, 121-127). Moreover, Ibaraa *et al.* showed that let-7 is a marker for differentiated cells and is undetected in stem cells (128).

miR-21 is a well described "oncogenic" miRNA. High expression of miR-21 has been reported in breast (129-131), glioblastomas (132-134), pancreatic (135-137) and colon cancer (138-140). miR-21 has anti-apoptotic properties by targeting several tumor suppressors, PTEN, PDCD4, BCL2, TIMP3, TGFβR2, SPRY3 and RECK (64, 103, 139, 141). miRNA profiling in five gliobastoma cell lines revealed increased expression

of miR-21, known to target hTERT, PTEN, PDCD4 and TIMP3 (142). Also, inhibition of miR-21 caused disruption in glioma growth and increased caspase activity (143).

One of the well-known polycistronic miRNA clusters is miR-17-92, which consists of six individual miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a (144). Overexpression of this cluster has been observed in several tumor types (63, 144, 145). Moreover, miR-17-92 suppresses c-myc induced apoptosis in colorectal adenoma and progenitor B-cells and thus is considered an oncomir (146, 147). Using miR-17-92 knockout mice, Ventura et al. showed that each of the miRNA components in the cluster may have its own specific functions apart from some common function with the entire cluster (148).

Recently, two other miRNA clusters formed from miR-200 family members (1st cluster consisting of miR-200a, miR-200b and miR-429 and 2nd cluster consisting of miR-200c and miR-141) have gained attention. miRNA profiling studies have showed that these clusters are downregulated in breast (149, 150), colon (99, 100, 151-153), pancreatic (154, 155), and prostate cancer (156, 157). One of the mechanisms by which miR-200 exerts its effect is through a double negative feedback loop between miR-200 family members and transcription factors ZEB1 and ZEB2 (99, 158-160). Inhibition of ZEB1 and ZEB2 by these miRNAs caused an increase in a key epithelial maker, E-cadherin, resulting in the altered acquisition of an epithelial phenotype (160, 161). Moreover, an extensive study conducted on NCL-60 cell lines suggested that miR-200 was a marker of epithelial phenotype (153). Several studies have also identified that

miR-200/ZEB association also affects pathways that play a key role in cancers, e.g., TGFβ (99, 162) and p53 (163-165).

1.6 Modulation of miRNAs by bioactive dietary agents

There is clinical, experimental and epidemiological evidence that suggests that diet is one of the most important modifiable determinants for developing a number of chronic diseases. Various natural dietary chemopreventive agents have been identified and shown to exert pleiotropic actions in cancer cells. Also, studies utilizing several dietary agents such as resveratrol, curcumin and EGCG have shown that these compounds have neuroprotective properties. Recent literature has suggested that environmental agents, specifically bioactive food components (BFC) and exercise play a role directly or indirectly in the modulation of miRNA expression (140, 151, 166-168).

1.6.1 Modulation of mRNA and miRNAs by fatty acids

There are compelling data indicating a functional link between dietary fat intake and colon cancer risk (47, 169). It is well known that n-3 PUFA are modulators of several genes known have a role in inflammation, lipid metabolism and energy utilization (170). Moreover, in order to elucidate the molecular mechanisms by which n-3 PUFA inhibit azoxymethane (AOM) induced colon carcinogenesis, we have shown that dietary n-3 PUFA uniquely alter colonic gene expression profiles (50). For example, n-3 PUFA modulate signaling pathways, which promote cell cycle reentry of stem progenitor cells and regulate mitogenic activity in the colon (171). Further studies are needed to understand how the colonic stem cell population responds to diet and carcinogen exposure.

In glioblastoma cells, following treatment with three different types of PUFAs (GLA, AA and DHA), several miRNAs including miR-16, miR-143, miR-22, miR-20b, miR-31, miR-145, miR-182, miR-183, miR-200c, miR-26a, miR-206, miR-140, miR-17, miR-29c and miR-34 were differentially expressed. Specifically, in PUFA treated cells, miR-143 was reduced, while miR-20b was elevated when compared to untreated cells (172). Vinciguerra *et al.* observed that unsaturated fatty acids (oleic, palmitoleic and linoleic acid) reduced PTEN expression in hepatocytes (173). They reported that treatment with oleic acid ($18:2^{\Delta 9}$) also upregulated miR-21 synthesis by activating the miR-21 promoter via a mTOR/NF-κB65-dependent mechanism. These findings were corroborated in the liver of Wistar rats fed a high-fat diet. In mice fed CLA and standard-or-high fat diets, a significant correlation was observed between miR-103 and miR-143; miR-103 and miR-107 and miR-221 and miR-222 (174). *In vitro* studies in breast cancer cell lines (MCF-7 and MDA-MB-231) showed that DHA inhibits expression of CSF-1 (colony stimulating factor-1). Moreover, PTEN regulated CSF-1 expression through PI3K kinase/Akt signaling via a transcriptional mechanism. Moreover, DHA treatment inhibited miR-21, which was associated with increased PTEN protein levels. This in turn attenuated expression of CSF-1. *In vivo* studies in mice breast tumors also demonstrated similar results as the *in vitro* study (175). To date, the effect of fatty acids on miRNAs in the context of colon cancer has not been investigated.

1.6.2 Modulation of miRNAs by butyrate

Diet-derived butyrate, a short chain fatty acid (SCFA) is produced via fermentation of dietary fiber in the distal intestine. With respect to epigenetic effects, butryate acts as a histone deacetylase inhibitor and can decrease proliferation and increase apoptosis in colorectal cancer cells (176-179). Studies have demonstrated that these effects are mediated in part through induction of p21 expression (59). Recent, evidence suggests that the protective effects of butyrate may be mediated in part by modulating miRNA expression. Hu *et al.* showed that on treatment of human colon cancer cells (HCT116) with butyrate, expression of miR-17, miR-20a, miR-20b, miR-93, miR-106a and miR-106b were significantly reduced (180). Also, p21 was determined to be a direct miR-106b target. These data indicate that SCFA regulate host gene expression by modulation of miRNAs implicated in intestinal homeostasis and in carcinogenesis. Another study by Humphreys *et al.* explored the effects of several histone deacetylase inhibitors (HDI) on miRNA expression in human colon cancer cell lines (HCT116 and HT-29). They found that these HDIs also decreased miR-17-92 cluster miRNAs, while their target genes, e.g., PTEN, BCL2L11, CDKN1A, were increased. When miR-17-92 cluster miRNAs were overexpressed in presence of the HDIs, the protective effects of HDIs were diminished (181).

1.6.3 Modulation of miRNAs by other dietary agents

There is evidence suggesting that several other bioactive agents such as vitamins A, B, D, E; polyphenols, curcumin, resveratrol, catechins, isoflavones, indoles and isothiocynates modulate cancer risk (182-196) by altering gene expression and signal transduction pathways. In the last five years, a plethora of studies have examined the

effect of these agents on miRNA expression in different types of cancer (155, 197-217). Seven microRNAs, let7a, miR-21, miR-26, miR-34, miR-125, miR-146 and miR-200 were shown to be modulated by at least five of the above mentioned dietary agents. These studies concluded that the bioactive dietary agents cause reduction in the expression of several miRNAs that are overexpressed in cancer, which subsequently modulated genes acting as tumor suppressors or oncogenes, thus conferring protection against cancer.

1.7 Colonic stem cells

The adult stem cells of the colon are of particular interest because they sustain self-renewal and are target cells for cancer initiation mutations (218, 219). Therefore, perturbations in the stem cell dynamics are generally believed to represent the earliest step towards colon tumorigenesis. In general, the intestinal epithelium is constantly being replaced, undergoing apoptosis and shedding (exfoliation) into the lumen (220). The replacement of epithelial cells is regulated by the stem cells residing at the base of the crypts. These cells are under constant microenvironmental (niche) influence (221, 222). Stem cells can undergo asymmetrical or symmetrical cell division to give rise to both stem cells and daughter cells. In the case of asymmetrical division, one of the daughter cells is identical to the original cell (maintains stemness), and the other daughter cell (also called progenitor cell) further differentiates and migrates to the top of the crypt. This hierarchical organization is also seen with other tissues that undergo constant renewal (223). Although the exact identity of the stem cells has been proven to be controversial over the last 30 years, in the last few years, significant progress has

been made to identify, isolate and characterize the stem cells of the colon. Currently, it has been proposed that there are two pools of stem cells located in an intestinal crypt (224). One of the pools consisting of fast cycling cells is located at the base of the crypt and recently, Lgr5 was identified as a functionally validated marker for these cells by crossing stem-cell-specific Lgr5-EGFP-IRES-creER^{T2} knockin mice to Apc$^{flox/flox}$ mice (225). Interestingly, lineage tracing has revealed that Lgr5^{+} stem cell activity in mouse intestinal adenomas (226). Lgr5 is a leucine–rich repeat containing G protein-coupled receptor 5 and is overexpressed in colon cancer stem cells (227). Moreover, the distribution of Lgr5^{+} cells within stem cell-derived adenomas indicate that a stem cell/progenitor cell hierarchy is maintained in early neoplastic lesions (228). Also, single Lgr5^{+} intestinal stem cells can generate continuously expanding, self-organizing epithelial structures reminiscent of the normal gut (229, 230). The other pool consists of slow cycling cells that are located at +4 position and Bmi-1 and Tert have been identified as markers of these cells (231-233)

1.7.1 Role of miRNAs in colonic stem cells

There is emerging evidence that suggests a role for miRNAs in regulating the post-transcriptional genetic programs in stem and progenitor cells. A recent study systematically examined miRNA expression profiles in adult tissue-specific stem cells and their differentiated counterparts (231). A common signature of miRNA expression in blood, muscle and neural stem cell populations was detected. This suggests that miRNA signatures mark the transition from self-renewing and quiescent stem cells to proliferative and differentiating progenitor cells, implying an extensive role of miRNAs

in regulating self-renewal, proliferation, and quiescence programs in the cells (234). Moreover, miRNA profiling in cancer stem cells isolated from human colon cancer cell lines (HT-29) using the CD133 surface marker revealed 11 overexpressed miRNAs and eight underexpressed miRNAs such as miRR-429, miR-155, and miR-320d, some of which may be involved in regulation of stem cell differentiation (235).

1.8 Summary and purpose of the study

Evidence emerging from many clinical and experimental studies shows that diets rich in n-3 PUFAs, found in fish oil, have chemoprotective effects against colon carcinogenesis. This protection has been shown to be conferred in part by modulating transcription and translation of apoptotic and Wnt related signaling pathways (171, 236). However, the precise molecular mechanism of action is still obscure. Recently, several studies have shown that transformation of adult stem cells is an extremely efficient route towards initiating intestinal cancer (228). However, the impact of the above mentioned chemoprevention agents on adult intestinal stem cell biology has not been determined. Therefore, the main objective of this study was to determine whether the effects of chemoprotective dietary n-3 polyunsaturated fatty acids (PUFA) are mediated in part by miRNA signatures. Specifically, the effects of omega-3 and omega-6 PUFA on the global targets of select miRNAs were investigated. In addition, the effects of the chemoprotective dietary agents on miRNA expression in adult colonic stem cells were documented. In an attempt to elucidate the molecular mechanisms of action the following hypothesis and aims were designed.

1.9 Hypotheses and specific aims

Hypothesis 1: The effects of chemoprotective n-3 PUFAs are mediated, in part, via modulation of miRNA signatures in the colon.

Aim 1. Determine the targets of miR-15b, miR-21 and miR-107

Aim 2. Identify protective and promotive intestinal microRNAs based on carcinogen and diet treatment in a rat colon cancer model.

Aim 3. Validate the targets of miR-19b, miR-26b and miR-203; and elucidate the effect of chemoprotective dietary agents (fish oil (DHA)+pectin (butyrate)) on the levels of these microRNAs.

Hypothesis 2: Chemoprotective diets containing n-3 PUFA plus pectin will protect the colon from carcinogen-induced microRNA and mRNA dysregulation in adult colonic stem cells.

Aim 1. Determine the effects of a chemoprotective diet on the global expression of microRNAs in stem cells isolated from Lgr5-EGFP mice injected with a colon carcinogen (AOM).

Aim 2. Compare the expression of microRNAs in adult colonic stem cells to "global" expression of microRNAs in the entire colonic crypt.

2. DETERMINATION OF TARGETS OF miR-15B, miR-21 AND miR-107*

2.1 Introduction

Colorectal cancer continues to pose a serious health problem in United States. Every year more than 108,000 cases are diagnosed and more than 50,000 deaths occur annually due to colon cancer (22). From a dietary perspective, a growing number of clinical and experimental studies indicate a protective effect of dietary fish oil, containing n-3 polyunsaturated fatty acids (PUFA), with respect to colon cancer risk (43, 50, 169, 237-243). Eicosapetaenoic acid ($20{:}5^{\Delta 5,8,11,14,17}$) and docosahexaenoic acid ($22{:}6^{\Delta 4,7,10,13,16,19}$) are typical *n*-3 PUFA (found in fish oil), defined according to the position of the first double bond from the methyl end of the molecule, which is designated 'n-3. In contrast, dietary lipids rich in n-6 PUFA (found in vegetable oils) e.g. linoleic aicd ($18{:}2^{\Delta 9,12}$) and (meats) arachidonic acid ($20{:}4^{\Delta 5,8,11,14}$), enhance the development of colon tumors (43, 44). These effects are both exerted at both the initiation and post-initiation stages of carcinogenesis (43, 244). In addition, it has been reported that consumption of fiber, a source of butyrate, may confer protection against colorectal cancer (245). Butyrate, a major source of energy for colonocytes is also known to have chemoprotective qualities in colon cancer and animal models (246).

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* Reprinted with permission from "*n*-3 Polyunsaturated fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon" by Davidson LA, Wang N, Shah MS, Lupton JR, Ivanov I, Chapkin RS, *Carcinogenesis*.12, 2077-2084, 2009. Copyright 2009 by Oxford Journals.

Consumption of fish oil and fiber together may reduce colon cancer risk in humans (237, 241). In a series of seminal experiments, we have demonstrated that pleiotropic bioactive components generated by fish oil (n-3 PUFA) and fermentable fiber (butyrate) act coordinately to protect against colon cancer in part, by enhancing apoptosis (59, 60, 239, 247, 248).

Aberrant expression of miRNAs are believed to be one of the key epigenetic events involved in colon tumor development. Over the last several years, there has been an increasing amount of evidence demonstrating the modulation of miRNA and their targets by several bioactive dietary agents in cancer. But no studies have examined the chemoprotective effects of n-3 and n-6 PUFA on miRNA expression in colon. Recently, we reported for the first time that combined fish oil and pectin treatment modulates a subset of miRNAs and their predicted targets (mRNA) implicated in the regulation of the colon stem cell niche and tumor evolution (140).

In order to determine the significantly altered miRNAs in various stages of colorectal cancer, Sprague Dawley rats were injected with azoxymethane (AOM), a colon carcinogen determined to generate colonic tumors. To observe the changes taking place in the early stages of colorectal cancer, rats were sacrificed and total RNA was extracted from mucosal scrapping from the colon after 10 weeks of AOM injections. It has been well documented that this time point high multiplicity aberrant crypt foci (ACF) are evident (50). Comparison of the miRNA expression profiles in rats injected with AOM versus saline, revealed that twenty-seven miRNAs were found to be overexpressed, while nineteen miRNAs were downregulated in AOM injected rats as

compared to the saline injected rats. To assess the protective effects of omega-3 PUFA, rats injected with either AOM or saline were fed with fish oil or corn oil, along with either pectin or cellulose. Ten weeks after the AOM injection, miRNA profiling was carried out. Initial analysis determined that five miRNAs,let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were downregulated in the corn oil AOM treated group as compared to the fish oil AOM treated group (140).

In order to understand the function of the differentially expressed miRNAs, we determined the gene targets of these diet modulated miRNAs. Hence, we carried out knockdown studies to document the changes taking at the protein level of the putative targets.

2.2 Materials and methods

2.2.1 Identification of established targets of significantly modulated diet miRNAs

Empirically established miRNA targets were identified using miRecords (http://mirecords.biolead.org/), an integrated resource for miRNA-target interactions.

2.2.2 Cell culture

HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum and 2 mM glutamax (Gibco, Carlsbad, CA) at 37^0C in 5% CO_2. Cells were plated at 1-2x10^5 wells per well in a 12-well plate on the day of transfection. Subsequently, cells were transfected with 5' FITC-labeled miRNA inhibitors (Exiqon, Denmark) anti-miR-107, anti-miR-21, anti-miR-15b (20 nM) using Hiperfect transfection reagent (Qiagen, Valencia, CA). Scrambled miR was utilized as a negative

control. Twelve hours after the transfection, the media was changed. The experiment was repeated in triplicate.

2.2.3 Total RNA isolation and Real time PCR

Twenty-four hours after transfection of miRNA inhibitors in HCT116 cells, media was removed and the cells were washed thrice with phosphate -buffered saline (PBS) (Gibco, Carlsbad, CA). 600ul of RNA lysis buffer provided with miRVana miRNA isolation kit was added to each well and the cells were scrapped with the help of cell scrapper. Total RNA was isolated using miRVana miRNA isolation kit following the manufacturer's protocol. RNA quantity and quality were measured by nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies, CA) respectively. Real time PCR was carried out by using miRNA Taqman PCR assay (Applied Biosystems) to determine the expression of mature miR-15b, miR-21 or miR-107 in treated and untreated samples. Normalization was carried out by using the $2^{\Delta\Delta CT}$ method relative to 18S rRNA.

2.2.4 Western blotting

In order to determine the change in protein expression after miRNA knockdown, western blotting was carried out. 2-4x10^6 cells were seeded in 100 mm dishes on the day of transfection. Transfection with 20 nM miRNA inhibitors was carried out as described above. Seventy-two hours after the transfection, media was removed and cells were washed thrice with PBS. 300 ul of lysis buffer containing 50 mM Tris-HCL (pH 7.2), 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid (pH 7.6), 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (pH 7.5), 50 µM NaF, 1% Triton-X, 100 µM sodium orthovanadate, protease inhibitor cocktail and 10 mM β-mercaptoethanol were

added to the cells. The lysate was then passed through a 27 gauge syringe and incubated on ice for 30 minutes. Subsequently, the lysate was centrifuged at 16,000g for 20 minutes. The supernatant was collected and the protein concentrations were determined by Bradford method. 20-80 μg of samples were loaded onto a 4-20 % Tris-Glycine gels (Invitrogen, Carlsbad, CA). After blotting, the membrane was incubated overnight with goat BACE1 antibody at 1:1000 (R&D systems, Minneapolis, MN), PTEN antibody at 1:1000 (Cell signaling Technology, Boston, MA) or Bcl-2 at 1:1000 (Stressgen, Ann Arbor, MI) and horseradish peroxidase linked (Jackson Immunoresearch Laboratories, West Groove, PA) secondary antibody at 1:10,000 dilution and chemoluminescent detection was performed. Unless noted, all the other reagents were from Sigma.

2.2.5 Statistics

The effect of two independent variables (treatment effects) was assessed using Student's t-test. The graphs were plotted using means and standard error of 4 cell culture wells (data points) obtained two different experiments (performed in triplicate). Standard error bars were plotted in order to document the variation in the population mean. *P*-values < 0.05 were considered to be statistically independent.

2.3 Results

2.3.1 miRNA functional target analysis

Established targets of let-7d, miR-15b, miR-107, miR-191 and miR-324-5p were identified using miRecords (http://mirecords.biolead.org/). miRecords is an integrated resource for miRNA-target interactions (249). Since insilico analysis is not accurate and generates numerous false positives (250), we opted to determine the change in expression of those targets that were already validated in other tissues **(Table 1)**. From a cancer perspective, miR-15b has been shown as a natural antisense interactor with Bcl-2, a well documented anti-apoptotic protein (251). Functional analyses have demonstrated that miR-107 contributes to the regulation of beta-site amyloid precursor protein-cleaving enzyme (BACE1) in Alzheimer's disease (252).

Table 1. **Diet modified miRNAs and their established mRNA targets**. Experimentally validated mIRNA targets were identified using the miRecords data base (http://mirecords.umn.edu/). This data base lists over 301 miRNAs and 902 target genes in 9 species.

miRNA	Established Target	Pathway/Function	Disease/Process	Reference
let-7d	Sept3	Cytokinesis	Brain GTPase controlling cytoskeletal and membrane organization	Jeyaseelan, 2008
miR-15b	Bcl2 CCNE1	Apoptosis Cell cycle	Gastric cancer cells Glioma cancer cells	Cimmino, 2005; Xia, 2008 Xia, 2009
miR-107	Bace1	Protease	Brain Voltage-gated Na^+ channels	Wang, 2008
miR-107	Serbp1	Plasminogen activation	Ovarian cancer	Beitzinger, 2007
miR-191	None identified	-	-	-
miR-324-5p	None identified	-	-	-

2.3.2 Colonic miRNA functional target analysis: BACE1 is a target of diet-modulated miR-107

To further assess targets for select diet-modulated miRNAs, we determined whether BACE1 is targeted by miR-107 in coloncytes. BACE1 was selected because it is highly expressed in HCT116 human colon cancer cells. Hence, we transfected cells with anti-miR-107 or control anti-miR. On average, transfection efficiency was >70% **(Figure 3)**. Twenty-four hours after knockdown, miR-107 levels were reduced by ~70% **(Figure 4)**. Correspondingly, 72 hours after transfection, BACE1 levels were increased by >100% in knockdown compared with negative control **(Figure 5)**. These results suggest that BACE1 is a target of diet-responsive miR-107 in the colon. In complementary experiments, we also determined whether Bcl2 is targeted by miR-15b. Since Bcl-2 has been shown to be a target of miR-15b in gastric cancer cells (251), we knocked down miR-15b. Twenty-four hours after transfection, miR-15b levels were reduced by ~90% as compared to the untreated cells and control anti-miR **(Figure 4)**. However, Bcl-2 levels were unchanged 72 hours following transfection **(Figure 5)**, suggesting that it may not be a target of miR-15b in the colon.

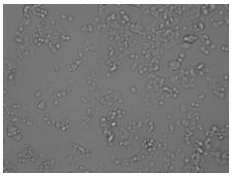
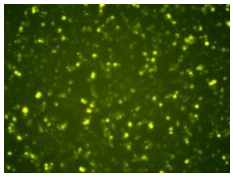
(A)  (B) 

Figure 3. Images after transfection of miRNA inhibitors. (A) Bright-field image of HCT116 cells (40X magnification). (B) Fluorescence microscopy of HCT-116 cells transfected with 20 nM miR-107 (40X magnification). Transfection efficiency was approximately 70%.

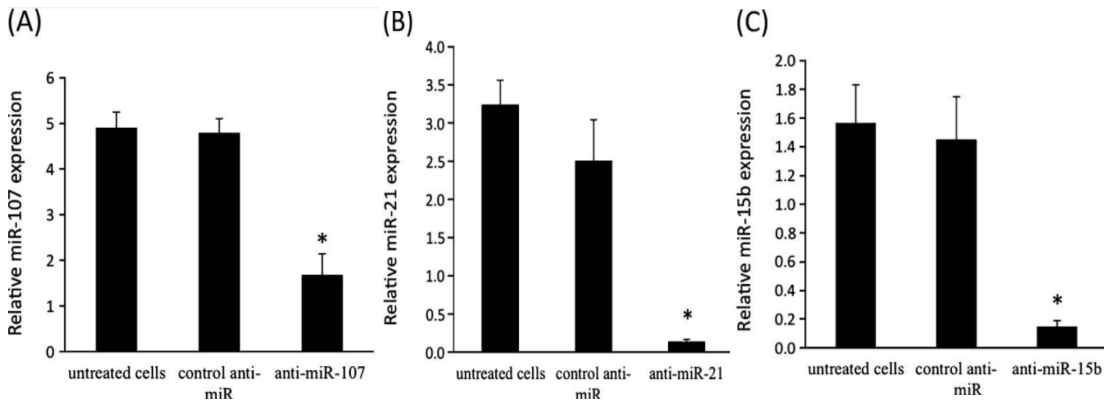


Figure 4. miRNA expression following transfection with miRNA inhibitors. HCT116 cells were transfected with either (**A**) 20 nM anti-miR-107, (**B**) 50 nM anti-miR-21 or (**C**) 50 nM anti-miR-15b or a control anti-miR. After 24 h, total RNA was isolated and analyzed for miRNA expression as described in the Materials and Methods. Asterisk indicates $P < 0.05$, $n = 4$ cultures from two separate experiments.

2.3.3 PTEN is a target of miR-21 in the colon

miR-21 is a well known oncogenic miRNA in the colon and was significantly upregulated in rat colon adenocarcinomas compared to normal colonic mucosa (253). We subsequently determined if PTEN is a target of miR-21 in the HCT116 colon cancer cell model system. PTEN levels were observed to be ~80% higher in the miR-21 knockdown samples compared with control samples **(Figure 5)**, confirming that PTEN is a functional target of miR-21 in the colon.

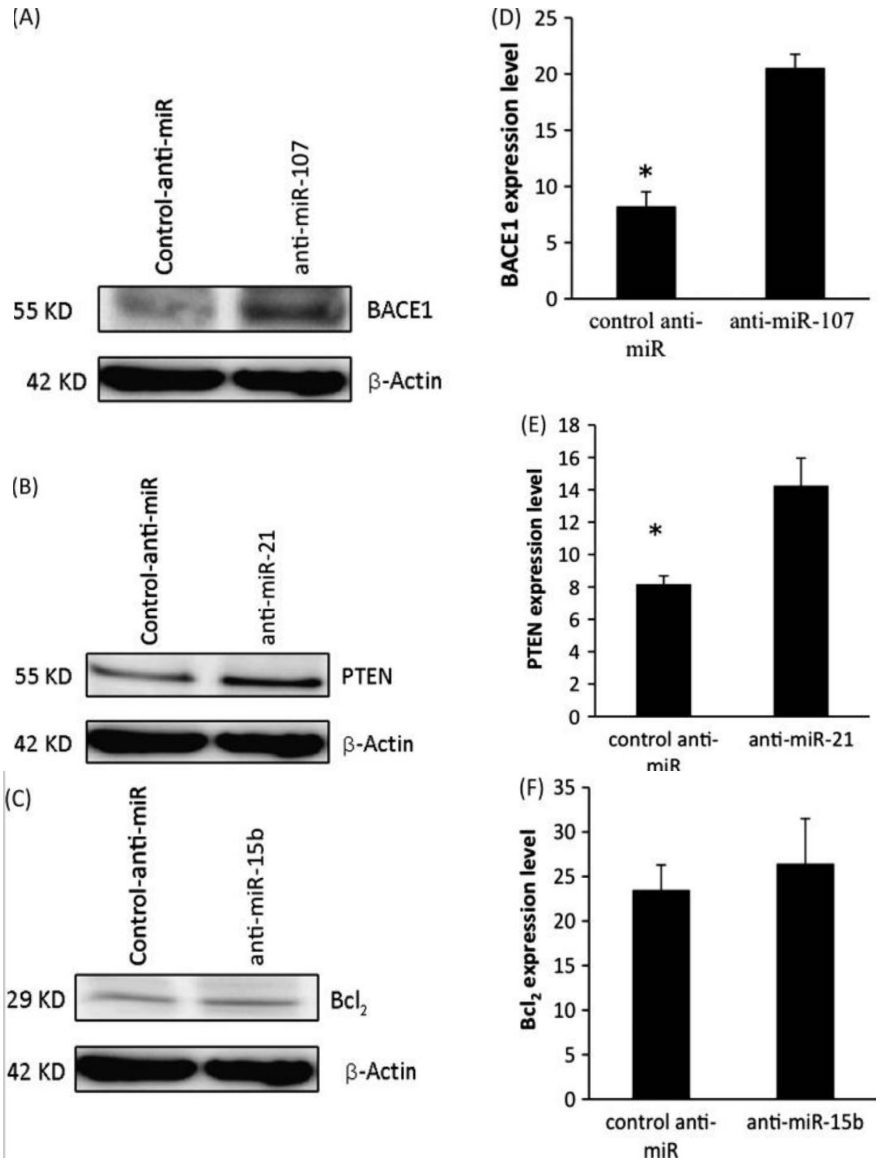


Figure 5. Target protein expression following knockdown of miRNAs. Representative immunoblots of HCT116 cells transfected with either (**A**) anti-miR-107, (**B**) anti-miR-21 or (**C**) anti-miR-15b or a control anti-miR. After 72 h, proteins were extracted to detect BACE1, PTEN or Bcl-2 levels by western blotting. Bar graphs represent the corresponding quantitative analysis of (**D**) BACE1, (**E**) PTEN and (**F**) Bcl-2 western blots. Asterisk indicates $P < 0.05$, $n = 4$ cultures from two separate experiments.

2.4 Discussion

In order to determine the targets of the diet modulated miRNAs, we used miRecords, an online database to identify the validated targets of miRNAs of interest. We report for the first time that upon knock down of miR-107, there was an increase in the protein levels of BACE1 in colonic epithelial cells. BACE1 is a validated target of miR-107 in Alzheimer's disease in human brain tissues (252) and has been shown to play a role in cancer (254). Also, we confirmed that PTEN is a target of miR-21. PTEN is frequently mutated or deleted in solid cancers, including colon cancer (255). It is the upstream regulator for both PI3K/AKT and MEK/ERK signaling pathways (256, 257). Also, miR-21 has been demonstrated to inhibit PTEN expression, which in turn causes a decrease in AKT and ERK activation, which results in tumor angiogenesis (258).

Upon inhibition of miR-15b, Bcl-2 protein levels did not change, even though it was shown to be a target in gastric cancer cells. There is evidence suggesting that miRNAs have tissue specific targets, which could be one of the reasons why Bcl-2 was unchanged (259).

In summary, these findings indicate the need to consider the impact of dietary bioactive agents when examining the role of miRNAs in the biology and pathobiology of the gastrointestinal tract. Also, using complementary analysis by comparing expression of significantly differentially modulated miRNAs and mRNA across treatments will help to shed some light on the underlying global epigenetic changes and further elucidate the biological effects of chemoprotective diets.

3. INTEGRATED miRNA AND mRNA EXPRESSION PROFILING IN A RAT COLON CARCINOGENESIS MODEL: EFFECT OF A CHEMOPROTECTIVE DIET*

3.1 Introduction

miRNAs are a diverse class of highly conserved small non-coding RNAs (~22 nucleotides long), shown to play a critical role in fundamental biological processes including cellular differentiation, apoptosis, cell proliferation and development. Currently, greater than 800 human and mouse miRNAs have been identified (4). MiRNAs regulate protein expression by acting through perfect or imperfect complementarity to 3' untranslated regions (UTRs) of their target mRNAs, resulting in post-transcriptional repression of gene expression (2, 3). With respect to chronic diseases, it has been shown that the miRNA gene-silencing pathway influences the processes of carcinogenesis and immune modulation (1, 2). With regard to mechanisms of oncogene activation, loss of miRNA complementary sites due to widespread shortening of 3' UTRs by alternative cleavage and polyadenylation selectively activates oncogenes and drives malignant transformation (260). Along these lines, several studies have shown that dysregulation of miRNAs and their mRNA targets contribute to the initiation and progression of colon carcinogenesis (100, 261). For example, miR-145 and miR-143 have been shown to act as tumor suppressors in the colon, while miR-21

*Reprinted with permission from "Integrated microRNA and mRNA expression profiling in a rat colon carcinogenesis model: effect of chemo-protective diet" by Shah MS, Schwartz SL, Zhao C, Davidson LA, Zhou B, Lupton JR, Ivanov I, Chapkin RS, *Physiol Genomics* 43, 640-654, 2009.

can act as an oncogene by repressing several tumor suppressor genes including PTEN, Pdcd4 and TPM1 (138, 262).

Several clinical and experimental studies have demonstrated that diets rich in n-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), found in fish oil, are protective against colon tumorigenesis (43, 239, 263, 264). In contrast, n-6 PUFAs such as linoleic acid (LA) and arachidonic acid (AA), found in vegetable oils, have been shown to enhance both the initiation and promotion of colon cancer (44, 253, 265). These findings are supported by a growing list of clinical and epidemiological studies indicating a functional link between dietary fat intake and colon cancer risk (47, 169, 237). In addition, it has been reported that the consumption of fiber, a source of butyrate in the intestinal lumen, may confer protection against colorectal cancers (245). Intriguingly, the protective effect of n-3 PUFAs with respect to colon tumor development is enhanced when a highly fermentable fiber, pectin, rather than a poorly fermentable fiber, cellulose, is added to the diet (59, 266). With respect to non-coding RNAs, we recently reported that these chemoprotective dietary agents favorably modulate carcinogen-directed non-coding miRNA signatures in the rat colon (140). Several recent studies have also documented the chemoprotective effects of other dietary agents such as folate, retinoids and curcumin (diferuloylmethane) on miRNA expression in different cancers (187, 201, 267). Collectively, these data suggest a pervasive effect of diet in miRNA mediated oncogenic transformation.

It has been shown that several tumor suppressors and proto-oncogenes can influence the formation of mature ribosomes and thereby regulate the activity of translation factors. Translational regulation plays an important role in repression/activation of gene expression during malignant transformation. In addition, some miRNAs which target mRNAs have been shown to be associated with polysomes and shuttle in the polysomal spectrum as a consequence of miRNA regulation (268-274). To date, a genome-wide perspective of the effects of miRNAs on actively translated (polysomal) mRNA populations in the colon has not been performed.

To elucidate the biological function of intestinal miRNAs, it is necessary to infer miRNA activity by combining gene expression with miRNA target prediction. Since miRNAs interact with complementary mRNAs (post-transcriptional level), resulting in either degradation or translational repression of their mRNA targets (275, 276), it is reasonable to expect that genome wide profiling of gene expression and miRNAs will allow for the investigation of genomic changes in cancer development. Therefore, when mRNA and miRNA levels are measured in the same sample, an integrative analysis can be performed to compare both profiles and determine their interactions. Various computational algorithms are currently used in order to predict the target genes of miRNAs. However, since a single miRNA can directly target greater than 200 genes and each mRNA may be regulated by several miRNAs (261, 277), computational challenges in miRNA-mediated regulation persist. As a result, there have been several approaches taken to analyze miRNA and gene expression data, such as performing

cluster analysis or computing correlation coefficients for miRNA and mRNA target expression (3, 278-280).

In the absence of comprehensive human data, the azoxymethane (AOM) chemical carcinogenesis model serves as one of the most definitive means of assessing human colon cancer risk (281, 282). Therefore, in this study, we determined the effect of carcinogen and diet on targets of colonic miRNAs in rats at 10 and 34 weeks post initiation. miRNA expression was quantified using a qPCR approach and mRNA expression was quantified using a Codelink^{TM} microarray platform. For the purpose of determining the effect of cancer progression and dietary chemoprevention on genomic profiles, we specifically examined the effect of carcinogen, n-6 vs n-3 fatty acid effects, and fat x fiber interaction on global colonic miRNA and mRNA expression profiles. Four complementary computational approaches were utilized to test the hypothesis that miRNAs and their post-transcriptionally regulated mRNA targets, i.e., both total mRNAs and actively translated mRNA transcripts (in polyribosome complexes), are differentially modulated by carcinogen and diet treatment. Specifically, gene enrichment analysis was used to obtain those miRNAs significantly enriched by the change in expression of their putative target genes across carcinogen and diet comparisons. This was complemented with canonical pathway analyses to further assess miRNA and mRNA interaction. In addition, cumulative distribution function (CDF) plots were used to evaluate the impact of diet/carcinogen on mRNA levels induced via miRNA alterations. For validation purposes, a subset of the gene targets was also examined at the protein level. Lastly, linear discriminant analysis (LDA) was used to

identify the best single, two and three-miRNA combinations for classifying diet effects and colon tumor development.

3.2 Materials and methods

3.2.1 Animals

Fifty-four weanling male Sprague Dawley rats (Harlan, Houston, TX) were acclimated for 2 wks in a temperature and humidity controlled facility on a 12 h light/dark cycle. The animal use protocol was approved by the University Animal Care Committee of Texas A&M University. The study was a 2 x 2 x 2 factorial design with two types of dietary fat (n-6 PUFA or n-3 PUFA), two types of dietary fiber (cellulose or pectin) and two treatments (injection with the colon carcinogen, AOM, or with saline). Animals (n=6 per group) were terminated 10 wks after AOM injection. For generation of colonic tumors, a second group of rats (n=6 per treatment) were continued on diet for 34 wks post-injection. All tumors were independently classified as adenocarcinomas by a board-certified pathologist.

3.2.2 Experimental diets

After a one week acclimation on standard pelleted diet, rats were assigned to one of four diet groups, which differed in the type of fat and fiber as previously described (140). Diets contained (g/100 g diet): dextrose, 51.00; casein, 22.40; D,L-methionine, 0.34; AIN-76 salt mix, 3.91; AIN-76 vitamin mix, 1.12; choline chloride, 0.13; pectin or cellulose, 6.00. The total fat content of each diet was 15% by weight with the n-6 PUFA

diet containing 15.0 g corn oil/100 g diet and the n-3 PUFA diet containing 11.5 g fish oil/100g diet plus 3.5 g corn oil/100 g diet to prevent essential fatty acid deficiency.

3.2.3 miRNA analysis

Total RNA enriched with miRNA was isolated using mirVana miRNA Isolation Kit (Ambion, Austin, TX). Expression of 368 mature miRNAs was determined using TaqMan Human MiRNA Panel Low Density Arrays (Applied Biosystems, CA). miRNA expression was normalized to RNU6B expression. Two-hundred fifteen miRNAs were disqualified due to low (close to background level) expression. The resultant readings were further quantile normalized within each experimental condition, and one-way ANOVA analysis (t-test) was performed followed by false-discovery rate correction. Adjusted p- and q-values were obtained, and significantly altered miRNAs ($q < 0.05$) with a fold change >1.3 or <0.7 were selected for further analysis.

3.2.4 mRNA analysis

Polysome and total RNA were isolated from diet and carcinogen-treated animals as previously described (283). Upon termination, each colon was cut open longitudinally, flushed clean with PBS, 1 cm from the distal colon was collected for fixation and embedding for immunohistochemical assays, an adjacent cm was taken for total RNA isolation and the remainder of the colon was used for polysome RNA isolation. For polysome RNA isolation, colons were incubated for 5 min at room temperature in PBS containing 100 μg/ml cycloheximide, an inhibitor of translation which locks the mRNA/ribosome complex, facilitating its isolation and preventing RNA degradation. Following incubation in PBS containing 100 μg/ml cycloheximide, colonic

epithelial cells were allowed to swell in LSB (20 mM Tris pH 7.5, 10 mM NaCl and 3 mM $MgCl_2$) containing 1 mM dithiothreitol and 50 U RNase inhibitor for 2 min followed by lysis in LSB containing 0.2 M sucrose and 1.2% Triton X-100. After removal of nuclei by centrifugation, the supernatant was layered over a 15-50% linear sucrose gradient (in LSB) and centrifuged at 247,000 x g for 2 h at 4°C in a swinging bucket rotor. Gradients were fractionated, aliquots were taken for absorbance at 254 nm, and three volumes of denaturation solution (Ambion Totally RNA kit) was immediately added to the remainder of each fraction. Samples were frozen at -80°C until RNA was isolated using the Totally RNA kit (Ambion) as per manufacturer's instructions followed by DNase treatment. Both total RNA and polysome RNA were analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, CA) to assess RNA integrity. CodeLink rat whole genome bioarrays (Applied Microarray) were used to assess gene expression (253).

Only genes with a G flag (good quality) from the microarrays were selected for further analysis. Expression values were median normalized per array. Similar to the case of miRNA, both total and polysomal RNA expression values were quantile normalized within each experimental condition. A within-treatment quantile-normalization was then applied to all non-outlying observations Mixed-effect ANOVA was applied to the data to obtain p-values. P values <0.05 were considered to be statistically significant(284).

3.2.5 miRNA target prediction

Since routine identification of an overlap between miRNA target prediction algorithms is discouraged (285), Target Scan (http://www.targetscan.org/) was used to identify putative miRNA targets in rat. This program predicts biological targets of miRNAs by searching for conserved 8-mer and 7-mer sites that match the seed region of the miRNA (286). The predictions are ranked based on the putative efficacy of targeting as calculated using context scores of the sites (287). Specifically, this algorithm generates P_{CT} scores for the probability of any seed match being selectively maintained due to miRNA targeting. The higher the P_{CT} score, the greater the probability that a miRNA could target a particular gene. In addition, the DIANA-microT 3.0 algorithm was utilized to verify Target Scan output (288).

3.2.6 Overview of analysis pipeline showing the computational analyses

In order to infer the regulatory activities of miRNAs in the colon, microarray expression data was integrated with miRNA target prediction. For this purpose, three main biological comparisons were examined: (A) carcinogen effects (tumor versus saline), (B) dietary fat effects in the presence of carcinogen (corn oil+AOM versus fish oil+AOM), and (C) dietary fat x fiber interaction in the presence of carcinogen (corn oil+cellulose+AOM vs fish oil+pectin+AOM). The global impact of diet and carcinogen on miRNAs and mRNA tissue profiles was systematically elucidated using four complimentary computational methods.

In the first approach, we used *Gene Set Enrichment Analysis* (GSEA) (http://www.broad.mit.edu/gsea/index.jsp). GSEA incorporates knowledge of known

gene networks/pathways and identifies significantly enriched miRNA putative target lists based on the change in putative target expression according to a t-test for differential expression (289, 290). In experiments described herein, the target list corresponds to genes in the total or polysomal expression data sets which are ranked by the respective t-test for differential expression. The gene sets are made up of the putative targets of miRNAs obtained from Target Scan. GSEA calculates an enrichment score (ES) that reflects the degree to which the targets within the gene set are over-represented in the respective gene list. A p-value is assigned to the ES score by a permutation test, which states whether the enrichment is significant or not. GSEA then calculates a normalized enrichment score (NES), which takes into account the number of genes within the independent gene set. Analysis of the biological role of putative targets of miRNAs was performed using **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) (http://david.abcc.ncifcrf.gov/) **(Figure 6(A)).**

In the second approach, initially, highly abundant miRNAs exhibiting an inverse trend with their mRNA targets based on fold change were examined. Treatment effects were examined relative to significantly altered total and polysomal mRNAs (p-value < 0.05). Next, miRNAs predicted to target mRNAs based on Target Scan were selected. Finally, the list of significantly altered total and polysomal mRNAs predicted to be targeted by several miRNAs was intersected with the list of significantly altered highly abundant miRNAs in order to obtain non-coding RNAs which were inversely related to their significantly altered target total and polysomal mRNAs. Network eligible molecules were also combined into canonical pathways that maximize connectivity.

Specifically, datasets containing significant differentially expressed genes and miRNAs (fold changes) for the three comparisons were uploaded into the Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) knowledge base. Separate core analyses were performed using miRNA and total mRNA datasets. The miRWalk (previously known as Argonuate) algorithm was used to predict the association of miRNAs with gene targets (291). Molecules from each dataset that met the 1.5 fold change cut-off and were associated with biological functions and/or diseases in the Ingenuity's Knowledge Base were used to generate networks. Right-tailed Fisher's exact test was used to calculate p-values determining the probability that biological functions and/or diseases assigned to each data set were not due to chance alone. For a general overview of this analysis, refer to **Figure 6(B).**

In the third approach, we examined the global influence of miRNA differential expression across the various diet/carcinogen treatments using cumulative distribution function (CDF) plots of fold change for putative mRNA target lists. For each comparison group (i.e., Tumor/Saline, CA/FA, and CCA/FPA), both polysomal and total mRNA expression data sets were created using appropriate samples (rows=probes, columns=rats). Each data set was individually median and quantile normalized using only rows containing all "good" probe readings. Average probe values for each of the two treatments in the data set were computed, and then used as expression data (e.g., probe fold change between treatments would be the ratio of these averages). Based on a previous analysis, differentially expressed miRNAs were identified for each comparison group, and for each miRNA, mRNAs were classified according to Target Scan by

conserved 8mer, conserved 7mer types + non-conserved 8mer and 7mer types, and "other". These groupings roughly correspond to "strong", "weak", and "non" targets (287). Distributions of fold change between treatments for each of these groups were estimated using those mRNAs that are available in the data set. A comparison of the empirical fold change distributions of conserved 8mer targets and "non" targets was made for selected miRNA's of known interest using the Kolmogorov-Smirnov test, and p-values and their associated q-values (false discovery rate levels) were computed.

In the final approach, we performed linear discriminant analysis (LDA) classification using miRNAs as features to discriminate between the conditions described in the comparisons (A), (B), and (C). For this analysis, the main goal was to determine if miRNA expression can be used to discriminate between the different experimental treatments: Tumor vs saline (T vs S), corn oil+AOM vs fish oil+AOM (CA vs FA) and corn oil+cellulose+AOM vs fish oil+pectin+AOM (CCA vs FPA). We have previously used a linear classifier algorithm for feature set identification (292, 293). For the purpose of identifying feature sets, we designed classifiers that categorize samples based on the expression values of the miRNAs from the intersection of the miRNAs altered in tumor versus (not altered) saline. Classifiers for miRNAs feature sets of sizes 1, 2, and 3 were identified. Generally, there are two reasons why it is desirable to design classifiers involving small numbers of features: (a) the limited number of samples often available in clinical studies makes classifier design and error estimation problematic for large feature sets (294) and (b) small miRNAs sets facilitate design of practical diagnostic panels. For similar reasons, simple classifiers are

preferable for small samples; indeed, for small samples, if good classification is possible, then a simple classifier such as linear discriminant analysis (LDA) using a small number of miRNAs will typically outperform a complex classifier (295).

Given a set of features on which to base a classifier, one has to address not only the classifier design from sample data but also the estimation of its error, i.e., the precision with which the error of the designed classifier estimates the error of the optimal classifier. When data are limited, an error estimator may have a large variance and therefore may often be low even if it is approximately unbiased. This can produce many feature sets and classifiers with low error estimates. The algorithm used in this study mitigates this problem by applying the bolstered error estimation (296). Basically, this approach "bolsters" the original empirical distribution of the available data by means of suitable bolstering kernels placed at each data point location. The error can be computed analytically in some cases, such as in the case of LDA. The result of the overall approach is a list of "best" feature sets from among all possible feature sets. Hence, the best feature set is the one possessing minimum classification error. Because we only have data and not the underlying feature-label distributions, the errors have been estimated from the data. This approach takes into account that, in small-sample settings, we do not have much confidence in any single feature set and that it is much more likely that, if there is an adequate sized collection of good performing feature sets, then there are likely to be some that perform well on the overall population (295).

The general overview of the analysis pipeline is shown in **Figure 6**

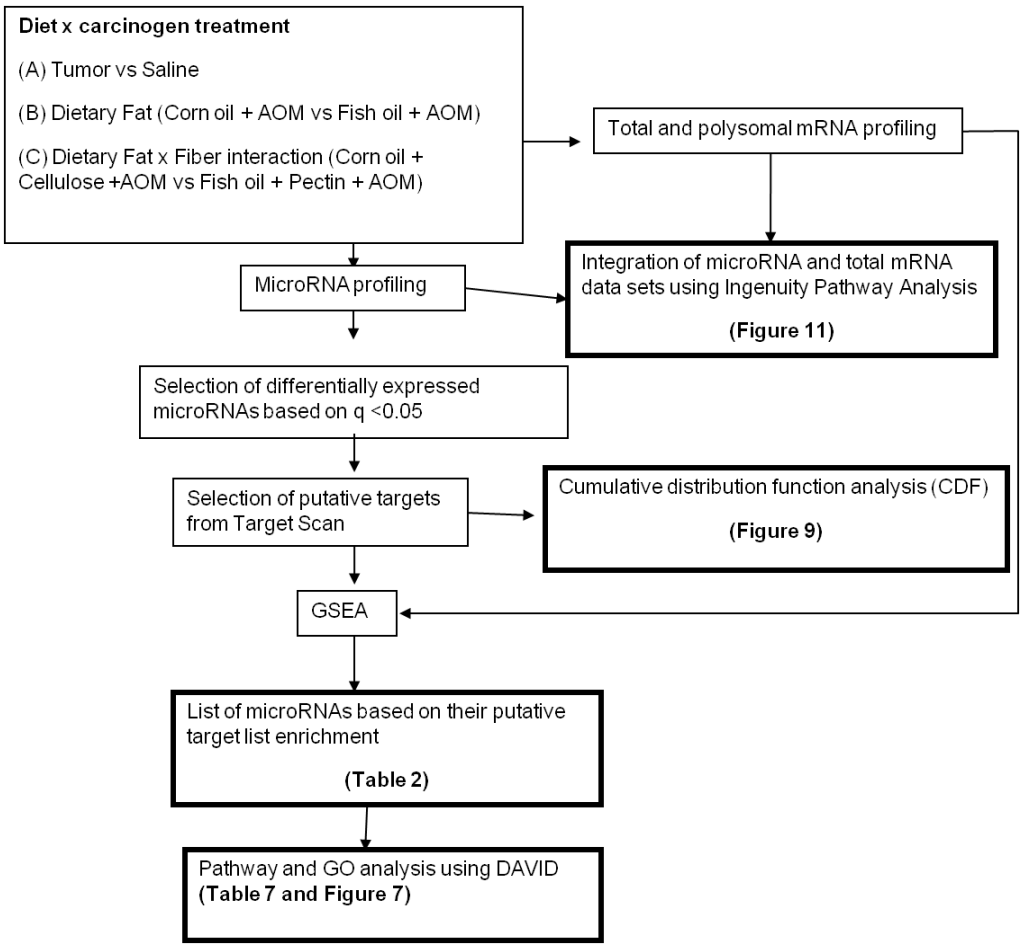


Figure 6. Overview of analysis pipeline. (A) Selection of enriched miRNAs based on the significant enrichment of their target mRNAs using GSEA.

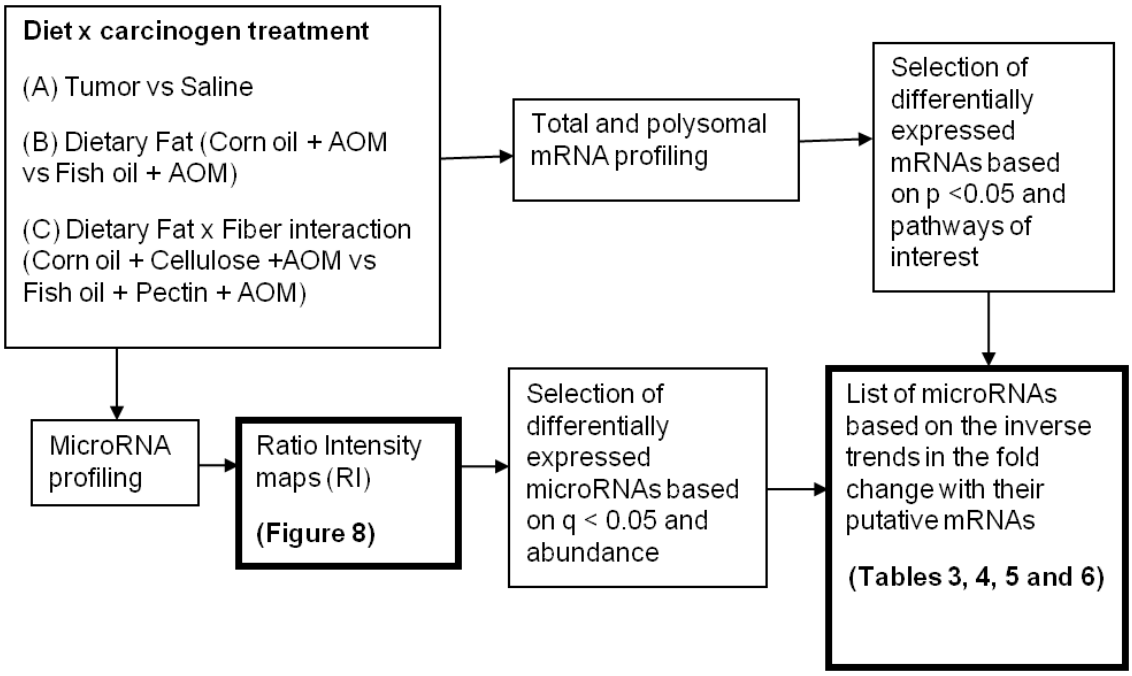


Figure 6. Continued. (B). Selection of microRNAs based on coherent relationship with their target mRNAs.

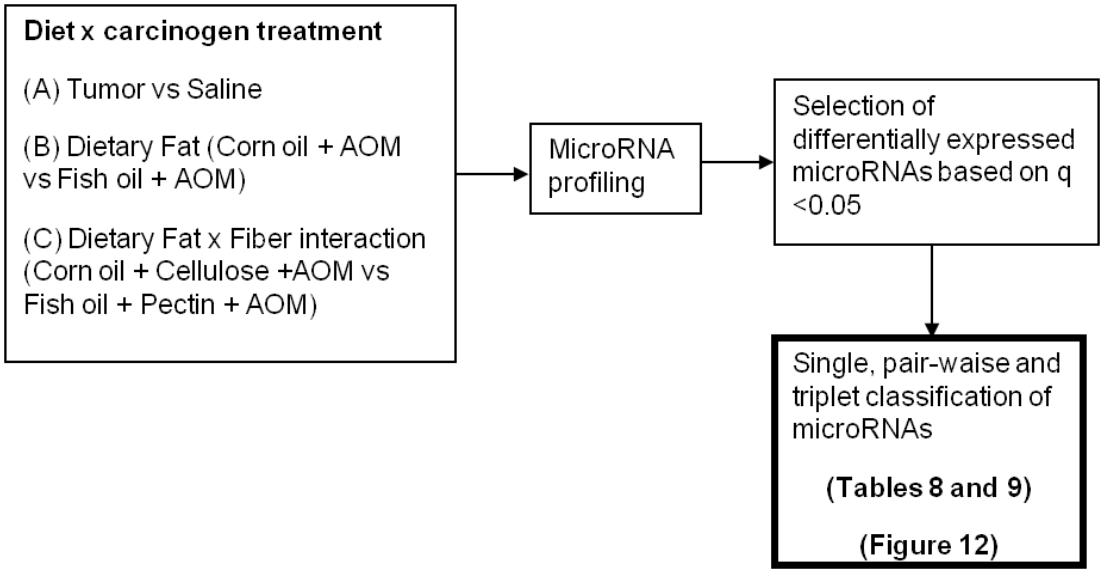


Figure 6. Continued**.** (C) Linear Discriminant Analysis (LDA) for classification of microRNAs

3.2.7 Immunoblotting

To generate colonic protein extracts, the mucosa was scraped and homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 100 mM sodium orthovanadate, 1% Triton X-100, 100 μM activated sodium orthovanadate, 40 μL/mL protease cocktail and 10 mM β-mercaptoethanol). Samples were then processed and subjected to polyacrylamide gel electrophoresis in precast 4-20% Tris-glycine mini gels (Invitrogen, CA). After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (PVDF) at 400 mA for 120 min. After the transfer, the membrane was then incubated in blocking solution (5% nonfat dry milk and 0.1% Tween 20 in PBS) at room temperature for 1 h with shaking. Following blocking, the membrane was incubated overnight at 4^0C with rabbit TCF4 at 1:1000 dilution (Cell Signaling Technology, MA), rabbit PDE4B at 1:200 dilution (Abcam, MA), rabbit PTK2B at 1:200 dilution (Santa Cruz Biotechnology, CA), rabbit IGF1R 1:200 dilution (Santa Cruz Biotechnology, CA) or mouse β-actin at 1:8000 dilution (Sigma-Aldrich, MO). Membranes were washed with PBS containing 0.1% Tween 20 and incubated with peroxidase conjugated anti-rabbit IgG secondary antibody at 1:8000 dilution (Jackson Immuno Research, PA) or goat anti-mouse at 1:30,000 (Kirkegaard & Perry, Gaithersburg, MD) as per manufacturer's instructions. Bands were then developed using Pierce SuperSignal West Femto maximum sensitivity substrate and imaged and quantified with a Fluor-S Max MultiImager system (Bio-Rad, Hercules, CA). β-actin was used as a loading control.

The effect of two independent variables (treatment effects) was assessed using Student's t-test. The graphs were plotted using means and standard error of values obtained from quantifying the immunoblot bands from five samples for each diet group. Standard error bars were plotted in order to document the variation in the population mean. *P*-values < 0.05 were considered to be statistically independent . The graphs were plotted using means and standard error of five different samples for each treatment. Standard error bars were plotted in order to document the variation in the population mean.

3.3 Results

3.3.1 miR-34a, miR-190, miR-193a, miR-214 and miR-215 are enriched based on target (total and polysomal) mRNA genome-wide expression analysis in carcinogen treated rats

We have recently demonstrated that several colonic miRNAs are significantly altered in carcinogen (AOM) compared to saline treated rats (140). To further elucidate the regulation of these miRNAs, we determined whether they targeted total mRNA and/or polysome mRNAs. Utilizing a computational approach, Gene Set Enrichment Analysis (GSEA), which incorporates knowledge of known gene networks/pathways and identifies significantly enriched miRNAs based on the change in expression of their putative targets, we identified five enriched miRNAs, miR-34a, miR-190, miR-193a, miR-214, miR-215, out of 46 candidate miRNAs **(Table 2(A))** in tumor versus saline treated rats. Examples of enrichment plots are shown in **Figure 7**. These non-coding

RNAs were significantly enriched based on the change in expression of their total and polysomal mRNA targets. By plotting the data as a ratio intensity (RI) profile map, miR-34a, miR-190, miR-193a, miR-214 and miR-215 were shown to be highly abundant in the colon **(Figure 8(A)).**

3.3.2 Diet influences miRNA enrichment in carcinogen injected rats

GSEA analysis was also used to further elucidate the impact of diet and carcinogen treatment on miRNAs. With respect to the dietary lipid source comparison in the presence of carcinogen (CA vs FA), five miRNAs (miR-132, miR-146b, miR-192, miR-206, miR-218) were significantly enriched based on the change in the expression of their total and polysomal mRNA targets **(Table 2(B))**. Of these, only miR-146b was highly abundant **(Figure 8(B)).** With regard to the effect of dietary fat x fiber interaction in the presence of carcinogen (CCA vs FPA), five miRNAs (miR-19a, miR-93, miR-98, miR-130b, miR-206) were significantly enriched based on the change in expression of their total and polysomal mRNA targets **(Table 2(C))**. In addition, three miRNAs (miR-26b, miR-30b, miR-206) were significantly enriched based on the change in the expression of their actively translated (polysome) mRNA targets **(Table 2(C))**. Of these, miR-26b and miR-30b were shown to be highly abundant in the colon **(Figure 8(C))**. Collectively, GSEA identified 16 miRNAs which were significantly enriched in three comparisons **(Table 2)**, and nine miRNAs targeted polysomal mRNA. These data indicate that both total and polysomal mRNAs are important for prediction of miRNA regulation with respect to experimental colon carcinogenesis. Interestingly, miR-206 was significantly enriched in both the dietary comparisons.

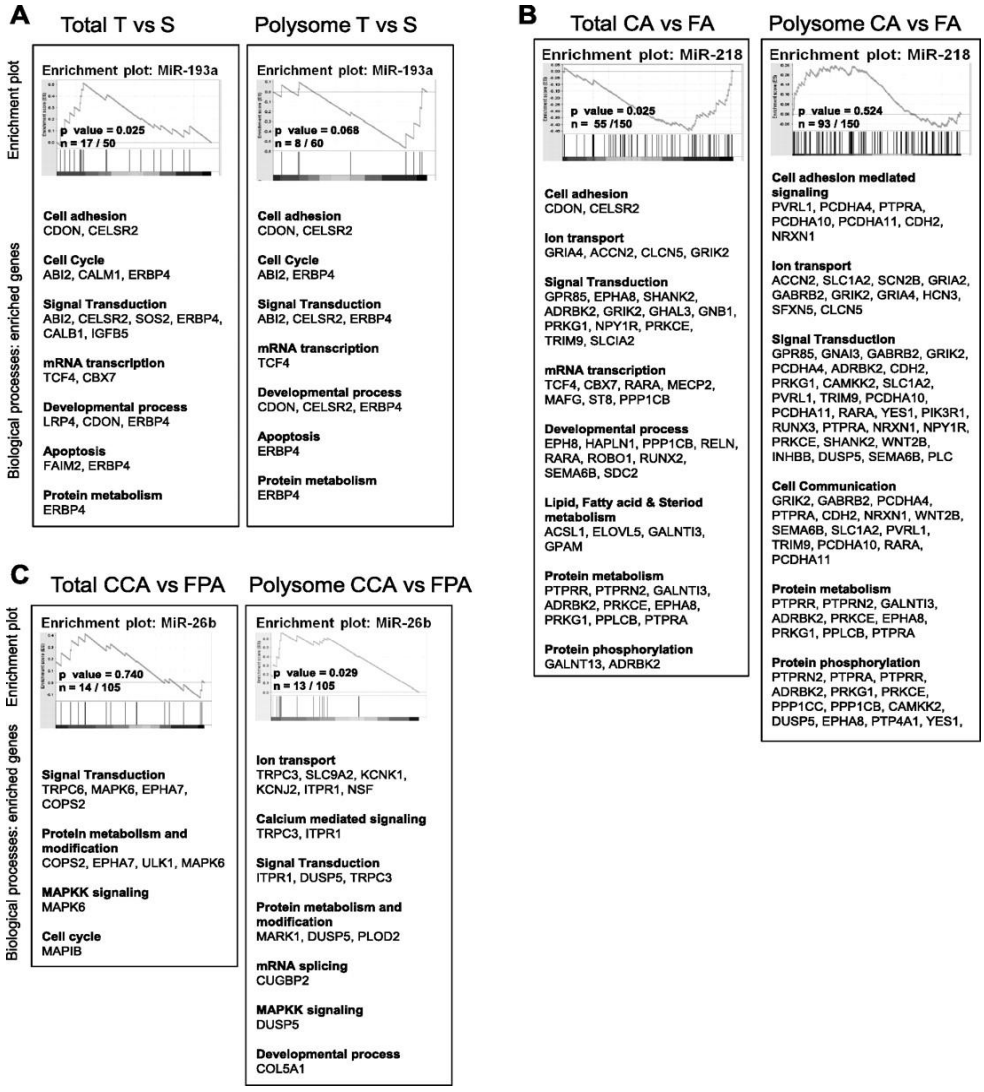


Figure 7. Enrichment plots of microRNAs in total vs. polysome mRNA datasets. Enrichment plots of microRNAs in total vs. polysome mRNA datasets. Polysome and total mRNA enrichment plots are shown for the three comparisons: tumor (T) vs. saline (S), corn oil + azoxymethane (CA) vs. fish oil + azoxymethane (FA) and corn oil + cellulose + azoxymethane (CCA) vs. fish oil + pectin + azoxymethane (FPA). Horizontal bars in graded color from left to right represent mRNA targets ranked from high expression in tumor vs. saline (A), dietary fat effects (B), and fat × fiber (C) interactions. At bottom of each panel is shown the biological processes in which the enriched genes play a role. The vertical gray lines represent the projection onto the ranked gene list of the targets for each microRNA (T over S, CA over FA, and CCA over FPA). The top curve corresponds to the calculation of the enrichment score (ES). The horizontal line indicates the 0 value for the ES. The greater the enrichment curve is shifted to the upper left of the graph, the higher the gene set enrichment in the T, CA, and CCA treatments. P values for the ES scores are shown in the ES plots along with the number of significantly enriched genes.

Table 2. Summary of colonic microRNAs significantly enriched using GSEA. Data represent significantly enriched microRNAs based on the differential expression of their putative targets in either total or polysomal mRNA datasets for the three comparisons: (i) Tumor (T) vs saline (S), (ii) corn oil+AOM (CA) vs fish oil+AOM (FA), and (iii) corn oil+cellulose+AOM (CCA) vs fish oil+pectin+AOM (FPA). Size, indicates the number of differentially expressed mRNA putative targets; ES, enrichment score; NES, normalized enrichment score.

(A) Tumor vs Saline Treatments

Total / Polysomal	microRNA	Size	ES	NES	p-value
Total T	miR-193a	17	0.511	1.688	0.025
Total T	miR-190	13	-0.545	-1.600	0.035
Poly T	miR-214	23	-0.482	-1.689	0.014
Poly T	miR-34a	13	-0.516	-1.591	0.022
Poly T	miR-215	1	-0.965	-1.312	0.029

(B) Corn oil/AOM vs Fish oil/AOM Treatments

Total / Polysomal	microRNA	Size	ES	NES	p-value
Total CA	miR-132	15	0.450	1.519	0.035
Poly CA	miR-218	55	-0.466	-1.592	0.030
Poly CA	miR-146b	2	-0.980	-1.517	0.008
Poly CA	miR-206	3	-0.809	-1.470	0.032
Poly CA	miR-192	9	-0.602	-1.463	0.047

(C) Corn oil/cellulose/AOM vs Fish oil/pectin/AOM Treatments

Total / Polysomal	microRNA	SIZE	ES	NES	p-value
Total CCA	miR-206	8	0.675	1.650	0.017
Total CCA	miR-93	17	0.512	1.549	0.029
Total CCA	miR-130b	25	0.440	1.485	0.046
Total CCA	miR-19a	109	0.329	1.398	0.028
Total FPA	miR-98	6	0.786	1.692	0.015
Poly CCA	miR-26b	13	0.674	1.559	0.029
Poly FPA	miR-206	4	-0.768	-1.480	0.029
Poly FPA	miR-30b	3	-0.830	-1.438	0.034

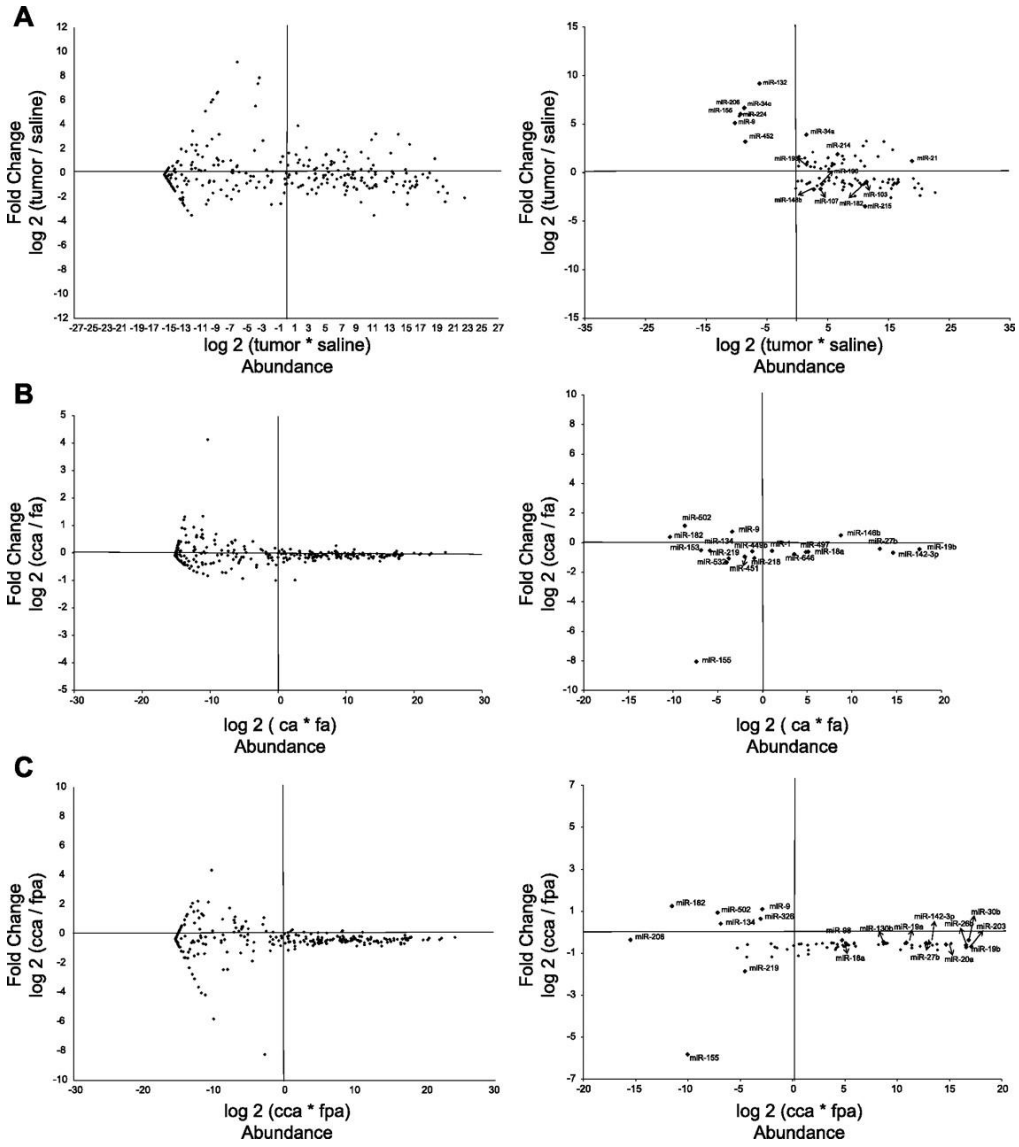


Figure 8. Ratio intensity (RI) profile maps for colonic microRNAs from rats fed specific diets and injected with carcinogen or saline (control). Ratio intensity (RI) profile maps for colonic microRNAs from rats fed specific diets and injected with carcinogen or saline (control). Treatment effects on the expression of microRNAs are shown. The x-axis shows the intensity (abundance) of each microRNA, and the y-axis shows the fold change across treatments. MicroRNA expression is normalized to RNU6B. Each graph is divided into 4 quadrants where the top right quadrant contains those microRNAs that are highly abundant and exhibit a high fold change. For each comparison, there are 2 graphs: left, documenting all 384 microRNAs, and right, showing selected microRNAs with a fold change >1.3 and <0.7.

3.3.3 Global assessment of miRNA targets using cumulative distribution function analysis

Using Target Scan, the putative targets of differentially expressed miRNAs were partitioned based on the miRNA-mRNA seed pairing, or lack thereof, in the 3' untranslated region (UTR). For each diet/carcinogen comparison, and for both total and polysomal mRNA, we compared CDF plots of fold change for each subset of the partitioned mRNA targets. This provides a visual examination of the global effects of colonic miRNA differential expression for the various diet/carcinogen comparisons. Three relevant examples, miR-214 for tumor vs saline, miR-18a for corn oil+AOM vs fish oil+AOM and miR-19a for corn oil+cellulose+AOM vs fish oil+pectin+AOM, are shown in **Figure 9**. For the majority of miRNAs, the lengths of the partitioned target lists were not sufficiently large to allow for statistically detectable differences between the associated CDFs. Overall, however, the qualitative visual representation of global miRNA provided from the CDF analysis was confirmatory of our GSEA findings.

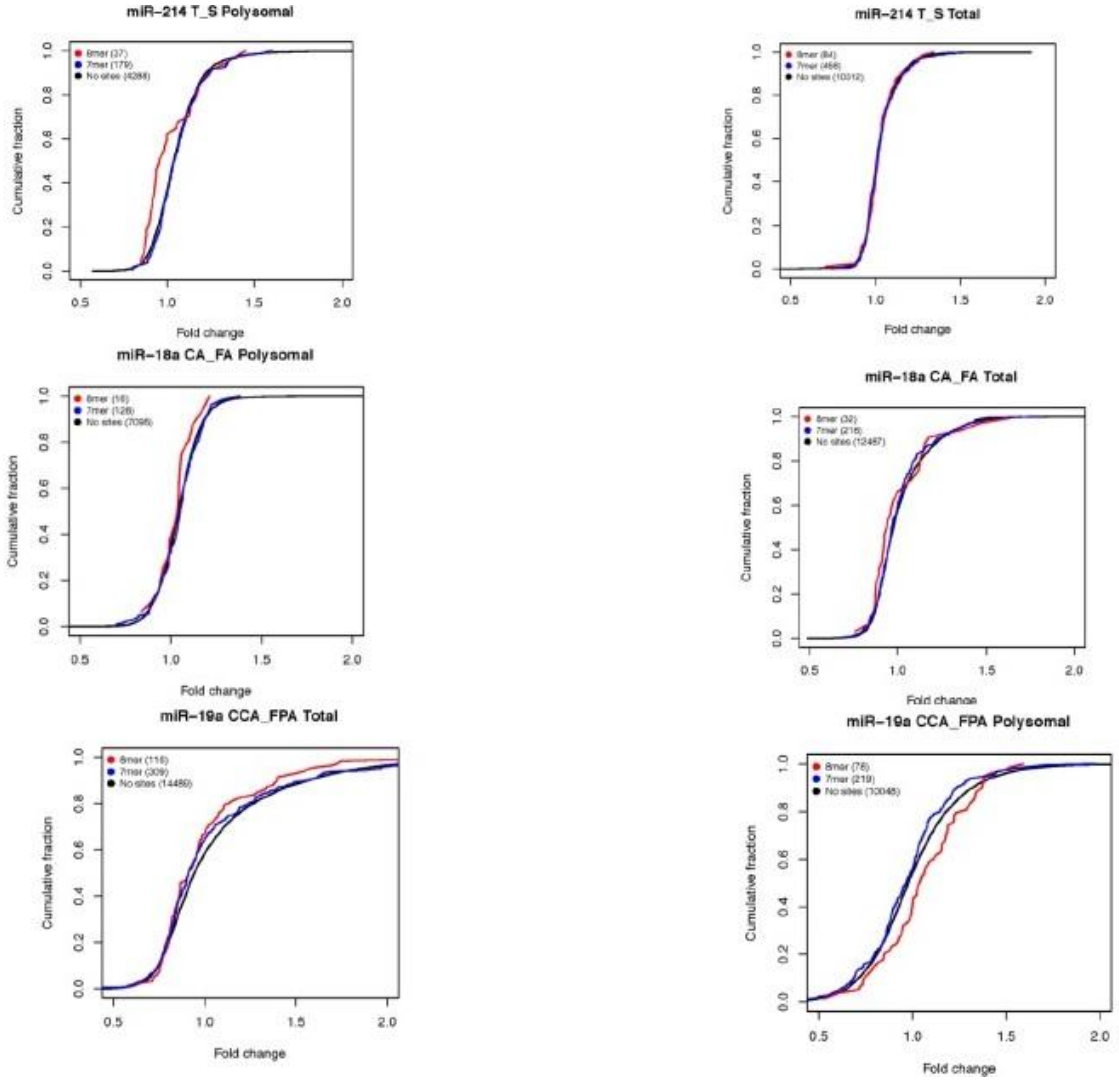


Figure 9. Cumulative distribution function analysis(CDF). CDFs represent the fold changes for putative miRNA target lists portioned by 8mer(red), 7mer(blue) and no miRNA-mRNA(black) seed pairing in 3' UTR. CDFs for total and polysomal mRNA are shown for miR-214 in tumor vs saline comparison, miR-18a in the corn oil+AOM vs fish oil+AOM comparison and miR-19a in the corn oil+cellulose+AOM vs fish oil+pectin+AOM grouping.

3.3.4 Identification of coherent colonic miRNA-mRNA modules

miRNAs have been shown to have an inverse relationship (coherent) with their mRNA targets (276). In order to infer the relative activity of colonic miRNAs, we examined expression changes in total and polysome mRNA target transcripts. For (A) T vs S, (B) CA vs FA and (C) CCA vs FPA treatment comparisons, miRNAs were selected based on three criteria: (i) significant fold change **(Tables 3 & 4),** (ii) high abundance **(Figure 8)**, and (iii) a shared coherent relationship (demonstrating inverse trends based on fold change) with their mRNA targets **(Tables 3 & 4).** With the aid of the prediction algorithm, Target Scan, we identified a subset of miRNAs whose levels were inversely altered relative to their putative mRNA targets **(Tables 3, 4 & 5).** With respect to total and polysomal mRNA data, we selected only those mRNAs which satisfied the above criteria. In the case of carcinogen treated animals (T vs S), miR-15b, miR-16, miR-103, miR-107, miR-141, miR-146b, miR-148b, miR-183, miR-193a, miR-195, miR-204 and miR-497 exhibited coherent responses relative to their putative targets **(Table 3).** Furthermore, miR-15b, miR-16, miR-103 and miR-107, all target RASSF5; while mir-141, miR-183 and miR-204 target TCF12; both of which are linked to the total mRNA datasets. miR-103 and miR-107 cluster together and are part of one miRNA family. In contrast, from the polysome mRNA dataset, YWHAB can be targeted by miR-148b; SLC11A2 is a putative target of both miR-195 and miR-497 (miR-195 cluster), FLl1 and GR1D1 can be targeted by miR-193 and miR-146b, respectively. With respect to the effect of dietary lipid source (CA vs FA), mir-18a, miR-19b, miR-27b, mir-93 and miR-497 exhibited a coherent response at the total and polysomal mRNA levels **(Table 4)**.

With regard to fat x fiber interaction in the presence of carcinogen (CCA vs FPA), miR-19b, miR-26b and miR-203 were inversely associated with the total mRNA expression levels of PTK2B, IGF2R, PDE4B and TCF4, respectively **(Table 4).** In order to further assess the biological processes impacted by carcinogen and diet, gene ontology analysis was carried out and the top terms for the GO categories for each of the miRNA target gene sets was determined **(Table 7).** The fold changes and significance of the miRNA target gene sets are listed in **Table 5.** We also processed protein samples from carcinogen-exposed animals fed corn oil+cellulose (control) or fish oil+pectin (chemoprotective) diets to determine if some of the predicted targets were altered at the protein level. Specifically, we examined PTK2B and PDE4B, both of which are predicted targets of miR-19b **(Table 4)**; TCF4, a predicted target of miR-203 **(Table 4);** and IGF1R, a predicted target of miR-19b **(Table 4).** As expected, based on mRNA data, PTK2B, PDE4B2 and TCF4 were upregulated by ~2-fold in CCA as compared to FPA treatment **(Figure 10).** In contrast, no change was observed in IGF1R levels.

Table 3. Selection of colonic microRNAs based on the inverse trend in fold change of putative targets in tumor vs saline treated rats. MicroRNAs were selected based on three criteria: (i) a significant fold-change, (ii) high abundance, and (iii) inverse association with putative targets of microRNAs.

Tumor vs Saline Effects

Treat-ment	miRNA	Fold-change miRNA	p-value miRNA	Significa-nt target genes	Fold-change target genes	p-value target genes	Pathway	miRNA family/ cluster
Total T-S	**miR-103**	0.51	<0.001	RASSF5	1.32	0.023	Apoptosis	miR-103/miR107 family
Total T-S	**miR-107**	0.27	<0.001	RASSF5	1.32	0.023	Apoptosis	miR-103/miR107 family
Total T-S	**miR-15b**	0.60	<0.001	RASSF5	1.32	0.023	Apoptosis	miR-15b-miR 16-2 cluster
Total T-S	**miR-16**	0.69	<0.001	RASSF5	1.32	0.023	Apoptosis	miR-15b-miR 16-2 cluster
Total T-S	**miR-183**	0.68	0.002	TCF12	1.45	0.048	Wnt signaling pathway	miR-96/182/183 cluster
Total T-S	**miR-204**	0.29	<0.001	TCF12	1.45	0.048	Wnt signaling pathway	miR-204 family
Total T-S	**miR-141**	0.32	<0.001	TCF12	1,45	0.048	Wnt signaling pathway	miR-200c/141 cluster
Poly T-S	**miR-148b**	0.44	<0.001	YWHAB	1.14	0.037	Apoptosis, Cell cycle signaling	miR-148/152 family
Poly T-S	**miR-195**	1.83	<0.001	SLC11A2	0.68	0.034	Intestinal iron absorption	miR-195 cluster
Poly T-S	**miR-497**	1.92	<0.001	SLC11A2	0.68	0.034	Intestinal iron absorption	miR-195 cluster
Poly T-S	**miR-193a**	2.46	0.001	FLI1	0.64	0.001	Wnt signaling pathway	miR-193 family
Poly T-S	**miR-146b**	9.17	<0.001	GRID1	0.54	0.001	Glutamic acid signaling	miR-146 family

Table 4. Diet effects on colonic microRNAs and their inversely associated gene targets in carcinogen-injected rats. Refer to Table 2 for legend details.

Corn Oil vs Fish Oil Effects

Treatment	miRNA	Fold-change miRNA	p-value miRNA	Significant target genes	Fold-change target genes	p-value target genes	Pathway	miRNA family/ cluster
Total CA-FA	**miR-19b**	0.45	<0.001	IGF1R	1.48	<0.001	Apoptosis	miR-17-92 cluster
Total CA-FA	**miR-19b**	0.45	<0.001	RUNX3	1.34	0.041	Runt-related transcription factor 3	miR-17-92 cluster
Total CA-FA	**miR-27b**	0.75	0.043	ATP2B1	1.42	0.024	Stem cell pathway	miR-23b/27b/24-1 cluster
Total CA-FA	**miR-27b**	0.75	0.043	LIMK2	1.23	0.032	Actin cytoskeleton reorganization	miR-23b-24-1 cluster
Total CA-FA	**miR-27b**	0.75	0.043	PARD6B	1.21	0.044	Asymmetrical cell division and cell polarization	miR-23b-24-1 cluster
Total CA-FA	**miR-27b**	0.75	0.043	ZADH2	1.22	0.017	Metabolic processes (oxidation reduction)	miR-23b-24-1 cluster
Total CA-FA	**miR-497**	0.64	<0.001	CASR	1.37	0.050	Calcium homeostatsis	miR-195 cluster
Total CA-FA	**miR-93**	0.76	<0.001	NPAS2	1.23	0.024	Signal transduction	miR-106b-25 cluster
Poly CA-FA	**miR-18a**	0.65	<0.001	LAT	1.37	0.016	Ras protein signal transduction	miR-17-92 cluster
Poly CA-FA	**miR-27b**	0.75	0.043	SLC6A6	1.35	<0.001	Amino acid transporter	miR-23b-24-1 cluster
Poly CA-FA	**miR-27b**	0.75	0.043	GATA2	1.17	0.045	Transcription regulation	miR-23b-24-1 cluster
Poly CA-FA	**miR-27b**	0.75	0.043	ATP2B1	1.42	0.024	Stem cell pathway	miR-23b-24-1 cluster

Table 4. Continued

Fat x Fiber Effects								
Treatment	**miRNA**	**Fold-change miRNA**	**p-value miRNA**	**Significant target genes**	**Fold-change target genes**	**p-value target genes**	**Pathway**	**miRNA family/ cluster**
Total CCA-FPA	**miR-19b**	0.64	0.035	PTK2B	1.772	0.032	Cell proliferation	miR-17-92 cluster
Total CCA-FPA	**miR-19b**	0.64	0.035	IGF2R	1.664	0.034	Apoptosis	miR-17-92 cluster
Total CCA-FPA	**miR-26b**	0.65	<0.001	PDE4B	2.43	0.050	Apoptosis	miR-26 family
Total CCA-FPA	**miR-203**	0.61	<0.001	TCF4	2.16	0.043	Apoptosis	—
Total CCA-FPA	**miR-27b**	0.75	0.047	ATP2B1	1.42	0.025	Stem cell pathway	miR-23b-24-1 cluster

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Table 5. Fold changes and significance of microRNA target gene sets in CCA vs FPA comparison. Only the highly abundant microRNAs (qPCR Ct value < 30) are included.

Total CCA vs FPA

microRNA	Fold Change	p-value	Significant target genes	Fold change	p-value
miR-98	0.77	0.043	SLC35D2	1.37	0.015
miR-98	0.77	0.043	IGF1R	1.67	0.009
miR-107	0.69	0.016	GALNT7	1.35	0.014
miR-107	0.69	0.016	ANKFY1	1.19	0.035
miR-107	0.69	0.016	ACVR2B	1.26	0.047
miR-107	0.69	0.016	RASSF5	1.45	0.041
miR-107	0.69	0.016	NEDD9	1.47	0.043
miR-107	0.69	0.016	YWHAH	1.14	0.049
miR-107	0.69	0.016	HTR4	1.46	0.048
miR-130b	0.72	0.014	EPS15	1.25	<0.001
miR-130b	0.72	0.014	SMAD5	1.57	0.022
miR-130b	0.72	0.014	SNX5	1.13	0.024
miR-130b	0.72	0.014	AKAP11	1.25	0.022
miR-130b	0.72	0.014	PTPRM	1.39	0.046
miR-130b	0.72	0.014	USP33	1.28	0.047
miR-130b	0.72	0.014	UBL3	1.24	0.055
miR-15b	0.72	0.027	IGF1R	1.67	0.009
miR-15b	0.72	0.027	ZADH2	1.37	0.016
miR-15b	0.72	0.027	SMAD5	1.57	0.022
miR-15b	0.72	0.027	AP1GBP1	1.29	0.031
miR-15b	0.72	0.027	GRIN1	1.56	0.043
miR-15b	0.72	0.027	GNA12	1.33	0.048
miR-15b	0.72	0.027	DNAJA2	1.33	0.056
miR-195	0.57	0.006	BTG2	1.45	0.049
miR-497	0.62	0.001	OSBPL7	1.35	0.034
miR-16	0.74	0.008	GORASP2	1.3	0.049
miR-20a	0.66	0.002	EZH1	1.42	0.023
miR-20a	0.66	0.002	RIPK5	1.51	0.031
miR-20a	0.66	0.002	BNIP2	1.49	0.038
miR-20a	0.66	0.002	NAGK	1.23	0.042
miR-20a	0.66	0.002	CXADR	1.21	0.051
miR-93	0.65	<0.001	PTGFRN	1.23	0.012
miR-93	0.65	<0.001	NPAS2	1.47	0.014
miR-93	0.65	<0.001	HDAC4	1.51	0.022
miR-93	0.65	<0.001	NEDD4L	1.16	0.057
miR-18a	0.65	0.015	PTGFRN	1.23	0.012
miR-18a	0.65	0.015	NOTCH2	1.45	0.013

Table 5. Continued

microRNA	Fold Change	p-value	Significant target genes	Fold change	p-value
miR-18a	0.65	0.015	NCOA1	1.32	0.011
miR-18a	0.65	0.015	LAT	1.55	0.018
miR-18a	0.65	0.015	WBP2	1.33	0.019
miR-18a	0.65	0.015	TMEM1	1.20	0.017
miR-18a	0.65	0.015	AEBP2	1.19	0.024
miR-18a	0.65	0.015	FNBP1	1.23	0.033
miR-18a	0.65	0.015	NEDD9	1.47	0.043
miR-19b	0.63	<0.001	GDA	1.26	0.011
miR-19b	0.63	<0.001	WBP2	1.33	0.022

Polysomal CCA vs FPA

microRNA	Fold Change	p-value	Significant target genes	Fold change	p-value
miR-130b	0.72	0.014	AKAP1	4.04	0.012
miR-130b	0.72	0.014	MTF1	1.52	0.013
miR-15b	0.72	0.027	GRID1	1.18	0.057
miR-15b	0.72	0.027	SYNJ1	1.49	0.048
miR-15b	0.72	0.027	SMAD5	1.25	0.046
miR-15b	0.72	0.027	GGA3	1.40	0.044
miR-15b	0.72	0.027	CYP26B1	1.52	0.042
miR-15b	0.72	0.027	CDS2	3.55	0.038
miR-15b	0.72	0.027	LYPLA2	1.58	0.031
miR-15b	0.72	0.027	CASR	1.71	0.022
miR-15b	0.72	0.027	DLGAP2	1.23	0.011
miR-15b	0.72	0.027	EIF4E	1.97	0.012
miR-15b	0.72	0.027	ABCG4	1.95	0.008
miR-15b	0.72	0.027	BCL9L	1.67	<0.001
miR-15b	0.72	0.027	IHPK1	1.56	<0.001
miR-16	0.74	0.008	ADRB2	1.37	0.043
miR-16	0.74	0.008	CDC42EP2	1.21	0.012
miR-16	0.74	0.008	RET	1.82	0.006
miR-497	0.62	0.001	LPHN1	2.04	0.034
miR-93	0.65	<0.001	PPP2R2A	1.41	0.046
miR-93	0.65	<0.001	EGR2	1.45	0.041
miR-93	0.65	<0.001	SLC9A2	1.4	0.022
miR20a	0.66	0.002	RIPK5	1.62	0.034
miR20a	0.66	0.002	MTF1	1.52	0.013

Table 5. Continued

microRNA	Fold Change	p-value	Significant target genes	Fold change	p-value
miR20a	0.66	0.002	SMOC2	1.66	0.007
miR-182	0.74	0.013	GRID1	1.18	0.057
miR-182	0.74	0.013	ACCN1	1.42	0.046
miR-182	0.74	0.013	NCAM1	1.37	0.036
miR-182	0.74	0.013	PLCG1	1.24	0.032
miR-182	0.74	0.013	LPHN1	2.04	0.034
miR-182	0.74	0.013	FOXO3	1.29	0.018
miR-182	0.74	0.013	SLC9A2	1.4	0.022
miR-182	0.74	0.013	PPP1R9B	1.39	0.013
miR-182	0.74	0.013	DLGAP2	1.23	0.011
miR-182	0.74	0.013	CDC42BPA	1.28	0.011
miR-182	0.74	0.013	TGFBI	1.34	0.008
miR-182	0.74	0.013	STK10	1.39	0.012
miR-183	0.74	0.009	SMPD3	2.1	0.037
miR-183	0.74	0.009	SLC6A6	1.64	0.012
miR-183	0.74	0.009	CX3CL1	1.49	0.008
miR-18a	0.65	0.015	MTF1	1.52	0.013
miR-18a	0.65	0.015	LAT	1.43	0.013
miR-18a	0.65	0.015	XYTLT2	1.46	0.002
miR-19b	0.63	<0.001	MAPK6	1.6	0.033
miR-196b	0.70	0.002	HABP4	1.34	0.042

Together, these data indicate that 25 highly abundant miRNAs exhibited a significant ($p<0.05$) inverse relationship with their putative polysome and total mRNA targets following diet and carcinogen exposure **(Table 3, 4 & 5).** Furthermore, miRNAs which were not highly abundant (low expressors), miR-219 and miR-9, also exhibited a significant ($p<0.05$) inverse relationship with their putative total and polysomal mRNA targets **(Table 6).**

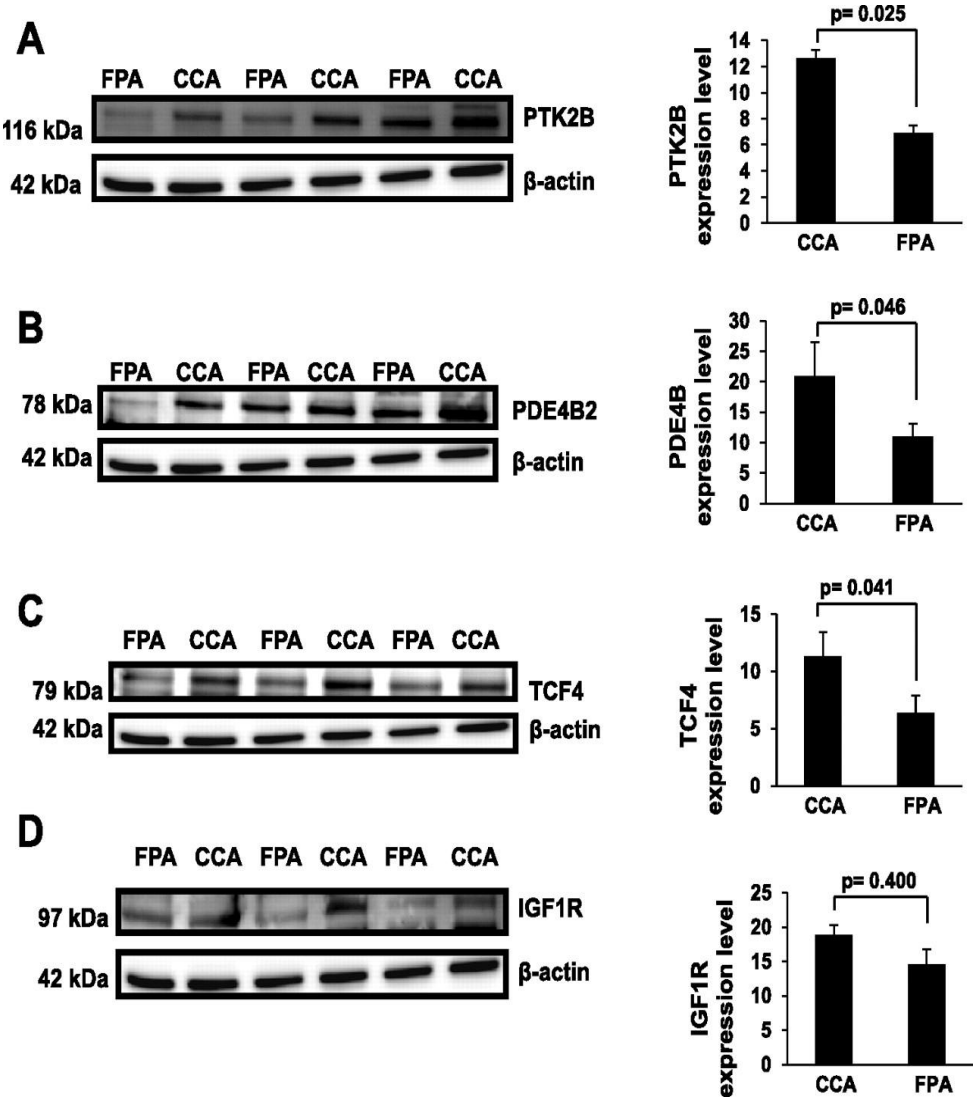

Figure 10. Suppression of PTK2B, PDE4B, and TCF4 in rats fed a chemoprotective diet. Suppression of PTK2B, PDE4B, and TCF4 in rats fed a chemoprotective diet. Expression of PTK2B (A), PDE4B (B), TCF4 (C), and IGF1R (D) in carcinogen-injected rats fed diets containing either (control) corn oil-cellulose or (chemoprotective) fish oil-pectin was analyzed by immunoblotting, n = 5 animals per treatment.

Table 6. Fold changes and significance of microRNA target gene sets in CCA vs FPA comparison. Only the low abundance microRNAs (qPCR Ct value > 30) are included.

Total CCA vs FPA

microRNA	Fold Change	p-value	Significant target genes	Fold change	p-value
miR-219	0.44	0.008	NCOA1	1.32	0.011
miR-219	0.44	0.008	DAZAP1	1.32	0.047
miR-9	1.68	<0.001	AAK1	0.60	<0.001
miR-9	1.68	<0.001	PAK6	0.59	0.042
miR-9	1.68	<0.001	NMT2	0.63	0.047
miR-9	1.68	<0.001	UHRF1	0.59	0.046
miR-9	1.68	<0.001	DIO2	0.39	0.058

Polysomal CCA vs FPA

microRNAs	Fold Change	p-value	Significant target genes	Fold change	p-value
miR-219	0.44	0.008	PIP5K1C	1.62	0.044
miR-219	0.44	0.008	GTPBP1	1.25	0.03
miR-9	1.68	<0.001	ARFGEF2	0.61	0.048
miR-9	1.68	<0.001	CPEB4	0.61	0.036
miR-9	1.68	<0.001	PGRMC2	0.69	0.028
miR-9	1.68	<0.001	CCNG1	0.77	0.019
miR-9	1.68	<0.001	STK3	0.69	0.018
miR-9	1.68	<0.001	CNOT6L	0.60	0.017
miR-9	1.68	<0.001	SHC1	0.69	0.015
miR-9	1.68	<0.001	TLK1	0.74	0.009
miR-9	1.68	<0.001	SYNJ2BP	0.61	0.007
miR-9	1.68	<0.001	EIF5	0.65	0.001
miR-9	1.68	<0.001	XPO4	0.74	<0.001

Table 7. Association of direct microRNA targets with biological processes in the fat x fiber x carcinogen comparison. GO analysis was performed to identify the enrichment of biological processes associated with the putative targets**.**

microRNA	ID	Term	p-value
miR-101	GO:0006468	protein amino acid phosphorylation	0.011
	GO:0016310	phosphorylation	0.016
	GO:0045859	regulation of protein kinase activity	0.018
	GO:0051338	regulation of transferase activity	0.022
	GO:0006796	phosphate metabolic process	0.025
	GO:0006793	phosphorus metabolic process	0.025
	GO:0010033	response to organic substance	0.032
	GO:0044237	cellular metabolic process	0.034
	GO:0042325	regulation of phosphorylation	0.038
	GO:0043687	post-translational protein modification	0.042
miR-107	GO:0007165	signal transduction	0.008
	GO:0007242	intracellular signaling cascade	0.029
	GO:0010817	regulation of hormone levels	0.049
	GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.099
miR-140	GO:0048468	cell development	0.004
	GO:0016043	cellular component organization	0.013
	GO:0044238	primary metabolic process	0.016
	GO:0043170	macromolecule metabolic process	0.030
	GO:0019538	protein metabolic process	0.030
	GO:0008152	metabolic process	0.035
	GO:0030154	cell differentiation	0.038
	GO:0048869	cellular developmental process	0.042
miR-15b	GO:0030154	cell differentiation	0.006
	GO:0048869	cellular developmental process	0.006
	GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0.013
	GO:0010557	positive regulation of macromolecule biosynthetic process	0.014
	GO:0031328	positive regulation of cellular biosynthetic process	0.015
	GO:0009891	positive regulation of biosynthetic process	0.016
	GO:0007166	cell surface receptor linked signal transduction	0.029

Table 7. Continued

microRNA	ID	Term	p-value
	GO:0022604	regulation of cell morphogenesis	0.040
	GO:0022414	reproductive process	0.016
miR-93	GO:0048519	negative regulation of biological process	0.044
miR-18a	GO:0010556	regulation of macromolecule biosynthetic process	0.023
	GO:0010468	regulation of gene expression	0.024

3.3.5 Functional analysis of differentially expressed miRNAs

To explore the functional relevance of select miRNA species and predicted targets in the context of colorectal cancer, miRNA and total mRNA data were analyzed using Ingenuity interactive pathway analysis (Ingenuity Systems, www.ingenuity.com) software. With respect to the effect of dietary fish oil treatment on carcinogen-injected rats, the top associated ($p<0.05$) networks were NFAT, adenocarcinoma, Wnt/beta-catenin signaling, phospholipase C and notch signaling. Moreover, *fatty acid metabolism* in mitochondria was the top ranked canonical pathway. With respect to the *fat x fiber* comparison, p38 Mapk signaling, RhoA signaling, and notch signaling genes were modulated ($p<0.03$). In addition, Wnt/beta-catenin, carcinoma, and phospholipase C were the top ranked associated network functions ($p<0.01$). For the dietary lipid (CA vs FA) comparison, fish oil feeding downregulated miRNAs -miR-18a, miR-19b, miR-27b and miR-93 targeted several differentially expressed targets which are involved in pathways related to colorectal cancer, e.g., ERK-MAPK, Wnt/β-catenin, PTEN and

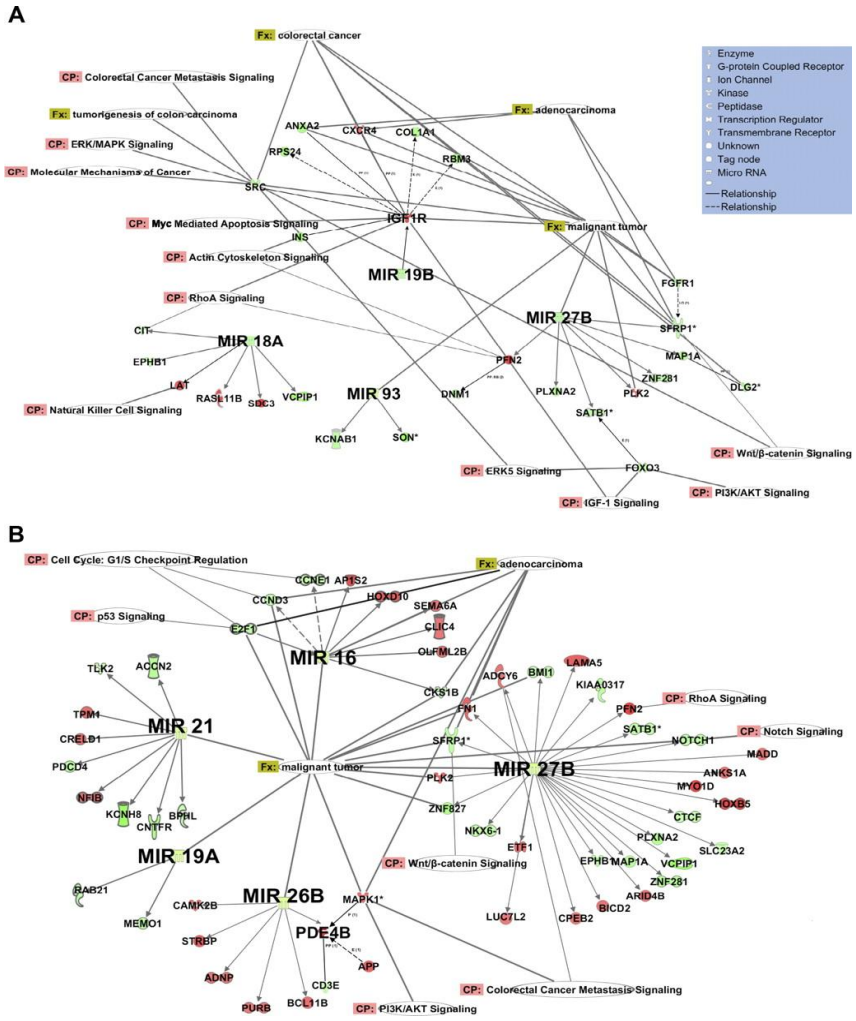

Figure 11. Functional analyses of differentially expressed predicted targets of significantly altered microRNAs. Functional analyses of differentially expressed predicted targets of significantly altered microRNAs. A: microRNAs miR-18a, miR-19b, miR-27b, and miR-93 were downregulated in CA vs. FA treatments (expression of CA divided by expression in FA). B: microRNAs- miR-16, miR-21, miR-26b, and miR-27b were downregulated in the CCA vs. FPA (expression of CCA divided by expression in FPA treatments). Their differentially expressed predicted targets (by mirWalk) are also shown. Signaling networks identified by IPA software are based on significant fold changes of the differentially expressed microRNAs. The intensity of the node (gene/microRNA) color indicates the degree of upregulation (red) or downregulation (green). Nodes are displayed using various shapes that represent the functional class of the gene product. Nodes without any color were not present in the input dataset but were present as a part of the network. Solid lines between genes/microRNAs represent a direct interaction based on experimental proven associations. Dashed line represent predicted interactions (indirect interactions) based on experimental evidence. Fx, functions and diseases; CP, canonical pathway. Symbols describing cell functions are shown in A.

apoptosis **(Figure 11(A))**. These miRNAs also shared a coherent relationship with their predicted total mRNA targets **(Table 4)**. Moreover, the association of miR-19b and IGF1R shown by IPA analysis was also observed in the coherent analysis **(Table 4)**. Up regulation of IGF1R can lead to downregulation of genes such as SRC, INS, COL1A1, CXCR4, all of which have been associated with colorectal cancer (297, 298)(25, 61). With regard to fat x fiber interaction in the presence of carcinogen (CCA vs FPA), fish oil-pectin combination downregulated miR-16, miR-21, miR-26b and miR-27b, which targeted several differentially expressed genes associated with adenocarcinomas, colorectal cancer, mTOR signaling, PI3K/AKT signaling, and apoptosis **(Figure 11(B))**. Fat x fiber effects on miR-26b and miR-27b were also observed in GSEA and coherent analyses **(Tables 2 & 4)**.

3.3.6 miRNA expression and treatment classification

For the purpose of classifying colon tumor development using miRNAs, we applied linear discriminant analysis (LDA). The number of miRNAs (features) for each linear classifier was limited to three, which allowed for an exhaustive search while avoiding errors associated with small-sample setting feature extraction. Using this approach, the top ten best single, 2- and 3-miRNA classifiers were queried as a means to distinguish phenotype; (i) T vs S, (ii) CA vs FA, and (iii) CCA vs FPA. We noted several cases where single miRNAs can provide good (in terms of the error estimate) classification **(Tables 8 & 9).** As expected, when considering these features as part of 2- or 3-gene classifiers, a significant decrease in the classification error was noted

Table 8. Classification of colonic microRNAs altered in tumors vs saline treated rats. Top 10 single, pair-wise and triplet-wise microRNA LDA classifiers are shown. $\varepsilon_{bolstered}$ denotes bolstered resubstitution error for the respective classifier. The classifiers are ranked according to the error measurement. $\Delta(\varepsilon_{bolstered})$ denotes the decrease in error for each feature set relative to its highest ranked subset of features.

Tumor vs Saline Effects

microRNA			$\varepsilon_{bolstered}$	$\Delta(\varepsilon_{bolstered})$
miR-21			0.001	
let-7c			0.001	
miR-200c			0.001	
miR-215			0.001	
miR-200a			0.001	
miR-30c			0.001	
miR-30e-3p			0.001	
miR-30b			0.001	
miR-422a			0.001	
miR-200b			0.020	
let-7g	miR-21		<0.001	<0.001
let-7a	miR-200c		<0.001	<0.001
let-7a	miR-215		<0.001	<0.001
let-7a	miR-200b		<0.001	<0.001
let-7c	miR-141		<0.001	<0.001
let-7c	miR-200c		<0.001	<0.001
let-7c	miR-215		<0.001	<0.001
let-7c	miR-200a		<0.001	<0.001
let-7c	miR-30c		<0.001	<0.001
let-7c	miR-30b		<0.001	0.020
let-7a	let-7c	miR-30c	<0.001	<0.001
let-7a	let-7c	miR-30b	<0.001	<0.001
let-7a	miR-21	miR-215	<0.001	<0.001
let-7a	let-7c	miR-215	<0.001	<0.001
let-7a	let-7c	miR-200c	<0.001	<0.001
let-7a	let-7c	miR-375	<0.001	<0.001
let-7a	miR-141	miR-30b	<0.001	<0.001
let-7a	let-7b	miR-200c	<0.001	<0.001
let-7a	let-7b	miR-215	<0.001	<0.001
let-7a	let-7b	miR-30b	<0.001	<0.001

Table 9. Classification of microRNAs altered by dietary effects in carcinogen treated rats. Top 10 single, pair-wise and triplet-wise microRNA LDA classifiers are shown. Refer to Table 4 for legend details.

Corn Oil vs Fish Oil Effects

microRNA			$\varepsilon_{bolstered}$	$\Delta(\varepsilon_{bolstered})$
miR-19b			0.133	
miR-93			0.152	
miR-497			0.181	
miR-18a			0.218	
miR-101			0.238	
miR-532			0.240	
miR-199b			0.266	
miR-146b			0.268	
miR-92			0.277	
miR-106b			0.285	
miR-19b	miR-21		0.117	0.016
miR-19b	miR-15b		0.137	0.014
miR-19b	miR-93		0.138	0.042
miR-19b	miR-30c		0.147	0.071
miR-18a	miR-497		0.154	0.084
miR-19b	miR-92		0.154	0.085
miR-93	miR-25		0.157	0.108
miR-93	miR-15b		0.157	0.110
miR-19b	miR-106b		0.158	0.119
miR-93	miR-106b		0.161	0.124
miR-19b	miR-21	miR-25	0.107	0.010
miR-19b	miR-22	miR-15b	0.109	0.027
miR-19b	miR-23	miR-92	0.110	0.028
miR-19b	miR-24	miR-106b	0.112	0.034
miR-19b	miR-25	miR-30c	0.126	0.028
miR-19b	miR-30c	miR-15b	0.132	0.021
miR-19b	miR-21	miR-93	0.136	0.021
miR-93	miR-324-5p	miR-15b	0.140	0.017
miR-93	miR-106b	miR-15b	0.140	0.018
miR-19b	miR-93	miR-106b	0.141	0.020

Table 9. Continued

Corn Oil Cellulose vs Fish Oil Pectin Effects

microRNA			$\varepsilon_{bolstered}$	$\Delta(\varepsilon_{bolstered})$
miR-27b			<0.001	
miR-30d			0.015	
miR-20a			0.018	
miR-200c			0.033	
miR-11			0.037	
miR-26b			0.048	
miR-93			0.060	
miR-30e-3p			0.066	
miR-195			0.069	
miR-196b			0.072	
miR-200c	miR-26a		<0.001	<0.001
miR-27b	miR-30e-3p		0.001	0.014
miR-20a	miR-30d		0.001	0.017
miR-200c	miR-203		0.001	0.031
miR-27b	miR-182		0.003	0.033
miR-30d	miR-196b		0.004	0.044
miR-146a	miR-27b		0.004	0.055
miR-27b	miR-186		0.004	0.061
miR-301	miR-20a		0.005	0.064
miR-20a	miR-26b		0.005	0.066
miR-146a	miR-27b	miR-30e-3p	<0.001	<0.001
miR-200c	miR-20a	miR-203	<0.001	<0.001
miR-200c	miR-26a	miR-148a	<0.001	0.001
miR-200c	miR-26a	miR-203	<0.001	0.001
miR-200c	miR-26a	miR-15b	<0.001	0.003
miR-200c	miR-203	miR-25	<0.001	0.004
miR-17-3p	miR-20a	miR-30d	<0.001	0.004
miR-107	miR-20a	miR-30d	<0.001	0.004
miR-200c	miR-26a	miR-200b	<0.001	0.004
miR-200c	miR-203	miR-182	<0.001	0.005

(Table 9). To identify sets of miRNAs that perform in a multivariate manner to provide strong classification, we specifically looked for pairs of miRNAs that performed better

than either of the miRNAs individually, and also triplets of miRNAs that performed well and substantially better than the best-performing pair among the three. To estimate the improvements of the classification performance, we introduced two quantities for each feature set: $\varepsilon_{bolstered}$ and $\Delta(\varepsilon_{bolstered})$. $\varepsilon_{bolstered}$ denotes the bolstered resubstitution error for the LDA classifier for the respective feature set of size *n (n = 1, 2, 3)*, and $\Delta(\varepsilon_{bolstered})$ denotes the decrease in error with respect to the highest ranked of its subset of features (in the list of features of size *n-1, n = 2, 3)*. The feature sets were ranked based on the value of $\varepsilon_{bolstered}$. The top T vs S (control) single-gene classifier (one-feature), miR-21 (ranked 1st in the list of single-miR classifiers) had an estimated classification error of 0.001. Interestingly, when this miRNA was combined with a poorly performing single-miRNA classifier, let-7g (ranked > 20 in the list of single-gene classifiers), it resulted in a two-non-coding gene classifier with a low estimated error, < 0.001 **(Table 9).** In addition, the let-7a, miR-141 and miR-375 triple classifier exhibited unique properties, as all three miRNAs performed poorly as single classifiers (ranked >15 in the list of single-gene classifiers). This shows that when combined, these miRNAs increase the power of classification with a low estimated error, <0.001 **(Table 8).** Moreover, let-7a and let-7c appeared frequently in the top 10 triple classifiers. For example, let-7 family members, let-7a and let-7c, performed well as classification features when combined with miR-215 and miR-21 **(Table 8)**, well known colon cancer miRNAs (4, 67). Interestingly, miR-215 was also significantly enriched as assessed by GSEA **(Table 2).** miR-141, found to share a coherent relationship with its putative

targets, also classified with the let-7 family. In summary, these miRNAs performed poorly as single classifiers, but when combined, they decreased the classification error **(Figures 12(A) &(B)).**

With respect to the classification of the n-3 PUFA-enriched chemoprotective diet, comparison of the FA vs CA treatments revealed that miR-19b, miR-21 and miR-93 appeared at the top of the single, double and triple classifier lists **(Table 9, Figures 12(C) & (D))**. These data combined with the fact that miR-19b and miR-93 exhibited a significant coherent relationship with their putative targets **(Table 4)**; suggest an important role for these non-coding RNA. In addition, miR-106b when combined with miR-93 and miR-19b produced a classifier with a low estimated error. With regard to the fat x fiber interaction, for the CCA vs FPA comparison, miR-200c appeared repeatedly in the single $\varepsilon_{bolstered}$ = (0.033), double (0.001) and triple (< 0.001) LDA classifier lists **(Table 9, Figures 12(E) & (F)).** In addition, miR-93 and miR-182 exhibited significant coherent relationships with their putative targets **(Table 4).**

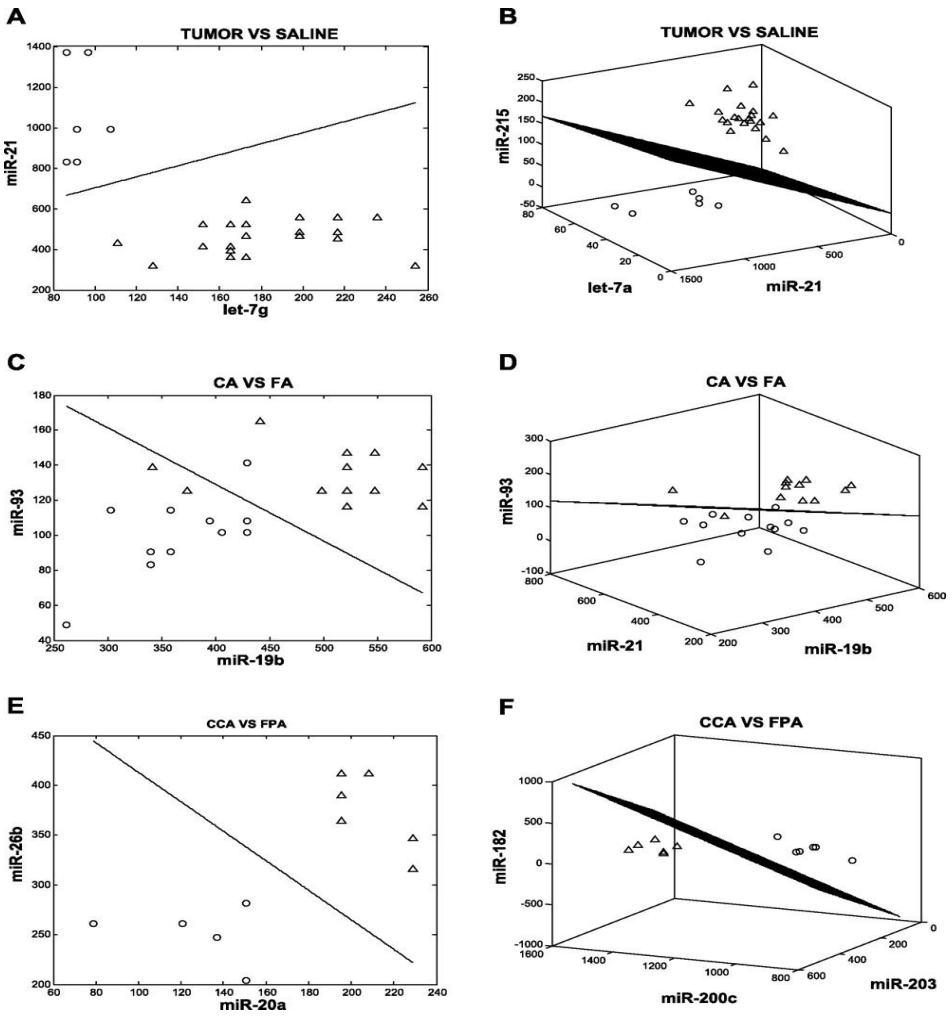

Figure 12. Linear discriminant analysis phenotype classification using microRNAs. Linear discriminant analysis phenotype classification using microRNAs. Panels represent examples of the top 2- and 3-microRNA classifiers. For example, tumor vs. saline comparison, miR-21 and let-7g (A), corn oil + AOM vs. fish oil + AOM comparison, miR-93 and miR-19b (C), and corn oil + cellulose + AOM vs. fish oil + pectin + AOM comparison, miR-26b and miR-20a (E) are the best performing 2-microRNA feature sets. Classification is between tumor, corn oil, corn oil cellulose (○) and saline, fish oil, fish oil pectin (△)-treated rats (refer to Tables 4 and 5 for additional details). The axes represent the normalized intensity values of the indicated microRNAs. Note the clear separation between the 2 groups in each comparison. The best-performing 3-microRNA feature sets are shown: tumor vs. saline comparison, miR-215, miR-21, and let-7a (B), corn oil + AOM vs. fish oil + AOM comparison miR-21, miR-93, and miR-19b (D), and corn oil + cellulose + AOM vs. fish oil + pectin + AOM comparison, miR-182, miR-200c, and miR-203 (F). The 3-dimensional LDA hyperplane discriminates between the tumor, corn oil, corn oil cellulose (○)- and saline, fish oil, fish oil pectin (△)-treated rats.

3.4 Discussion

We have recently demonstrated that n-3 PUFA uniquely modulate carcinogen-directed non-coding miRNA signatures in the colon (140). In this study, in order to further elucidate the global biological effects of chemoprotective diets on miRNAs, we used an integrated global approach to assess total and polysomal mRNA targets. With regard to the computational methodology to predict target genes of miRNAs and infer functionality, we systematically assessed the complex regulatory factors mediating miRNA–mRNA interaction. The large-scale prediction of targets across the whole genome allowed us to achieve a higher degree of specificity of prediction and to infer the regulatory activities of miRNAs in the colon. Indeed, a number of studies have utilized statistical/systems biology methodologies to identify miRNAs which may target genes in response to a pathophysiological state (299-301). However, there is a paucity of integrative miRNA and mRNA expression studies which focus on early stage colon cancer development and chemoprevention. Since mammalian miRNAs predominantly act to decrease target mRNA levels (276), our analyses focused on total and polysomal mRNA targets during the early promotional phase of malignant transformation.

We utilized four complementary approaches to elucidate the global effects of diet and carcinogen on miRNAs and total/polysomal mRNA profiles within the colon. Initially, GSEA was used to probe the effects of three biological conditions: (A) carcinogen (tumor versus saline), (B) dietary fat effects in the presence of carcinogen (CA versus FA) and (C) dietary fat x fiber interaction in the presence of carcinogen (CCA vs FPA). We subsequently identified 18 miRNAs which were significantly

enriched in the three treatments based on the change in the expression of total or polysomal mRNA targets. Out of 18 miRNAs, eight were significantly enriched, as assessed by their total mRNA targets, while nine miRNAs were significantly enriched with respect to their polysomal mRNA targets. In addition, miR-206 was significantly enriched with respect to both its polysomal and total mRNAs targets. These data suggest that polysome trafficking of transcripts and miRNAs plays an important role in experimental colon carcinogenesis. The notion that miRNAs localize to polyribosomes is consistent with the translational down-regulation of specific mRNAs (302, 303). We argue, therefore, that studies employing polysome purification/ribosome profiling and microarray analysis are needed in order to fully elucidate the mechanisms of translational deregulation associated with colon tumor development.

For the purpose of inferring the relative activity of each miRNA, we also examined the expression level of target transcripts, assuming that if the target mRNA is down-regulated by treatment, then the miRNA activity is likely to be enhanced. With the aid of miRNA-mRNA target prediction algorithms, Target Scan and DIANA-microT, we identified the miRNA-mRNA coherent pairs using only those miRNAs which were highly abundant, significantly altered and shared a coherent relationship with their mRNA targets. We avoided using the intersection of overlapping computational algorithms because enrichment is weak at best (285, 304). In addition, Target Scan performs well in predicting targets for miRNAs (305, 306). With respect to carcinogen effects on colonic miRNAs, miR-15b, miR-16, miR-107, miR-141, miR-204 have been also shown to be down regulated in human colon cancer (18, 49, 77, 86).

Although there is no literature regarding the association of miR-146 and miR-148b with colorectal cancer, they are altered in CNS tumor derived cell lines (307)and thyroid tumors (308).

Consistent with the inverse trend of putative targets in carcinogen-modulated miRNAs **(Table 3)**, miR-15b/miR-16 and miR-107 have been linked with RASSF5 (Ras-Association Domain Family) in other cancers (309, 310). RASSF5 is a member of the RASSF family, which has been shown to be a tumor suppressor and is pro-apoptotic (311, 312). Our data also suggest that TCF12, which is up-regulated in tumor samples, may be targeted by miR-141, miR-183, and miR-204. TCF12 is a transcription factor abundantly expressed in Paneth cells of adult small intestinal crypts (313). Interestingly, the activation of TCF12 may play a role in Paneth cell differentiation in colonic neoplasms (314). Therefore, TCF12 targeting by miR-141, mir-183 and miR-204 may be associated in mediating the differentiation of the adult stem cells and disruption in this mechanism may contribute to colonic neoplasia. Recently, it has been reported that miR-204 represses SIRT1 whose expression is regulated during embryonic stem cell differentiation (315). We also noted a down-regulation of FLI1 and up-regulation of its targeting miR-193a, which may participate in tumor progression by activating β-catenin transcription (316). Next, we applied linear discriminant analysis (LDA) to classify treatment effects on colonic miRNAs. The number of gene features for each linear classifier was limited to 3, which allowed for an exhaustive search. Using this technique, a number of miRNAs were strong single classifiers, including miR-21, a well-known oncomir in colon cancer (138). In contrast, several miRNAs did not classify

well, however, when paired with other poor single classifier), the pair-wise combination was strong with low estimated errors.

With respect to the effect of dietary lipid source (CA vs FA), miR-18a, mR-19b, miR-27b, miR-93 and miR-497 were down-regulated in corn oil+ AOM compared to fish oil+AOM treated rats, reaffirming that n-3 PUFA modulate the effect of carcinogen with respect to both miRNA and target gene levels (140). With respect to disease progression, several studies have reported the bivalent role for the miR-17-92 cluster, i.e., it has both tumor suppressor and oncogenic properties (317-319). miR-18a and miR-19b are a part of the miR-17-92 cluster, and repression of mR-18a leads to an increase in cell proliferation and promotes anchorage independent growth in HT-29 colon adenocarcinoma cell line (320). Interestingly, even though miR-18a has been shown to be up-regulated in several cancers, studies have indicated that the expression level of the members of miR-17-92 cluster depends on the cell type (321-323). Furthermore, miR-19b was reported to be down-regulated in Crohn's disease (324). Other notable observations include miR-27b, which shared a coherent relationship with total mRNAs ATP2B1, LIMK2, PARD6B, ZADH2 and polysomal mRNAs SLC6A6, GATA2 and ATP2B1. Interestingly, n-3 PUFA fed animals injected with carcinogen exhibited an increase in miR-19b levels and decreased IGF1R levels which would favorably modulate apoptosis and cell cycle activity within the colon. These findings are consistent with previous reports that fish oil blocks AOM-induced colon tumorigenesis by increasing apoptosis and suppressing cell proliferation (50, 244). Overall, the data are noteworthy, because in the absence of comprehensive human data,

the AOM chemical carcinogenesis model serves as a highly relevant means of assessing human colon cancer initiation and progression (281, 325).

With regard to dietary combination chemotherapy, i.e., fat x fiber interaction in the presence of carcinogen (CCA vs FPA), we observed a coherent relationship between miR-15b, miR-16, miR-18a, mir-19b, miR-20a, miR-26b, miR-27b, miR-93, miR-98, miR-107, miR-130b, miR-182, miR-183, miR-195, miR-196b, miR-203, and miR-497, and their respective mRNA targets **(Table 5)**. Of these, miR-19b, mir-26b, miR-27b and miR-203 showed a strong coherent response with respect to total mRNAs, PTK2B, IGF2R, PDE4B, ATP2B1 and TCF4, respectively. All four miRNAs were down-regulated in corn oil+cellulose+AOM (CCA) compared to fish oil+pectin+AOM (FPA) treated rats. Further, we examined a subset of these targets at the protein level and verified that PTK2B, PDE4B2 and TCF4 were also upregulated by ~2-fold in CCA as compared to FPA treatment, while the expression of their miRNAs were downregulated **(Figure 10).** This is noteworthy, because TCF4 is a well-known transcription factor involved in Wnt signaling, and mutations in TCF4 are linked to colon cancer development (326). We have previously demonstrated that colonic β-catenin signaling, an upstream mediator of TCF4, is suppressed in fish oil/pectin fed, AOM injected rats (247). Therefore, fish oil+pectin is capable of blocking the AOM-mediated down-regulation of miR-203, thereby suppressing TCF4 and preventing the further subversion of Wnt signaling. PTK2B protein tyrosine kinase, a predicted target of miR-19b has been shown to be involved in several signaling cascades and is overexpressed in malignant gastrointestinal stromal tumors (327). The level of PTK2B was upregulated

in CCA as compared to FPA at both the mRNA and protein level. PDE4B2 (variant of PDE4B) which is a predicted target of miR-26b, was also up-regulated in CCA animals. This phosphodiesterase limits cAMP-associated apoptosis in a number of cancers (328, 329). In the integrative computational analysis, we also noted that IGF1R and IGF2R were predicted targets of miR-19b. Interestingly, there was no change in IGF1R at the protein level in CCA vs FPA comparison. Collectively, these findings support the claim that pleiotropic bioactive components generated by fermentable fiber (*butyrate*) and fish oil (*DHA* and *EPA*) work coordinately to protect against colon tumorogenesis (46, 58).

In summary, we have documented the combined effects of dietary bioactive agents and carcinogen on miRNA and mRNA expression profiles in the rat colon. The fact that global miRNA expression patterns in human and rat AOM-induced tumors are similar (140), support the utility of this model. Four complementary computational approaches were utilized to demonstrate that miRNAs and their putative mRNA targets, i.e., both total mRNAs and actively translated mRNA transcripts in polyribosome complexes, are differentially modulated by carcinogen and chemoprotective diets. Furthermore, immunoblot analyses were carried out and oncogenic targets of miR-19b, miR-26b and miR-203 were down-regulated by the chemoprotective fish oil+pectin containing diet. These miRNAs will be the focus of future experiments involving knockdown and overexpression of strategies. We also conclude that polysomal profiling is tightly correlated with miRNA changes when compared with total mRNA profiling. To our knowledge, this represents the first integrated analysis of miRNA and mRNA expression at an early stage of colon cancer development.

4. IDENTIFICATION OF miR-26B AND miR-203 GENE TARGETS IN COLON CANCER CELLS

4.1 Introduction

miRNAs are a diverse class of noncoding RNAs involved in the regulation of several key cellular processes such as signal transduction, apoptosis, cell proliferation, morphogenesis and the pathogenesis of cancer (1, 2). Structurally, these are 19-24 nucleotide RNAs which are first transcribed as long primary miRNA, and then processed into 60-70nt miRNA precursor (pre-miRNA) by nuclear RNAse III Drosha. The pre-miRNA complex is then transported to the cytoplasm where it is further processed by Dicer into a miRNA duplex. This miRNA duplex is then taken up by the RISC complex, and generally the guiding strand then binds to complementary sequences at the 3' UTR of the mRNA and thus blocks translation of mRNA into proteins (2, 3).

A plethora of studies have demonstrated that miRNAs and their targets are aberrantly expressed in colon cancer (63-67). For example, expression of miR-21, miR-143 and miR-145 have been shown to be differentially expressed in human colonic tumors as compared to normal colon. These miRNAs have been shown to target several key signal transduction pathways that directly modulate malignant transformation (107, 109, 110).

Over several decades, there is substantial experimental, epidemiological and clinical evidence indicating that fish oil containing diets, rich in n-3 polyunsaturated fatty acids (PUFAs), e.g. docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA),

are protective against colon tumorigenesis (45-48). It is likely that EPA and DHA function through multiple pathways to decrease colon cancer risk, including modulation of cyclooxygenase (COX) activity, changes in cellular oxidative stress, modification of gene expression and alterations in membrane dynamics and cellular surface receptor function. Work from our laboratory has demonstrated a role for n-3 PUFA in increasing the level of oxidative stress in colonocytes to activate apoptosis (330, 331). We have also demonstrated fundamental differences in gene expression profiles in rats fed a fish oil enriched diet compared to corn oil or olive oil enriched diets (50). In addition, we have demonstrated that the chemoprotective effect of fish oil is enhanced when a highly fermentable fiber (pectin), capable of elevating colonic luminal butyrate levels, is added to the diet (59, 244, 248, 332).

Recently, we examined the effects of colon carcinogenesis on non-coding RNAs in a rat model by cataloguing mucosal miRNA expression following diet and carcinogen exposure (140). For this purpose, global miRNA and target mRNA expression analyses were carried out. We demonstrated that the unique combination of chemoprotective dietary fish oil (containing EPA and DHA) plus pectin (fermented to butyrate in the large intestine) modulated a subset of mucosal miRNAs-miR-19b, miR-26b and miR-203 and their predicted target genes -IGF1R, PTK2B, PDE4B and TCF4. Specifically, miR-19b, miR-26b and miR-203 were significantly downregulated by the corn oil cellulose diet in presence of carcinogen (CCA) as compared to fish oil pectin diet in the presence of carcinogen (FPA), whereas their predicted targets PTK2B, IGF1R, PDE4B and TCF4 were inversely upregulated. Immunoblotting analyses further revealed that the

targets were also modulated at the protein level (151). It is noteworthy that these diet modulated miRNAs are also aberrantly expressed in colon tumorigenesis and other types of cancers (333).

To validate the predicted miRNA:mRNA associations observed in our earlier study (151), we conducted a series of loss and gain of function analyses by knocking down or overexpressing select miRNAs. The global abundance of protein levels in the human colon cancer cell line (HCT116) model was subsequently assessed. As shown below **(Table 10)** , 100% homology exists between human (hsa), mouse (mmu) and rat (rno) for miR-19b, miR-26b and miR-203. The sequences in **Table 10** were obtained from miRBase database (www.mirbase.org/). The accession number corresponding to each miRNA is noted in the parenthesis (334).

Table 10. Alignment of miR-19b, miR-26b and miR-203 sequences found in human , mouse and rat. The sequences of miR-19b, miR-26b and miR-203 were obtained from miRBase database (334) in the three species. Hsa, human; mmu, mouse; rno, rat.

(1) miR-19b:

(MIMAT0000074) hsa: UGUGCAAAUCCAUGCAAAACUGA
(MIMAT0000513) mmu: UGUGCAAAUCCAUGCAAAACUGA-miR-19b-2
(MIMAT0000788) rno: UGUGCAAAUCCAUGCAAAACUGA-rno-mir-19b-1

(2) miR-26b:

(MIMAT0000083) hsa: UUCAAGUAAUUCAGGAUAGGU
(MIMAT0000534) mmu: UUCAAGUAAUUCAGGAUAGGU
(MIMAT0000797) rno: UUCAAGUAAUUCAGGAUAGGU

(3) miR-203:

(MIMAT0000264) hsa: GUGAAAUGUUUAGGACCACUAG
(MIMAT0000236) mmu: GUGAAAUGUUUAGGACCACUAG
(MIMAT0000876) rno: GUGAAAUGUUUAGGACCACUAG

Our present study was designed to determine the function of select diet and colon carcinogen modulated miRNAs and to validate their respective miRNA:mRNA association. Specifically, in order to determine the direct gene targets of miR-19b, 26b and 203, luciferase reporter assays were carried out. In addition, microRNA physiological function was assessed by examining effects on apoptosis and cell proliferation. We hypothesized that PTK2B, PDE4B and TCF4 are direct targets of miR-19b, miR-26b and miR-203, respectively.

4.2 Materials and methods

4.2.1 Cell culture

HCT116 cell line (p53+/+) was obtained from Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD (335). These cells were cultured in McCoy's 5A medium supplemented with 10% defined fetal bovine serum (Thermo Scientific, Wilmington, DE) and 2 mM glutamax (Gibco, Carlsbad, CA) at 37^0C in 5% CO_2. A detailed protocol is included in **Appendix B**.

4.2.2 Transfection

Cells were plated at 1-2x10^5 cells per well in a 12-well plate on the day of transfection. Subsequently, 70% confluent cells were transfected with 5' FITC-labeled miRNA inhibitors (Exiqon, Denmark) anti-miR-19b, anti-miR-26b or anti-miR-203 (30 nM) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Scrambled miR was utilized as a negative control. Twelve hours following transfection, the media was changed. Transfection efficiency was assessed by images collected with a Zeiss 510

META NLO Multiphoton Microscope System consisting of an Axiovert 200 MOT microscope (Carl Zeiss Microimaging, Thornwood, NY). For overexpression of miRNAs, cells were transfected with miRNA mimics (30 nM) (Dharmacon, Lafayette, CO) for miR-19b, miR-26b, miR-203 or scrambled miR. Each experiment was performed in triplicate. The working protocols can be found in **Appendices C-F**. The sequences of the miRNA mimics, inhibitors and scrambled controls are described in **Appendix A**.

4.2.3 Total RNA isolation and Real Time PCR

Forty-eight hours following transfection with miRNA inhibitors in HCT116 cells, media was removed and the cells were washed with phosphate buffered saline (PBS) (Gibco, Carlsbad, CA). RNA lysis buffer (600 µl) provided with miRVana miRNA isolation kit (Ambion, Austin) was added to each well and cells were removed using a cell scraper. Total RNA was isolated using the miRVana miRNA isolation kit as per the manufacturer's protocol. RNA quantity and quality were measured by Nanodrop (Thermo Scientific, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA), respectively. Real time PCR was carried out using miRNA Taqman PCR assays (Applied Biosystems) to determine the expression of mature miR-19b, miR-26b or miR-203 in treated and untreated samples. The detailed protocols for RNA isolation can be found in **Appendix L**. Normalization was carried out using the $2^{\Delta\Delta CT}$ method relative to 18S rRNA.

4.2.4 Western blotting

In order to determine the change in protein expression associated with miRNA knockdown, western blotting was carried out. 2-4x10^6 cells were seeded in 100 mm dishes on the day of transfection. Transfection with 30 nM miRNA inhibitors was carried out as described above. Forty-eight hours following transfection, media was removed and cells were washed thrice with PBS. Subsequently, lysis buffer (300 µL) containing 50 mM Tris-HCl (pH 7.2), 250 mM sucrose, 2 mM ethylenediaminetetraacetic acid (pH 7.6), 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid (pH 7.5), 50 µM NaF, 1% Triton-X, 100 µM sodium orthovanadate, protease inhibitor cocktail and 10 mM β-mercaptoethanol was added to the cells. The lysate was then passed through a 27 gauge needle and incubated on ice for 30 minutes. Subsequently, the lysate was centrifuged at 16,000g for 20 minutes. The supernatant was collected and the protein concentration determined by the Bradford method (336). Samples (20-80 µg) were loaded onto 4-20% Tris-Glycine gels (Invitrogen, Carlsbad, CA). After blotting, the membrane was incubated overnight with rabbit polyclonal PDE4B antibody at 1:200 (Abcam, Cambridge, MA), rabbit TCF4 monoclonal antibody at 1:1000 (Cell signaling Technology, Boston, MA) or rabbit PTK2B antibody at 1:1000 (Cell Signaling Technology, Boston, MA) and horseradish peroxidase linked (Jackson Immunoresearch Laboratories, West Groove, PA) secondary antibody at 1:7000 dilution and chemoluminescent detection was performed. Unless noted, all other reagents were from Sigma. Detailed protocols for total cell lysate and western blotting are described in the **Appendix M**.

4.2.5 Dual luciferase reporter assay

Cells were plated at 0.2 x 10^5 cells per well in a 96 well plate. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with *Renilla* reporter plasmid containing 3' UTR of PTK2B or PDE4B (Switchgear Genomics, Menlo Park, CA) or dual luciferase reporter plasmid containing wild type or mutated 3' UTR of TCF4 (Genecopoeia, Rockville, MD). For PTK2B and PDE4B constructs, firefly reporter plasmids for normalization were also cotransfected. A control 3' UTR plasmid with a random non-conserved and non-repetitive 3' UTR sequence (scrambled 3'UTR) was utilized as a negative control for reporter plasmids. miR-19b, miR-26b, miR-203 or scrambled controlled miRNA mimics (30 nM) were subsequently co-transfected with cells according to the manufacturer's instructions. Twenty-four hours after transfection, dual luciferase reporter assay (Promega) was carried out according to the manufacturer's instructions, and firefly and renilla activity were measured using a SpectraMax L Luminescence Microplate Reader (Molecular Devices Sunnyvale, CA). Relative firefly luciferase activity was normalized to Renilla luciferase activity. To assess the direct binding of PDE4B with miR-26b, morpholinos (0.5 μM, also called target protectors) (Gene Tools, Philomath, OR) against PDE4B were also co-transfected along with a reporter plasmid containing 3' UTR of PDE4B. All experiments were performed in triplicate. Detailed protocols are described in **Appendices G-H and J-K**. The sequences of the miRNA inhibitors, miRNA mimics and 3' UTR constructs are described in **Appendix A**.

4.2.6 Apoptosis assay

Cells were plated at 0.2 x 10^5 cells per well in a 96 well plate and miRNA inhibitors or control inhibitors were transfected as described above, and 48 hours later, apoptosis was measured using a cellular fragmentation enzyme linked immune-sorbent assay (ELISA) (Roche). Floating cells were harvested, washed, lysed and centrifuged to sediment nuclei. Supernatants containing mono- and oligonucloesomes were incubated with substrate and subsequently analyzed by ELISA as previously described (337). To determine the effect of treatment on cell proliferation, equal numbers of cells were seeded in a 96 well plate and 48 hours later, the number of cells were counted using a hemocytometer. All experiments were performed in triplicate.

4.2.7 Statistics

The effect of more than two independent variables (treatment effects) was assessed using the one-way analysis of variance test (ANOVA), and differences among the means were evaluated using Tukey's and Bonferroni post-hoc of contrast. P values <0.05 were considered to be statistically significant. The graphs were plotted using means and standard error of 6 to 8 cell culture wells (data points) obtained from each treatment collected from three different experiments (performed in triplicate). Standard error bars were plotted in order to document the variation in the population mean.

4.3 Results

4.3.1 PTK2B is not a direct target of miR-19b

To determine if PTK2B levels are modulated following miR-19b knockdown, HCT116 cells were transfected with anti-miR-19b inhibitor or with scrambled control miRNA inhibitor (negative control). The predicted pairing of the target 3' UTR gene region with miR-19b is shown in **Figure 13(A)**. With a transfection efficiency of >70% (±3.86), 48 hours following knockdown, miR-19b levels were significantly decreased by ~90% as compared to scrambled control and non-transfected control **(Figure 13(B))**. Dose dependent knockdown of miR-19b was carried out to determine the optimal concentration of miR-19b inhibitor (**Appendix Q1**). Correspondingly, PTK2B protein levels were significantly increased by approximately 40% in miR-19b knockdown samples compared with negative control and non-transfected control **(Figure 13(C, D)).** Alternatively, HCT116 cells were transfected with miR-19b mimic or scrambled miR mimic control and 48 hours later, expression of miR-19b was significantly ($P < 0.05$) increased by greater than 95% **(Figure 13(E))**. Dose dependent overexpression of miR-19b was carried out to determine the optimal concentration of miR-19b mimic (**Appendix Q4**). Following transfection, PTK2B protein levels were significantly ($P < 0.05$) decreased by approximately 60% in cells overexpressing miR-19b compared to the negative control and non-transfected control **(Figure 13(F,G))**. These results indicate that PTK2B is inhibited by miR-19b. In order to further determine if PTK2B is a direct target of miR-19b, a 3'UTR reporter plasmid containing the miRNA binding site of PTK2B **(Figure 13(A) and 13(A))** was utilized. HCT116 cells were cotransfected with

miR-19b mimic, 3'UTR PTK2B *Renilla* reporter plasmid (PTK2B 3' UTR) and normalized to control-firefly reporter plasmid (transfection control). Separately, a control plasmid, containing a 3'UTR with a scrambled sequence (scrambled 3' UTR) that is non-conserved and non-repetitive, was co-transfected with miR-19b mimic in HCT116 cells. This served as a negative control. Twenty-four hours later, luciferase activity was unaffected relative to the negative control **(Figure 14(B))**. These data indicate that PTK2B is not a direct target of miR-19b**.**

4.3.2 PDE4B is a direct target of miR-26b

To determine if PDE4B is directly targeted by miR-26b, HCT116 cells were transfected with anti-miR-26b inhibitor or with scrambled control miRNA inhibitor (negative control). Dose dependent knockdown of miR-26b was carried out to determine the optimal concentration of miR-26b inhibitor (**Appendix Q2**). Forty-eight hours following knockdown, miR-26b levels were significantly decreased ($P < 0.05$) by 75% as compared to control **(Figure 15(B)),** and correspondingly, PDE4B protein levels were significantly ($P < 0.05$) increased by 40% in miR-26b knockdown samples compared with negative control and non-transfected control **(Figure 15(C) and (D)).** In addition, in HCT116 cells transfected with miR-26b mimic or scrambled miR mimic control for 48 h, miR-26b levels were significantly ($P < 0.05$) increased by 95% **(Figure 15(E))** and PDE4B protein levels were significantly ($P < 0.05$) decreased by 45% in miR-26b overexpressed samples compared to the negative control and non-transfected control **(Figure 15(F) and (G))**. Dose dependent overexpression of miR-26b was carried out to

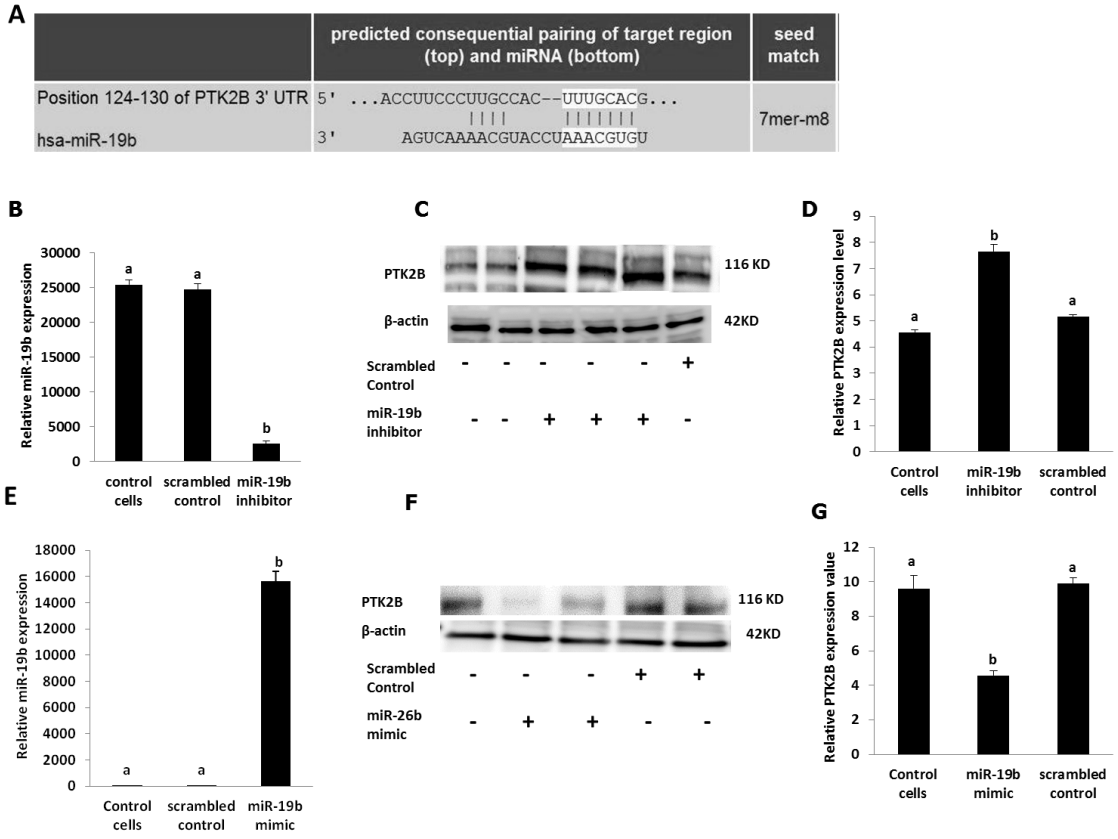

Figure 13. PTK2B expression following knockdown or overexpression of miR-19b expression. (A) Predicted binding of miR-19b to the 3' UTR of PTK2B as assessed by Target Scan (http://www.targetscan.org/). (A) Knockdown of miR-19b was carried out by transfecting miR-19b inhibitor or scrambled miRNA inhibitor (control) in HCT116 cells. 48 h following transfection, miR-19b expression was measured by qRT-PCR. (C) PTK2B protein levels were measured by western blotting after miR-19b knockdown and β-actin was used as a loading control as described in the Materials and methods. (D) Quantification of PTK2B levels in control (no transfection), scrambled control or miR-19b knockdown samples from immunoblot images. (E) Overexpression of miR-19b was carried out by transfecting miR-19b mimic or scrambled miRNA mimic control and 24 h later, miR-19b expression was measured by qRT-PCR. (F) PTK2B protein levels were measured by western blotting following miR-19b overexpression, and β-actin was used as a loading control. (G) Quantification of PTK2B levels in control (no transfection), scrambled control or miR-19b overexpressed samples from immunoblot images. Data represent means ± S.E. from six-eight replicate values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $P < 0.05$.

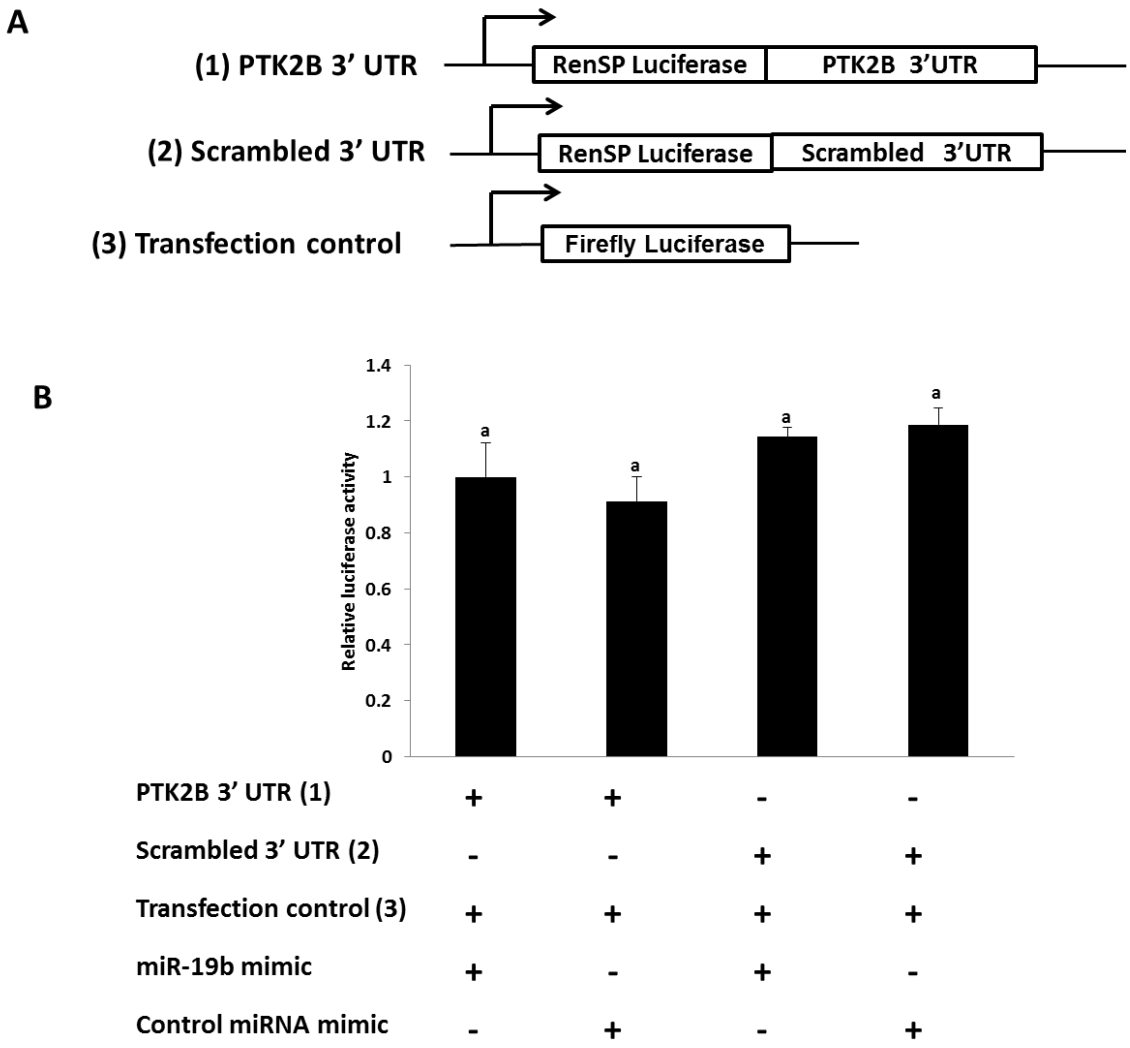

Figure 14. Validation of PTK2B as a gene target of miR-19b. (A) Schematic representation of the 3'UTR PTK2B *Renilla* reporter plasmid, scrambled 3'UTR *Renilla* reporter plasmid and normalization using a control-firefly reporter plasmid (transfection control). (B) Luciferase activity was determined 24 h following transfection. All luciferase values were normalized to firefly luciferase (transfection control). Data represent means $\pm$ S.E. from nine replicate values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $P < 0.05$.

determine the optimal concentration of miR-26b mimic (**Appendix Q5**). These results indicate that PDE4B is downregulated by miR-26b. In order to determine if PDE4B is a direct target of miR-26b, a 3'UTR reporter plasmid containing the miRNA binding site of PDE4B **(Figure 15(A) and 16(A))** was utilized. HCT116 cells were cotransfected with miR-26b mimic, 3'UTR PDE4B *Renilla* reporter plasmid (PDE4B 3' UTR) and a normalization control-firefly reporter plasmid (transfection control). Separately, a control 3' UTR plasmid with a random sequence (scrambled 3'UTR) or a non-conserved and non-repetitive 3' UTR sequence were co-transfected with miR-26b. This served as a negative control. Twenty-four hours later, luciferase activity was significantly ($P < 0.05$) decreased by 50% relative to the negative control **(Figure 16)**. These data demonstrate that PDE4B is a direct target of miR-26b. In order to confirm this finding, target protectors were utilized. Target protectors are morpholinos which bind to target mRNA and mask the binding site, blocking the targeting of miRNAs, thereby increasing luciferase activity (338). HCT116 cells co-transfected with a morpholino targeting PDE4B mRNA, PDE4B 3' UTR Renilla reporter plasmid, a normalization control-firefly reporter plasmid (transfection control) and miR-26b mimic increased luciferase activity significantly ($P < 0.05$) as compared to HCT116 cells co-transfected with miR-26b mimic, 3'UTR PDE4B *Renilla* reporter plasmid (PDE4B 3' UTR) and a normalization control-firefly reporter plasmid (transfection control) **(Figure 16(B))**. Separately, as a negative control, PDE4B target protector control was co-transfected with PDE4B 3' UTR plasmid. This confirmed that PDE4B is a direct target of miR-26b.

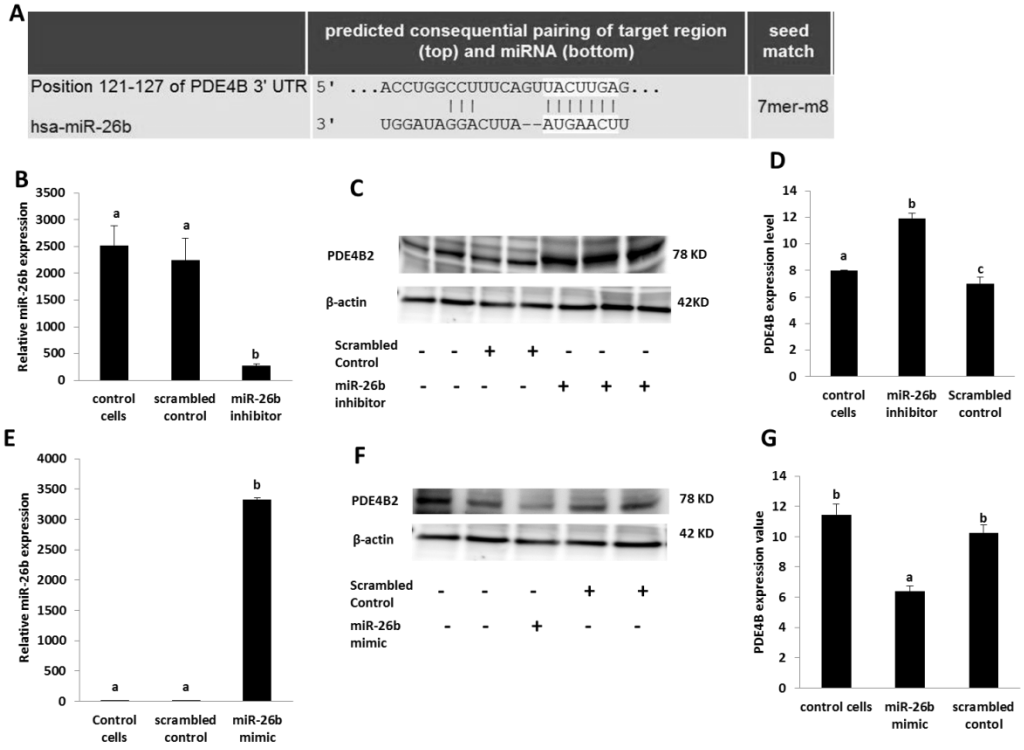

Figure 15. PDE4B expression following knockdown or overexpression of miR-26b expression. (A) Predicted binding of miR-26b to the 3' UTR of PDE4B as assessed by Target Scan (http://www.targetscan.org/). (B) Knockdown of miR-26b was carried out by transfecting HCT116 cells with miR-26b inhibitor or scrambled miRNA inhibitor (control) for 48 h. miR-26b expression was subsequently measured by qRT-PCR. (C) PDE4B protein levels were measured by western blotting following miR-26b knockdown. β-actin was used as a loading control as described in the Materials and methods. (D) Quantification of PDE4B protein levels in control (no transfection), scrambled control or miR-26b knockdown cultures from immunoblot images. (E) Overexpression of miR-26b was carried out by transfecting miR-26b mimic or scrambled miRNA mimic control. 24 h post transfection, miR-26b expression was measured by qRT-PCR. (F) PDE4B protein levels were measured by western blotting following miR-26b overexpression and β-actin was used as a loading control. (G) Quantification of PDE4B protein levels in control (no transfection), scrambled control or miR-26b overexpressed samples from immunoblot images. Data represent means ± S.E. from six-eight replicate values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $p < 0.05$.

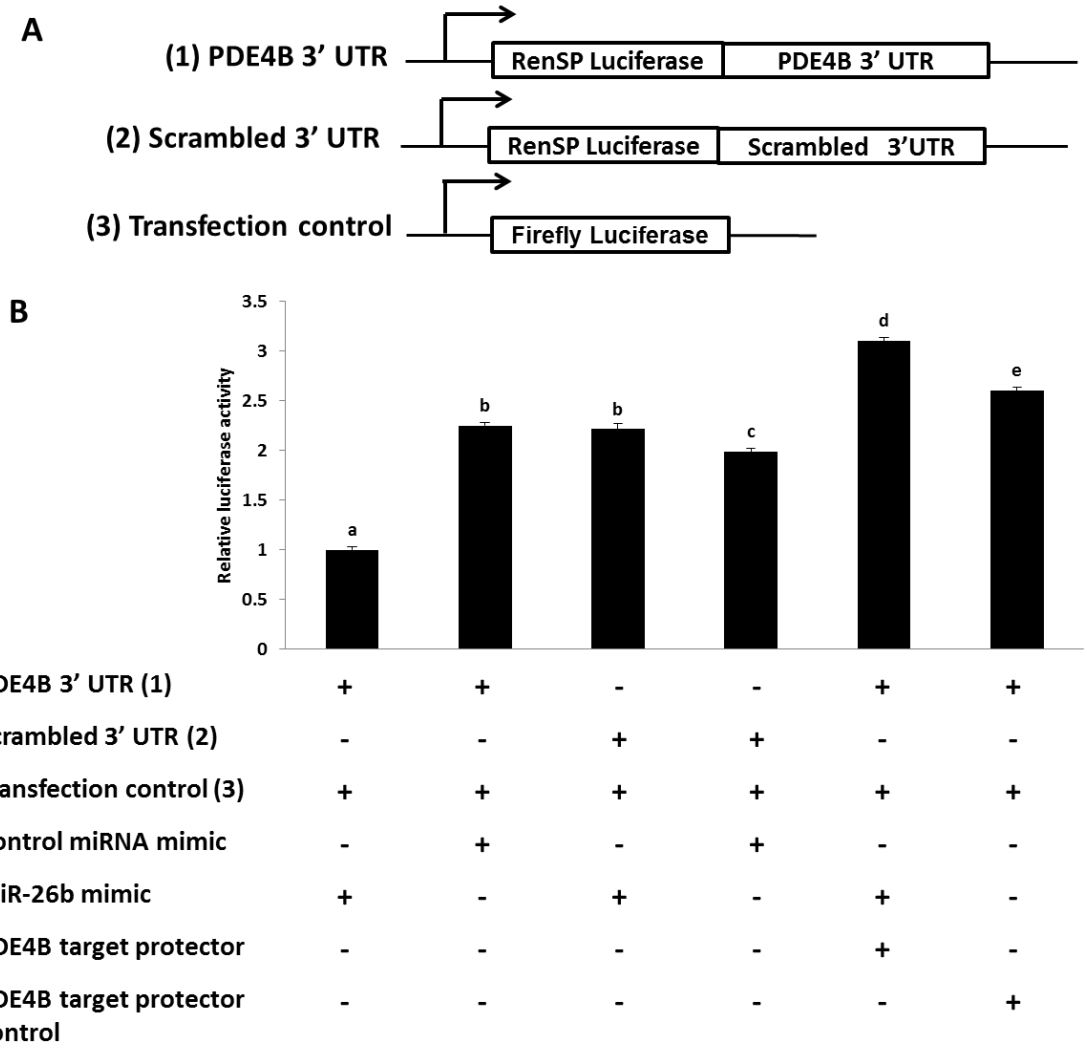

Figure 16. Validation of PDE4B as a gene target for miR-26b. (A) Schematic representation of the 3'UTR PTK2B *Renilla* reporter plasmid, scrambled 3'UTR *Renilla* reporter plasmid and normalization control-firefly reporter plasmid used in the study. (B) Luciferase activity was determined 24 h after transfection. All luciferase values were normalized to firefly luciferase (transfection control). Data represent means $\pm$ S.E. from nine values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $p < 0.05$.

4.3.3 TCF4 is a direct target of miR-203

To determine if TCF4 levels are modulated following miR-203 knockdown, HCT116 cells were transfected with anti-miR-203 inhibitor or with a scrambled control miRNA inhibitor (negative control). Dose dependent knockdown of miR-203 was carried out to determine the optimal concentration of miR-203 inhibitor (**Appendix Q3**). Forty-eight hours following knockdown, miR-203 levels were significantly ($P < 0.05$) decreased by 50% as compared to the controls **(Figure 17(B)).** Correspondingly, TCF4 protein levels were significantly increased ($P < 0.05$) by 43% in miR-203 knockdown samples compared with the negative control and non-transfected control **(Figure 17(C)** and **(D)).** In comparison, in HCT116 cells transfected with miR-203 mimic or scrambled miR mimic control, miR-203 levels were significantly ($P < 0.05$) increased by greater than 95% **(Figure 17(E))**. Dose dependent overexpression of miR-203 was carried out to determine the optimal concentration of miR-203 mimic (**Appendix Q6**). Correspondingly, TCF4 protein levels were significantly decreased ($P < 0.05$) by 55% in miR-203 overexpressed samples compared with negative control and non-transfected control **(Figure 17(F) and (G))**. These results indicate that TCF4 is downregulated by miR-203. In order to determine if TCF4 is a direct target of miR-203, a 3'UTR reporter plasmid containing the miRNA binding site of TCF4 **(Figure 17(A) and 18(A))** was utilized. HCT116 cells were cotransfected with miR-203 mimic and 3'UTR TCF4 firefly reporter plasmid. In separate experiments, as a negative control, a control 3' UTR plasmid with a random sequence (scrambled 3'UTR) was co-transfected with miR-203 mimic. Twenty-four hours later, luciferase activity was significantly decreased ($P < 0.05$)

by ~55% relative to the negative control. These findings demonstrate that TCF4 is a direct target of miR-203. In order to confirm this result, the binding sites of miR-203 in 3' UTR of TCF4 were mutated. The miR-203 binding site in the 3' UTR of TCF4, i.e., "catttcaa", was mutated to "gcgatatc" **(Figure 18(B))**. When a mutated 3' UTR version of TCF4 was co-transfected with the miR-203 mimic, there was no change in luciferase activity compared to the miRNA target clone control vector **(Figure 18(C)),** confirming the specificity of miR-203 binding.

4.3.4 miR-19b and miR-26b induce apoptosis whereas miR-203 reduces cell proliferation

Studies have shown that dietary fish oil and pectin diet combination promotes apoptosis (59, 247). We demonstrated earlier that levels of miR-26b and miR-203 were reduced by the dietary combination of corn oil and cellulose (CCA) as compared to chemoprotective diet, fish oil combined with pectin (FPA), in the presence of AOM (151). Hence, in order to elucidate how alterations in miRNAs in the colon alter cell phenotype, HCT116 cells were transfected with miR-19b, miR-26b mimic or scrambled control miRNA. A 66% increase ($P < 0.05$) in apoptotic index was observed when miR-19b and miR-26b were overexpressed compared to untreated control or scrambled control **(Figure 19(A)**, whereas there was a modest increase of ~25% ($P < 0.05$) in apoptotic index and a significant decrease in cell number when miR-203 was overexpressed **(Figure 19(B))**. Cell proliferation was not determined for miR-19b and miR-26b since studies have demonstrated that these miRNAs do not alter cell proliferation (339, 340).

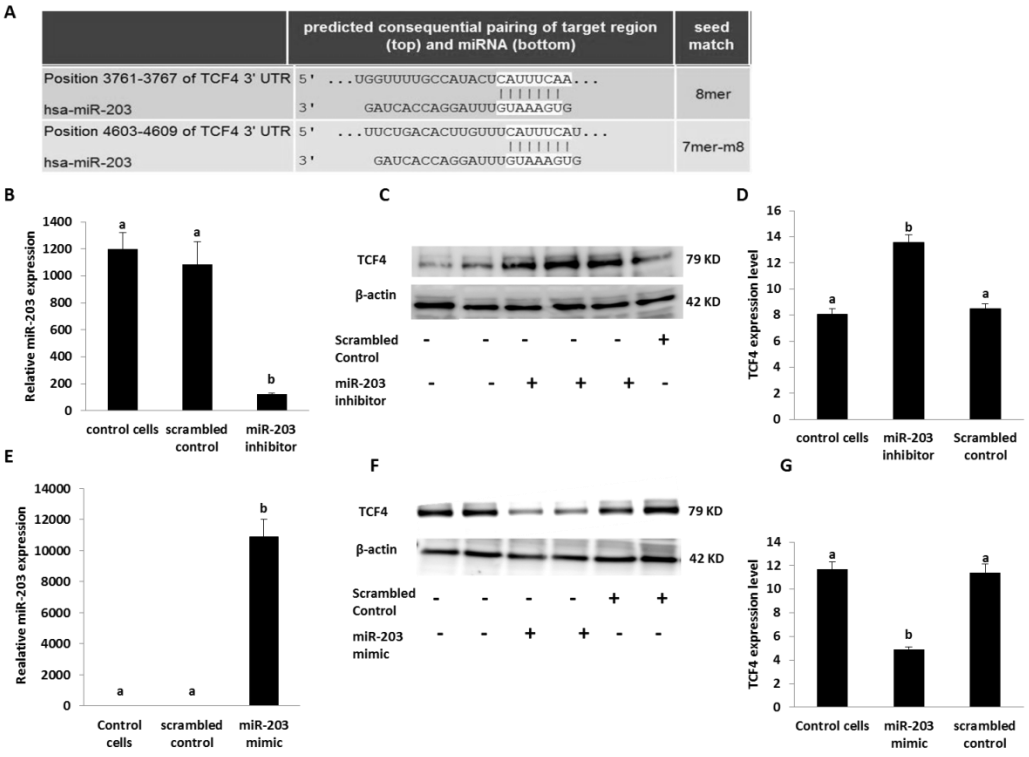

Figure 17. TCF4 expression following knockdown or overexpression of miR-203 expression. (A) Predicted binding of miR-203 to the 3' UTR of TCF4. (A) Knockdown of miR-203 was carried out by transfecting miR-203 inhibitor or scrambled miRNA inhibitor (control) in HCT116 cells. 48 h following transfection, miR-203 expression was measured by qRT-PCR. (C) TCF4 protein levels were measured by western blotting after miR-203 knockdown and β-actin was used as a loading control as described in the Materials and methods. (D) Quantification of TCF4 levels in control (no transfection), scrambled control or miR-203 knockdown samples from immunoblot images. (E) Overexpression of miR-203 was carried out by transfecting miR-203 mimic or scrambled miRNA mimic control. miR-203 expression was measured by qRT-PCR 24 h later. (F) TCF4 protein levels were measured by western blotting after miR-203 overexpression and β-actin was used as a loading control. (G) Quantification of TCF4 levels in control (no transfection), scrambled control or miR-203 overexpressed samples from immunoblot images. Data represent means $\pm$ S.E. from six- eight replicate values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $P < 0.05$.

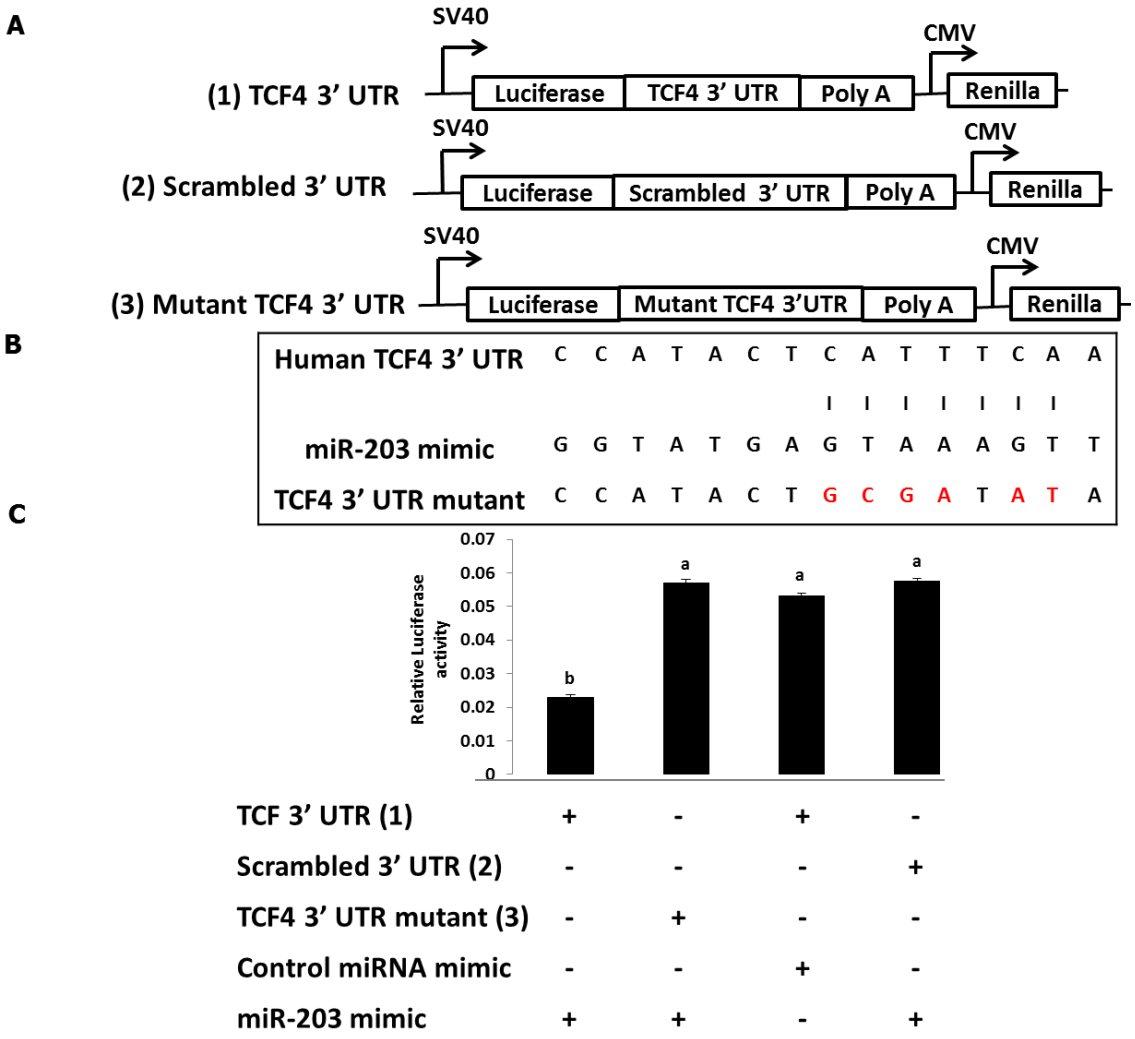

Figure 18. Validation of TCF4 as a target gene for miR-203. (A) Schematic representation of the 3'UTR TCF4 *Renilla* reporter plasmid, scrambled 3' UTR *Renilla* reporter plasmid and 3'UTR mutant TCF4 *Renilla* reporter plasmid. (B) Schematic representation of 3'UTR of TCF4 showing the putative miR-203 target site. The letters in red indicate the mutated base pairs. (C) Luciferase activity was determined 24 h after transfection. All luciferase values were normalized to *Renilla* luciferase. Data represent means $\pm$ S.E. from nine values obtained from three separate experiments. Mean values not sharing common letters are significantly different, $p < 0.05$.

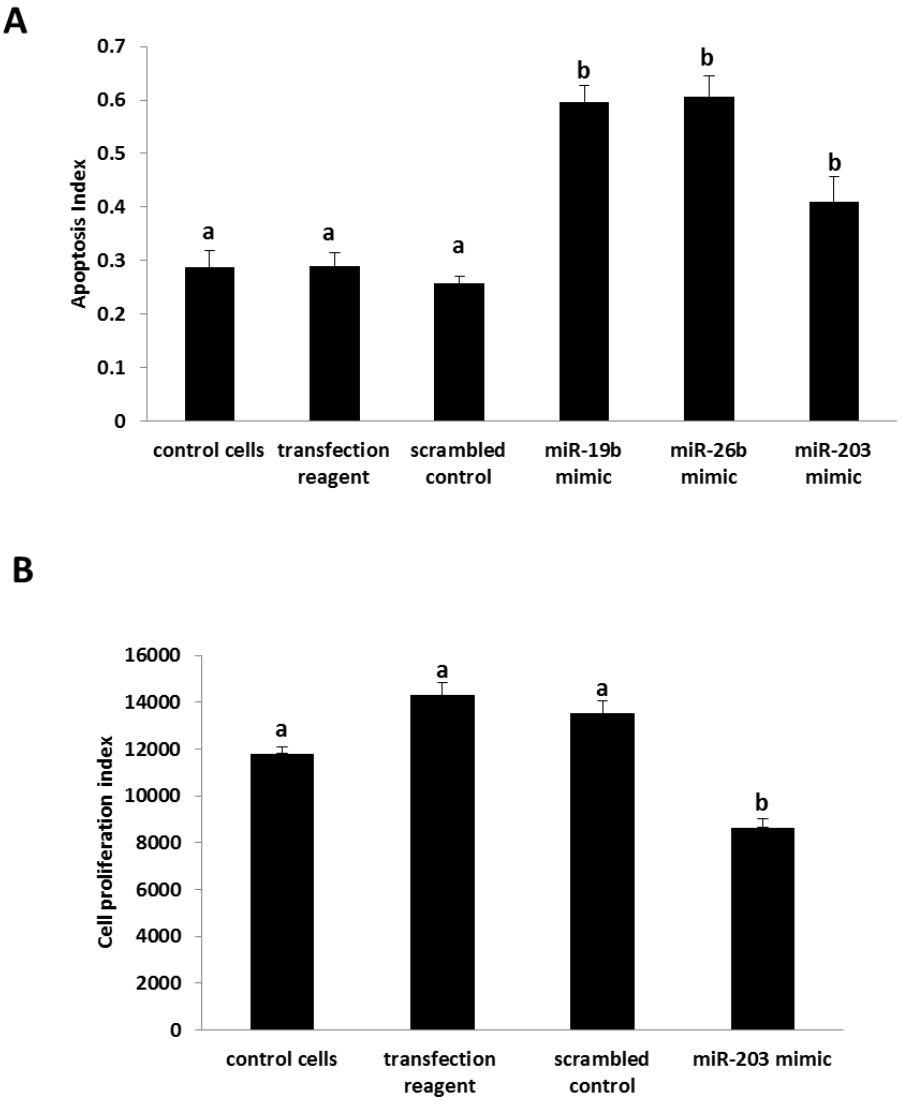

Figure 19. Effects of miR-19b, miR-26b and miR-203 on colonocyte phenotype. (A) HCT116 cells were transfected with miR-19b, miR-26b, miR-203 (30 nM) or scrambled control mimic. After 72 h, the apoptosis index was measured. (B) HCT116 cells were transfected with either miR-203 mimic or scrambled control mimic for 72 h and total cell number was counted. Data represent means $\pm$ S.E. from six culture wells obtained from two separate experiments.

4.4 Discussion

We have recently demonstrated that n-3 PUFA uniquely modulate carcinogen-directed miRNA signatures in the colon (140). Also, previous studies have shown that the bioactive compounds associated with dietary fish oil (DHA and EPA) and fermentable fiber (butyrate) synergize to protect against colon carcinogenesis, primarily by enhancing apoptosis (59, 60, 239, 332, 341). With regard to dietary combination, i.e., fat x fiber interaction in the presence of carcinogen (CCA vs FPA), we observed a coherent relationship between miR-19b, miR-26b, miR-203 and PTK2B, PDE4B and TCF4, respectively (151). Since these miRNAs may exhibit distinct functions in different cell types, their role in colonic epithelial cells warrants investigation. Therefore, to further elucidate the biological effects of diet and carcinogen on miRNAs and their targets, an integrated targeted approach was used.

In this study, by manipulating miRNA levels and performing luciferase reporter assays, we demonstrated that PDE4B and TCF4 are direct targets of miR-26b and miR-203, respectively. Even though PTK2B protein levels were modulated by miR-19b, there was no change in luciferase reporter activity, suggesting the absence of direct binding between miR19b and PTK2B. These data suggest that miR-19b modulates PTK2B by modulating an upstream target(s) of PTK2B.

We have previously documented the ability of dietary fish oil/pectin combination to increase miR-26b expression in the colon (151). This is noteworthy, because the suppression of miR-26b expression has been reported in various cancers including colon cancer, hepatocellular carcinoma, breast cancer and prostate cancer (342-344). In

addition, miR-26b status has been associated with the invasiveness and metastasis of colorectal cancer cells (342). The increased expression of PDE4B (cAMP-specific 3',5'-cyclic phosphodiesterase 4B) has been reported in colorectal cancer (345) and is known to act by limiting cAMP-associated apoptosis in a several types of cancer (328, 329). We observed that apoptosis was induced with increasing levels of miR-26b, which is consistent with published reports (342-344). In addition, previous studies from our lab have shown that fish oil - pectin combination act synergistically to induce apoptosis, both *in vivo* and *in vitro*, by recruiting a p53 independent, oxidation sensitive, Ca^{2+} - mediated intrinsic mitochondrial pathway (59, 346). Based on this evidence, we propose a putative mechanism of miR-26 action in the colon (**Figure 20)**. By increasing mucosal levels of miR-26b, fish oil-pectin combination decreases PDE4B expression, which promotes cyclic adenosine monophosphate (cAMP) formation (347). This can lead to reduction of phosphorylation at threonine 308 and serine 473 of Akt (i.e. pAKT) (348) and activation of protein kinase A (PKA) (347). From a chemoprotective standpoint, PKA can promote apoptosis (349)**.** Moreover, decreased pAKT activity can result in a reduction in PIP_3 levels and decrease the lipid kinase activity of PI3K (348). Interestingly, we have observed that other dietary chemoprotective agents, e.g., curcumin, are capable of blocking intestinal dextran sodium sulphate-induced increase in PDE4B (170). Also, EZH2, one of the targets of miR-26b has been shown to be modulated by curcumin in pancreatic cancer cells (343, 350), suggesting that miR-26b and its targets may be modulated by chemoprotective bioactive dietary agents.

Collectively, these findings suggest that miR-26b acts as a "tumor suppressor" by targeting PDE4B.

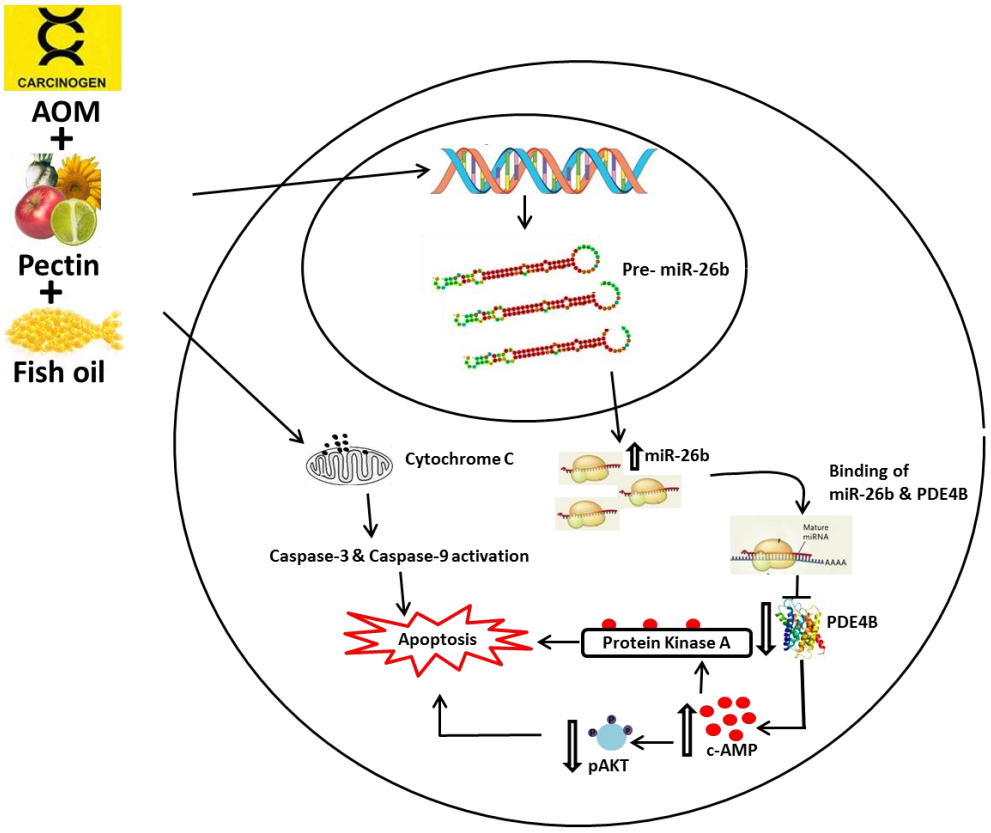

Figure 20. Proposed mechanism of action of miR-26b. Colonocytes from fish oil and pectin fed mice exposed to carcinogen exhibit an increased steady-state level of miR-26b, which directly targets and reduces PDE4B expression. This results in an increase in cAMP levels, which may antagonize pAKT levels and promote apoptosis. Increased levels of cAMP may also activate Protein Kinase A, which can promote apoptosis and delete mutated cells.

The increased expression of miR-203 in HCT116 cells was associated with decreased levels of one of its predicted targets, TCF4. miR-203 has been reported to be

downregulated in a number of cancers and was upregulated in the colonic mucosa of rats fed a fish oil + pectin diet (151, 351-356). miR-203 downregulation appears to be mediated by epigenetic silencing. For example, studies in breast cancer and multiple myeloma cells have shown that the miR-203 promoter is hypermethylated (351, 357). Moreover, Lena *et al.* demonstrated that miR-203 is downregulated during the epithelial commitment of embryonic stem cells (353). Through overexpression or knockdown of miR-203 levels in HCT116 cells, we showed that TCF4 levels were inversely correlated to the level of miR-203 expression. Also, luciferase reporter assays showed that miR-203 directly binds to TCF4 and mutation of the miR-203 binding site in the TCF4 3' UTR abolished the effect of miR-203. TCF4 is a well-known transcription factor expressed in the intervillus pocket of the developing intestine and is required to establish proliferative progenitor cells of prospective crypts in the embryonic small intestine (358). TCF4 is regulated by the Wnt signaling pathway and controls stem cell fate in the intestine (359). Indeed, it is well appreciated that an increase in TCF4 levels promotes TCF4-β-catenin interaction, which can lead to the inappropriate activation of a TCF4 target gene program (360). Previously, we have demonstrated that colonic-β-catenin signaling, an upstream mediator of TCF4, is suppressed in carcinogen injected rats fed a fish oil + pectin (FPA) as compared to control diet (247). Hence as shown in **Figure 21 (B)**, we predict that CCA combination will enhance hypermethylation of miR-203 promoter resulting in the reduced expression of mature miR-203. Hence, TCF4 protein levels will be higher, thus increasing cell proliferation. Alternatively, as shown in **Figure 21(A),** FPA combination will not induce hypermethylation of the miR-203 promoter,

thus decreasing cell proliferation by increasing expression of miR-203. Further work is needed in order to determine how FPA modulates the expression of mature miR-203.

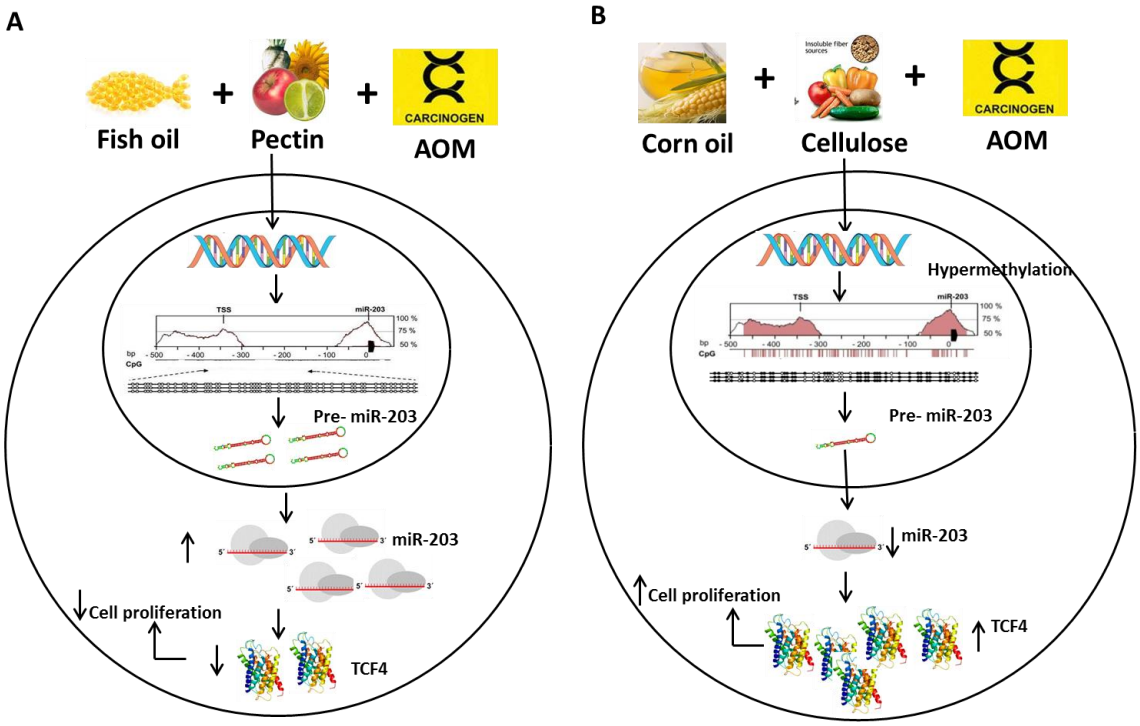

Figure 21. Proposed chemoprotective mechanism of action of miR-203. (A) In the presence of carcinogen (AOM), fish oil + pectin diet, transcription of miR-203 is mantained, regulating TCF4 levels. (B) In the presence of carcinogen (AOM), corn oil + cellulose (control) diet, miR-203 expression is repressed, thereby decreasing the formation of pre-miR-203, ultimately reducing the levels of mature miR-203. This elevates TCF4 levels, which promotes cell proliferation and epithelial cell stemness.

In summary, the chemoprotective effects of combined fish oil and pectin feeding antagonize the oncogenic effects of carcinogen, in part by modulating the expression of miR-19b, miR-26b and miR-203 and their targets.

5. MODULATION OF COLONIC STEM CELL MICRORNA EXPRESSION BY DIET

5.1 Introduction

The multicellular epithelium of the intestine is replaced every 4-6 days (361, 362). The massive rate of cell proliferation in the crypts is maintained by cells undergoing mitotic division at the base of the crypts and in transit, prior to undergoing differentiation. This renewal process is maintained by multipotent intestinal stem cells (ISCs). The exact identity of intestinal stem cell has proven controversial over the last 30 years, and mechanisms that regulate homeostasis are not fully elucidated, although plausible candidates have been proposed (363). Recently, advances have been made in the identification of adult stem cells that replenish the intestinal stem cell every four to six days (225, 228, 229, 364, 365). Barker *et al.* demonstrated for the first time that colon stem cells can be identified by specific expression of Lgr5/GPR49, a G protein-coupled receptor (225). $Lgr5^+$ ISCs are long-lived, rapidly cycling cells in the crypt base columnar (CBC) region giving rise to all the epithelial subtypes in the small intestine and colon.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and poses a serious health problem in United States (22). Current treatment consists of surgical resection of the tumor, chemotherapy, radiotherapy and palliative therapy (advanced stages). However, as many as 40% of patients may exhibit relapse within 5 years of treatment, generally in the form of metastases (366). Colorectal cancers contain subsets

of cancer stem cells (CSC), which are identified by the expression of surface markers such as CD133 (221, 367) and/or CD44 (368) and can effectively propagate the disease after implantation in an immunodeficient host. A recent study showed that there is a common gene signature between EphB2 and Lgr5 sorted stem cells obtained from healthy intestine and colon tumors, suggesting that the intestinal stem cell program defines a cancer stem cell niche within colorectal tumors and may play a role in colorectal cancer relapse (369). Hence, it has been suggested that the intestinal stem cell program defines a cancer stem cell niche within colorectal tumors and also plays a role in CRC relapse (369). Based on emerging evidence, cancer stem cells are primary therapeutic targets for colorectal cancer recurrence (370).

Recent evidence indicates that intestinal crypt homeostasis results from neutral competition between symmetrically dividing $Lgr5^{+}$ stem cells (371). By crossing stem-cell-specific Lgr5-EGFP-IRES-creER^{T2} knock in mice to $Apc^{flox/flox}$ mice, it was unequivocally shown that crypt $Lgr5^{+}$ stem cells are the cells-of-origin of intestinal cancer (228). Hence, perturbations in adult stem cell dynamics are generally believed to represent an early step in colon tumorigenesis. In addition, cell differentiation has been demonstrated to be an important event in carcinogenesis. The progression and aggressiveness of colorectal tumors has been shown to have an inverse relationship with the level of differentiation of tumor cells. Several studies have demonstrated that miRNA are key regulators of pluripotency and differentiation (372).

miRNAs are a type of noncoding RNAs that act through partial complementarity to 3' untranslated regions (UTRs) of target mRNAs, and regulate gene expression post-

transcriptionally (2, 3). Recently, it has been demonstrated that genes translationally repressed by more than one third also display detectable mRNA destabilization (373, 374). With respect to epigenetic mechanisms involved in colon tumor development, it is believed that non-coding miRNAs control the expression of approximately one-third of the mammalian messenger mRNA (mRNAs) (2). miRNAs play an important role in regulating stem cell renewal and differentiation by repressing the translation of selected mRNAs in stem cells and differentiated daughter cells (375-379). Also, a few studies have demonstrated the role of miRNAs in the maintenance of colon cancer stem cells (235, 380). Therefore, it is highly likely that the search for specific miRNA expression signatures of colorectal cancers will better define specific subtypes and hence can potentially influence cancer therapy (235, 342).

The overwhelming majority of colorectal cancers are initiated by activating mutations/deletions in the Wnt signaling pathway (381, 382). From a physiological standpoint, this pathway is essential for the maintenance of the intestinal stem cell niche, as exhibited by mice lacking the TCF4 transcription factor, lacking RNF43 deletion or expressing transgenically the secreted Dickkopf-1 Wnt inhibitor (383-386). Therefore, environmental factors that are capable of modulating Wnt signaling will likely have a unique and central role in the physiology and pathology of the intestinal stem cell. Along these lines, our laboratory (247) and others (387-389) have demonstrated that nutritional bioactives (n-3 fatty acids, folate, fermentable fiber) can modulate Wnt signaling by repressing coloncyte nuclear beta-catenin levels. In addition, we have recently demonstrated that a chemoprotective diet containing n-3 fatty acids may favorably

influence the stem cell niche and therefore suppress colon cancer development (171, 390). Our lab was the first to examine the effects of colon carcinogen on rat mucosal miRNA expression profiles and to unravel the effects of bioactive dietary components on miRNA expression in the colon (151, 283). We demonstrated that the novel combination of fish oil/pectin protects the colon from carcinogen-induced miRNA dysregulation (151). However, to date, the effects of chemoprotective dietary components on colonic stem cell non-coding miRNA signatures have not been determined, nor has the miRNA expression profile in colon stem cells been documented. Hence, with the identification of Lgr5 as the first definitive intestinal stem cell marker in mice, we used the highly novel, recently described, Lgr5-EGFP-IRES-cre ER^{T2} *knock in* mouse model to visualize and isolate intestinal stem cells, examine their response to chemotherapeutic agents by measuring miRNA expression and establish their stem cell-specific miRNA expression profile. This was contrasted with components of the stem cell "niche" i.e. daughter cells, which contribute to the physiological microenvironment consisting of specialized cells that physically anchor the stem cell and provide the necessary factors to maintain its stemness. We hypothesized that a chemoprotective diet containing n-3 PUFA (fish oil) and butyrate (pectin) would modulate the stem cell miRNA profile resulting in a favorable shift in programmed cell death (apoptosis) and self-renewal in colonic crypts. Our overall goal is to better understand how the colonic stem cell population responds to environmental factors such as diet and carcinogen. Hence, we investigated the effects of disease progression on miRNAs in colonic stem cells by feeding mice injected with azoxymethane (AOM, a colon carcinogen) a diet

containing n-3 PUFA and a fermentable fiber (fish oil + pectin) diet or n-6 PUFA and a poorly fermentable fiber (corn oil + cellulose).

5.2 Materials and methods

5.2.1 Animals

All procedures involving animals followed guidelines approved by the Institutional Animal Care and Use Committee at Texas A&M University in accordance with EU Directive 2010/63/EU for animal experiments. Lgr5-EGFP-IRES-creER^{T2} originally described by Barker *et al.* (225) (10-15 wk of age) were used. The study was 2 x 2 factorial design with two types of dietary fat fiber combination –corn oil (n-6 PUFA) + cellulose and fish oil (n-3 PUFA) + pectin and two treatments (injection with colon carcinogen, AOM or saline). Animals (n=5 per group) were terminated eight weeks after the last AOM injection. **Figure 22** shows the timeline of the treatments.

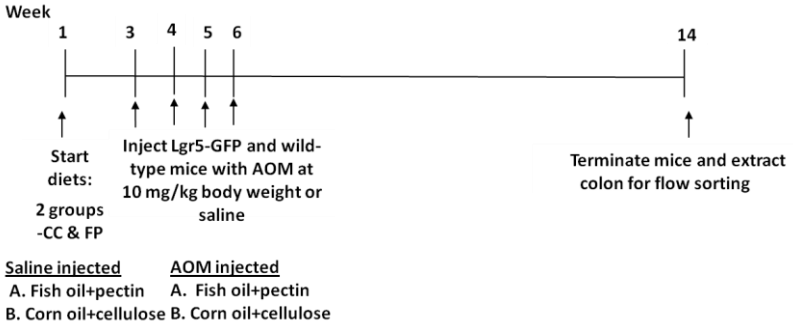

Figure 22. Timeline of treatment. Lgr5-EGFP-IRES-creER^{T2} mice were provided with either corn oil / cellulose or fish oil / pectin diets for 2 weeks prior to treatment. The mice were then injected four times with AOM or saline at 10 mg/kg body weight for four weeks, one injection per week. Eight weeks after the last injection, the mice were terminated and the colon was extracted for stem cell isolation. CC, corn oil cellulose; FP, fish oil pectin.

5.2.2 *Experimental diets*

Lgr5-EGFP-IRES-creER^{T2} mice were assigned to one of the two diet groups (fish oil / pectin or corn oil / cellulose), which differed only in the type of fat and fiber. Diets contained (g/100 g diet): dextrose, 51.00; casein, 22.40; D,L-methionine, 0.34; American Institute of Nutrition (AIN)-76 salt mix, 3.91; AIN-76 vitamin mix, 1.12; choline chloride, 0.13; pectin or cellulose, 6.00. The total fat content of each diet was 15% by weight with the n-6 PUFA diet containing 15.0 g corn oil/100 g diet (Dyets, Bethlehem, PA) and the n-3 PUFA diet containing 11.5 g fish oil/100g diet (Omega Protein, Houston, TX) plus 3.5 g corn oil/100 g diet to prevent essential fatty acid deficiency. All diet ingredients except oils were obtained from Bio-serv (Frenchtown, NJ). To prevent the formation of oxidized lipids, diets were stored at -20^{0}C and provided fresh to animals every day.

5.2.3 *Determination of aberrant crypts*

Mice were euthanized by CO_2 overdose, followed by cervical dislocation. The colon was removed and 1 cm of the distal colon was fixed in 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) for immunohistochemistry (IHC). The remainder of the colon was prepared for determination of aberrant crypt foci (ACF). Enumeration of ACF was conducted as previously described (391). Briefly, the total number of ACF and high multiplicity aberrant crypt foci (HM-ACF) were counted from each colon for each treatment (n=10 per treatment). HM-ACF were considered to contain four or more aberrant crypts per focus (392).

5.2.4 Stem cell isolation from colonic crypt using fluorescence activated cell sorting

Twenty-four hours prior to termination, 200 ul of 10 mg/mL tamoxifen (Sigma, CO) dissolved in corn oil (without *tert*-Butylhydroquinone (TBHQ)) was administered to each mouse via intraperitoneal injection using a 27 gauge syringe. The final dose of tamoxifen was 2 mg per mouse. As a negative control, a mixed solution containing 198 ul of corn oil and 2 ul of 100% ethanol was injected. Colonic crypts were isolated by the method of Sato et al (229) with minor modification. The intact colons were everted on a disposable mouse gauge needle (Instech Laboratories) and incubated with 20 mM EDTA in PBS at 37°C for 30 min. Following transfer to chilled Ca/Mg free HBSS, colons were vigorously vortexed to release crypts. The crypts were then incubated with 50 ul of DNase (stock concentration- 20 units/ml) in 10 ml of trypsin solution and single cells were then passed through a 40 micron cell strainer. The cells were counted and resuspended to a final cell density of 2 x10^6 cells/mL. FACS (Fluorescence activated cell sorting) was then carried out to sort the GFP^{high} expressing stem cells, GFP^{low} expressing daughter cells and $GPF^{negative}$ cells isolated from the colon using a BD FACS Aria II cytometer /sorter (BD Biosciences). Cells from wild type mice were used to set the gates for sorting **(Figure 23(A)).** For a detailed protocol, see Appendix N.

5.2.5 Total RNA isolation and miRNA profiling

Three populations of cells (GFP^{high}, GFP^{low} and $GPF^{negative}$) from each colon were directly sorted into miRVana lysis solution provided by the mirVana miRNA Isolation Kit (Ambion, Austin, TX). Total RNA enriched with miRNA was isolated according to the manufacturer's instructions **(Figure 23(B))**. The quantity of isolated RNA was

measured using Nanodrop (Thermo Scientific, Wilmington, DE) and by Quant-iT^{TM} Ribogreen RNA Assay Invitrogen, CA). RNA quality was also assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). For protocol details, see Appendix L.

5.2.6 miRNA profiling

The expression of 384 mature miRNAs was determined using the TaqMan Rodent Panel Low Density Arrays Panel A (Applied Biosystems, CA) according to the manufacturer's instructions. For a detailed protocol, see Appendix O.

5.2.7 Statistics

Two-hundred sixty-eight miRNAs were disqualified due to low (close to background level) expression.. The resultant readings were then normalized by global mean, global median, RNU6B (housekeeping gene) or a group of miRNAs whose expression were not altered in the treatments (Appendix N.7). On comparing the four methods, global median was found to be an appropriate normalization method, since the overall variance was reduced. After normalization, the data were then analyzed by the Mann-Whitney or Wilcoxon signed rank non-parametric tests based on the comparisons(393). *P* values were obtained and significantly different miRNAs ($P \leq 0.05$) with a fold change >1.3 or <0.7 were selected for further analyses. Fold change was calculated for different comparisons by taking the arithmetic mean of one treatment divided by the other. Standard error bars were plotted in order to document the variation in the population mean. The analysis pipeline is described in Appendix O.

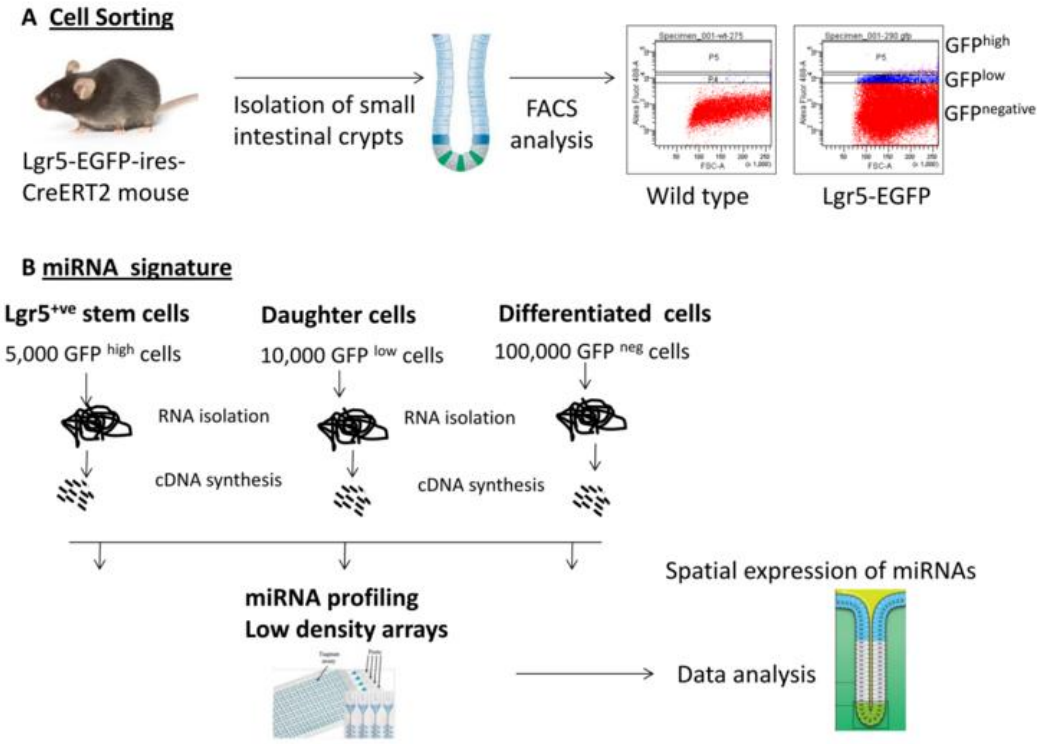

Figure 23. Experimental design. (A) Lgr5^{high} (stem cells), Lgr5^{low} (daughter cells) and Lgr5^{negative} (differentiated cells) from Lgr5-EGFP-IRES-creER^{T2} mice were sorted. (B) Using the TaqMan low density array (microfluidic card) platform, miRNA profiling was carried out on FACS sorted samples and data analyses were performed to determine the expression of miRNAs in the three different cell populations.

5.3 Results

5.3.1 Enumeration of aberrant crypts foci (ACF) in carcinogen injected mice fed bioactive dietary agents

Whereas AOM-injected mice developed aberrant crypt foci, their saline treated counterparts did not. Therefore, all aberrant crypt results represent AOM-injected groups only. AOM injected mice fed the corn oil + cellulose diet exhibited a greater number of total ACF ($P < 0.05$) and (high multiplicity) HM-ACF ($P < 0.05$) compared to fish oil +

pectin + AOM treatment **(Figure 24)**. This demonstrates that the fish oil + pectin diet suppressed the carcinogenic effects of AOM.

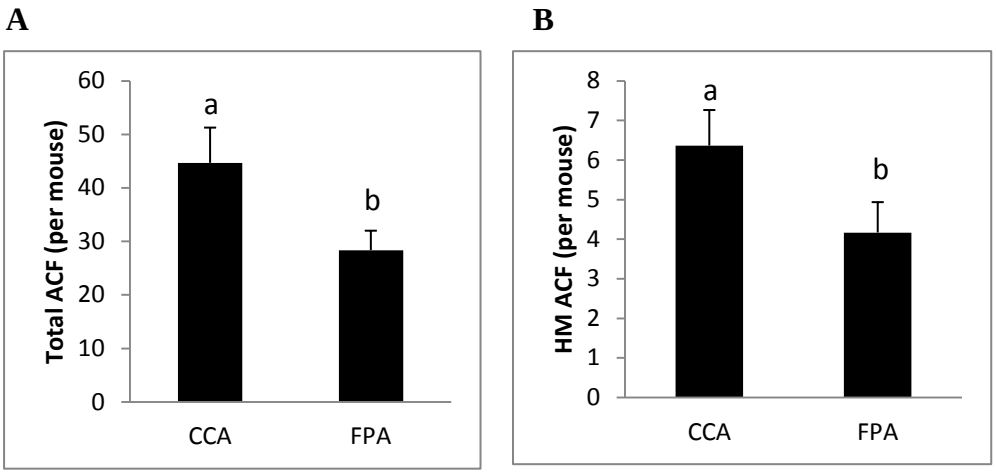

Figure 24. Effect of diet on aberrant crypt foci (ACF). (A) Mice injected with AOM and fed with a corn oil + cellulose diet (CCA) had increased total ACF and (B) HM ACF compared to those fed with the fish oil + pectin diet (FPA). All mice were injected with AOM. Bars with different letters are significantly different (P <0.05). Data represent means ± SEMs from 11 mice per treatment.

5.3.2 Assessment *of global miRNA expression in colon adult stem cells*

To globally assess the miRNA profiles in stem/progenitors cells, FACS sorting was used to isolate stem cells (GFP^{High}), daughter cells (GFP^{low}) and differentiated cells ($GFP^{negative}$). Using a TaqMan™ PCR approach, 111 mature miRNAs (critical threshold (ct) <35) were detected in all three cell types (n = 20 samples per each cell type) **(Table 11)**. In order to identify the spatial expression of miRNAs, expression profiles in intestinal stem cells vs. differentiated cells (GFP^{high} vs. $GFP^{negative}$), daughter stem cells

vs. differentiated cells (GFP^{low} vs. $GFP^{negative}$) and stem cells vs. daughter stem cells (GFP^{high} vs. GFP^{low}) were compared. Expression profiles were used to identify miRNA programs that mark the transition from stem cells to differentiated cells **(Table 12, Figure 25).** As seen in **Table 12**, out of 111 miRNAs expressed in the colon, twenty-nine were differentially expressed. Sixteen miRNAs were expressed at higher ($P<0.05$) levels in GFP^{high} cells relative to GFP^{low} cells. In contrast, thirteen miRNAs were expressed at lower ($P<0.05$) levels in GFP^{high} cells compared with $GFP^{negative}$ cells.

In order to determine whether there was evidence of differential expression of miRNAs in daughter cells, miRNA expression between GFP^{low} vs. $GFP^{negative}$ were compared. Twenty six miRNAs were significantly affected by location. Nine miRNAs were expressed at higher ($P<0.05$) levels in GFP^{low} compared to $GFP^{negative}$ cells (**Table 13)**. In contrast, seventeen miRNAs were expressed at lower ($P<0.05$) levels in GFP^{low} compared with $GFP^{negative}$ cells.

Table 11. Ct values of 113 miRNAs expressed in mouse colonic epithelial cells. Expression of miRNAs was quantified by reverse transcription using miRNA-specific primers followed by real-time PCR TaqMan low-density arrays. Ct values represent means of 60 samples, mmu, mouse; rno, rat; Ct, cross threshold.

miRNA	Ct values	miRNA	Ct values	miRNA	Ct values
mmu-miR-31	11.44	mmu-miR-99a	26.67	mmu-miR-224	28.92
rno-miR-190b	14.92	mmu-miR-671-3p	26.81	mmu-let-7g-	28.94
mmu-miR-872	17.72	mmu-miR-215	26.81	mmu-miR-103	28.95
mmu-miR-124	20.88	mmu-miR-151-3p	26.9	mmu-miR-320	29
mmu-miR-124	20.88	mmu-let-7b	26.96	mmu-miR-218	29.02
mmu-miR-128a	20.99	mmu-miR-203	26.97	mmu-miR-30d	29.2
mmu-miR-429	21.89	mmu-miR-484	27.02	mmu-miR-125b-5p	29.2
mmu-miR-148b	22.68	mmu-miR-29a	27.11	mmu-miR-125b-5p	29.2
mmu-miR-324-5p	22.88	mmu-miR-29b	27.24	mmu-miR-205	29.63
mmu-miR-322	23.04	mmu-miR-340-5p	27.41	mmu-miR-18a	29.63
mmu-miR-142-3p	23.43	mmu-miR-139-5p	27.43	mmu-miR-195	29.68
mmu-miR-142-3p	23.43	mmu-miR-15b	27.43	mmu-miR-28	29.7
mmu-miR-192	23.51	mmu-miR-139-5p	27.43	mmu-miR-574-3p	29.83
mmu-miR-200a	23.75	mmu-miR-93	27.49	mmu-miR-101a	29.83
mmu-miR-423-5p	23.88	mmu-let-7e	27.55	mmu-miR-29c	30.01
mmu-miR-375	23.94	mmu-miR-20b	27.71	rno-miR-345-3p	30.02
mmu-miR-10b	24.03	mmu-miR-27b	28.01	mmu-miR-301b	30.12
mmu-miR-19b	24.16	mmu-miR-30a	28.04	mmu-miR-146b	30.29
mmu-miR-30c	24.31	mmu-miR-27a	28.04	mmu-miR-146b	30.29
mmu-miR-92a	24.36	mmu-miR-148a	28.06	mmu-miR-744	30.34
mmu-miR-191	24.43	mmu-miR-222	28.13	mmu-miR-331-3p	30.37
mmu-miR-30b	24.75	mmu-miR-141	28.18	mmu-miR-186	30.38
mmu-miR-24	24.78	mmu-miR-188-5p	28.19	mmu-miR-196b	30.39
mmu-miR-126-3p	24.79	mmu-miR-106b	28.22	mmu-miR-340-3p	30.44
mmu-miR-17	24.91	mmu-miR-19a	28.23	mmu-miR-301a	30.45
mmu-miR-17	24.91	mmu-let-7d	28.23	mmu-miR-130b	30.49
mmu-miR-194	24.92	mmu-miR-25	28.24	mmu-miR-193b	30.68
mmu-miR-200b	24.99	rno-miR-196c	28.46	mmu-miR-155	30.77
mmu-miR-34b-3p	25.05	mmu-miR-130a	28.46	mmu-miR-155	30.77
mmu-miR-20a	25.16	mmu-miR-100	28.5	mmu-miR-152	30.8
mmu-miR-106a	25.16	mmu-miR-30e	28.57	mmu-miR-23b	30.98
mmu-miR-200c	25.38	mmu-miR-182	28.57	mmu-miR-183	31.39
mmu-let-7c	25.74	mmu-miR-328	28.59	mmu-miR-125a-5p	31.84
mmu-miR-16	25.98	mmu-let-7i	28.68	mmu-miR-181a	31.94
mmu-miR-10a	26.11	mmu-miR-26b	28.84	mmu-miR-181a	31.94
mmu-miR-145	26.32	mmu-miR-140	28.86	mmu-miR-181c	34.76
mmu-miR-26a	26.33	mmu-miR-140	28.86		

Table 11. continued

miRNA	Ct values	miRNA	Ct values	miRNA	Ct values
mmu-miR-21	26.48	mmu-miR-146a	28.87		

Comparison of the significantly differentially expressed miRNAs between GFP^{high} vs. $GFP^{negative}$, and GFP^{low} vs. $GFP^{negative}$ cells revealed that seven miRNAs that were commonly downregulated and ten were upregulated in both stem cells and daughter cells compared to differentiated cells **(Figure 25).** These data suggest that stem cells and their daughter cells exhibit a similar miRNA profile. In contrast, miRNA profiles in stem cells and differentiated cells were quite distinct.

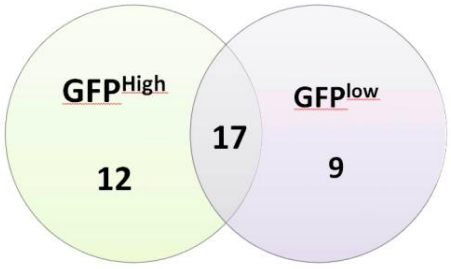

Figure 25. Common differentially expressed miRNAs in GFP^{high} and GFP^{low} cells. The Venn diagram indicates the number of differentially expressed miRNAs in GFP^{high} vs. $GFP^{negative}$ cells and GFP^{low} vs. $GFP^{negative}$ cells (n=20 mice per cell type).

Table 12. Differentially regulated miRNAs in GFP^{high} compared with GFP^{negative} cells. Expression of miRNAs were quantified as described in Table 11. GFP^{high} stem cells (n=20); GFP^{negative} cells (n=20). Only miRNAs with P <0.05 are shown. mmu, mouse; rno, rat.

Upregulated in GPF^{high}			Downregulated in GPF^{high}		
miRNA	Expression ratio GFP^{high}/ GFP^{negative}	*P*-value	miRNA	Expression ratio GFP^{high}/ GFP^{negative}	*P*-value
mmu-miR-155	2.63	0.034	mmu-miR-652	0.31	0.001
mmu-miR-342.3p	2.29	0.000	mmu-miR-27a	0.42	0.001
rno-miR-345-3p	2.24	0.040	mmu-miR-215	0.43	5.72E-06
rno-miR-190b	1.99	0.027	mmu-miR-30d	0.51	0.024
mmu-miR-17	1.72	6.29E-05	mmu-miR-7b	0.52	0.040
mmu-miR-191	1.68	0.000	rno-miR-224	0.53	0.002
mmu-miR-20b	1.46	0.011	mmu-miR-532 5p	0.53	0.008
mmu-miR-106a	1.46	0.024	mmu-miR-21	0.56	0.012
mmu-miR-218	1.45	0.003	mmu-miR-124	0.63	0.031
mmu-miR-186	1.42	0.008	mmu-miR-30a	0.63	0.001
mmu-miR-125a-5p	1.39	0.031	mmu-miR-29a	0.64	0.019
mmu-miR-92a	1.29	0.017	mmu-miR-200b	0.65	0.008
mmu-miR-146b	1.27	0.044	mmu-let-7g	0.66	0.040
mmu-miR-200a	1.27	0.019	mmu-miR-103	0.69	0.031

Table 13. Differentially regulated miRNAs in GFP^{low} compared with GFP^{negative} cells.
Expression of miRNAs were quantified as described in Table 11. GFP^{low} stem cells (n=20); GFP^{negative} cells (n=20). Only miRNAs with P <0.05 are shown. mmu, mouse; rno, rat.

Upregulated in GFP^{low}			Downregulated in GPF^{low}		
miRNA	Expression ratio GFP^{low}/ GFP^{negative}	*P*-value	miRNA	Expression ratio GFP^{low}/ GFP^{negative}	*P*-value
mmu-miR-155	2.88	0.029	mmu-miR-27a	0.39	5.34E-05
mmu-miR-342-3p	2.23	0.001	mmu-miR-128a	0.41	0.008
mmu-miR-106a	1.70	0.007	mmu-miR-652	0.43	0.002
mmu-miR-20b	1.63	0.001	mmu-miR-215	0.47	0.001
mmu-miR-31	1.55	0.013	mmu-miR-29a	0.54	0.012
mmu-miR-17	1.51	0.000	mmu-miR-484	0.55	1.53E-05
mmu-miR-218	1.37	0.004	mmu-miR-30d	0.58	0.001
mmu-miR-93	1.30	0.015	mmu-miR-200c	0.62	0.001
mmu-miR-92a	1.28	0.009	mmu-miR-532-5p	0.62	0.036
			mmu-miR-30a	0.63	0.001
			mmu-miR-21	0.64	0.030
			mmu-miR-331-3p	0.66	0.027
			mmu-miR-365	0.66	0.040
			rno-miR-224	0.68	0.036
			rno-miR-196c	0.68	0.044
			mmu-miR-28	0.69	0.035
			mmu-miR-200b	0.69	0.015

5.3.3. miR-125a-5p, miR-190 and miR-191 are differentially expressed in stem cells compared to daughter cells

In order to determine if certain miRNAs were uniquely expressed in stem cells, miRNA in stem cells (GFP^{high}) and daughter stem cells (GFP^{low}) were compared. We observed that miR-125a-5p, miR-190 and miR-191 were enriched in the stem cells compared to daughters cells **(Figure 26)**. Interestingly, miR-190, miR-191 and miR-125a-5p were also significantly ($P < 0.05$) upregulated in stem cells compared to differentiated cells **(Table 13)**. This suggests that miR-190, miR-191 and miR-125a-5p may be required by stem cells and their expression decreases as cells differentiate.

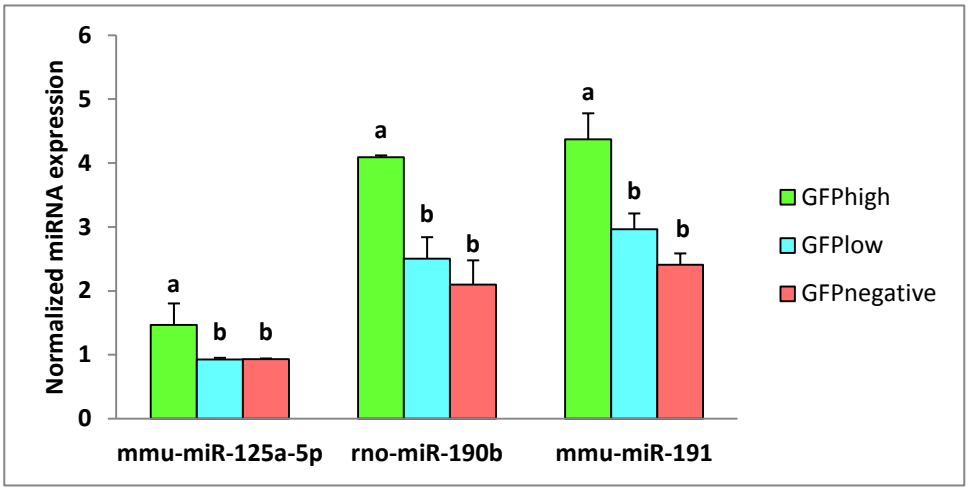

Figure 26. miR-125a-5p, miR-190b and miR-191 are intestinal stem cell specific miRNAs. Data represent normalized miRNA expression (means ± SEM, for details, see Materials and methods). miRNA profiling in stem cells (GFP^{high}), daughter cells (GFP^{low}) and differentiated cells ($GFP^{negative}$) by real time PCR was used to quantify miR-125a-5p, miR-190b and miR-191 expression. Bars with different letters are statistically different ($P < 0.05$). (n=20 per cell type). mmu, mouse; rno, rat.

5.3.4. Diet modulates stem cell miRNAs following exposure to carcinogen and saline (control)

To determine the effects of chemoprotective diets on stem cell miRNAs, single cells isolated from the colon of carcinogen injected mice fed corn oil + cellulose (CCA) or fish oil + pectin (FPA) diet were FACS sorted to obtain stem cells ,daughter cells and differentiated cells. Global miRNA profiling was subsequently performed. As shown in **Table 14**, seventeen miRNAs were differentially expressed ($P \leq 0.05$), fifteen of which were upregulated and two miRNAs were downregulated in CCA vs. FPA treatment. These miRNAs were not significantly changed in the stem cells in presence of saline (CCS vs. FPS) **(Table 14)**. Moreover, miR-21, that was downregulated in stem cells compared to differentiated cells, was upregulated in stem cells, specifically by CCA **(Figure 27)**.

With respect to dietary fat x fiber effects in presence of carcinogen (CCA vs. FPA) in differentiated cells ($GFP^{negative}$), nineteen miRNAs were significantly ($P \leq 0.05$) altered (**Table 15)**. Out of the nineteen miRNAs, miR-19b and miR-18a were significantly ($P \leq 0.05$) downregulated in CCA compared to FPA. This is consistent with a previous study where colonic scraping was used to assess miRNA expression in CCA vs. FPA treatments in rats (151). These miRNAs were not altered in the differentiated cells ($GFP^{negative}$) when fat x fiber effects were analyzed in presence of saline (i.e CCS vs. FPS) **(Table 15)**

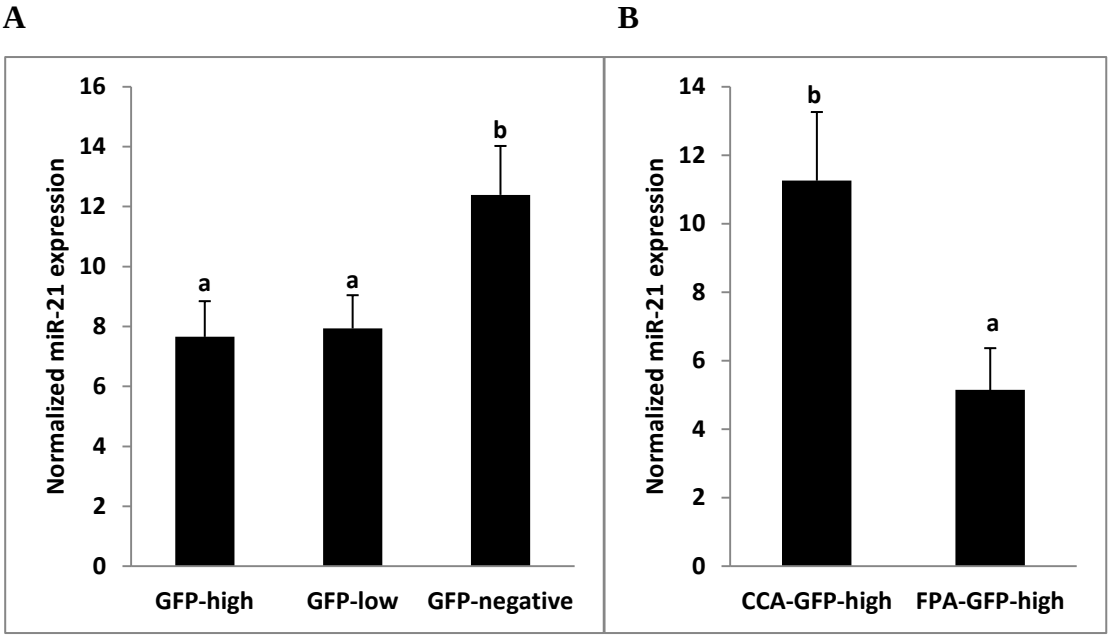

Figure 27. Diet and carcinogen modulate miR-21 in intestinal stem cells. (A) Expression of miR-21 in stem cells (GFP^{high}), daughter cells (GFP^{low}), and in differentiated cells ($GFP^{negative}$), n=20 mice per cell type. (B) Expression of miR-21 in intestinal stem cells (GFP^{high}) from Lgr5-EGFP-IRES-creER^{T2} *knock in* mice injected with azoxymethane (AOM) and fed a corn oil + cellulose diet (CCA) or fish oil + pectin diet (FPA), n=5 mice per treatment). Bars with different letters are statistically different ($P < 0.05$). Data represent means $\pm$ SEMs of normalized miRNA expression (for normalization and statistical analyses details, please refer to Materials and methods).

Table 14. Effect of diet on miRNA expression in GFP^{high} sorted cells. Expression of miRNAs was quantified as described in Table 11. CCA, Corn oil + cellulose + azoxymethane (AOM) (n = 5); FPA, Fish oil + pectin + AOM (n = 5); CCS, Corn oil + cellulose + saline (n = 5); FPS, Fish oil + pectin + saline (n = 5); GFP^{high}, stem cells. Only miRNAs with $P \leq 0.05$ are shown.

Carcinogen injection

miRNA	Expression ratio CCA-GFP^{high} / FPA-GFP^{high}	*P*-value
mmu-miR-106b	2.46	0.028
rno-miR-190b	2.32	0.050
mmu-miR-27b	2.24	0.027
mmu-miR-21	2.19	0.047
mmu-miR-31	2.01	0.043
mmu-miR-26b	2.00	0.028
mmu-miR-200a	1.83	0.009
mmu-miR-26a	1.68	0.016
mmu-miR-29c	1.59	0.016
mmu-miR-182	1.55	0.025
mmu-miR-30c	1.47	0.047
mmu-miR-203	1.47	0.016
mmu-miR-30a	1.40	0.047
mmu-miR-19b	1.33	0.034
mmu-miR-24	1.29	0.047
mmu-miR-181a	0.56	0.028
mmu-miR-101a	0.49	0.050

Saline Injection

miRNA	Expression ratio CCS-GFP^{high} / FPS-GFP^{high}	*P*-value
mmu-miR-188-5p	5.25	0.027
mmu-miR-218	0.57	0.016
mmu-miR-125a-5p	0.52	0.047
mmu-miR-574-3p	0.47	0.028
mmu-miR-200c	0.47	0.047
mmu-miR-222	0.43	0.016
mmu-miR-429	0.31	0.034
mmu-miR-106a	0.12	0.047

Table 15. Effect of diet and carcinogen combination on miRNA expression in GFP^{negative} sorted cells. Expression of miRNAs was quantified as described in Table 11. CCA, Corn oil + cellulose + azoxymethane (AOM) (n = 5); FPA, Fish oil + pectin + AOM (n = 5); CCS, Corn oil + cellulose + saline (n = 5); FPS, Fish oil + pectin + saline (n = 5); GFP^{neg}, differentiated cells. Only miRNAs with $P \leq 0.05$ are shown.

Carcinogen Injection

miRNA	Expression ratio CCA-GFP^{neg} / FPA-GFP^{neg}	*P*-value
mmu-miR-29b	3.84	0.025
mmu-miR-142-3p	3.30	0.043
mmu-miR-99a	2.38	0.050
mmu-miR-188-5p	2.31	0.050
mmu-let-7e	2.14	0.016
mmu-miR-27a	1.84	0.034
mmu-miR-186	1.47	0.050
mmu-let-7c	1.33	0.047
mmu-let-7d	1.26	0.034
mmu-miR-10a	0.63	0.034
mmu-miR-19b	0.61	0.009
mmu-miR-484	0.54	0.028
mmu-miR-365	0.45	0.014
mmu-miR-103	0.45	0.034
mmu-miR-19a	0.43	0.028
mmu-miR-18a	0.41	0.050

Saline Injection

miRNA	Expression ratio CCS -GFP ^{neg} / FPS-GFP^{neg}	*P*-value
rno-miR-345-3p	2.90	0.014
mmu-miR-146a	1.77	0.009
rno-miR-196c	1.50	0.028
mmu-miR-140	1.43	0.016
mmu-miR-200b	0.61	0.028
mmu-miR-181a	0.56	0.047
mmu-miR-126-3p	0.37	0.025

5.4. Discussion

Recent findings lend support to the concept that in some circumstances, carcinogen exposure can perturb homeostasis within the stem cell compartment and promote malignant transformation (394). "Adult" somatic cells of the colon are of particular interest because they sustain self-renewal capacity and are targets for cancer initiating mutations (218, 219). Lgr5 is regarded as a specific marker for identification of intestinal stem cells (225). Therefore it is important to know precisely how intestinal stem cell populations respond to environmental factors, specifically bioactive dietary agents.

To date, the effect of n-3 PUFA/pectin on miRNAs in stem cells during initiation of colon cancer has not been documented. Hence, in this study we used the Lgr5-EGFP-IRES-creER^{T2} *knock in* mouse model to examine miRNA expression profiles in stem cells, daughter and differentiated cells. Moreover, since transformation of adult stem cells is an extremely efficient route towards initiation of intestinal cancer (228), Lgr5-EGFP-IRES-creER^{T2} *knock in* mice were injected with azoxymethane (AOM, a colon carcinogen) and the response to a chemoprotective n-3 PUFA / fermentable fiber diet was assessed. The AOM-induced colon tumor model was selected because it provides a clear distinction between tumor initiation and promotion (43).

We hypothesized that the miRNA expression profiles would differ between stem cells and differentiated cells. Of the 384 rodent miRNAs identified, twenty nine miRNAs in stem cells were significantly altered compared to differentiated cells **(Table**

12). Seventeen out of the twenty-nine miRNAs shared similar patterns of expression in daughter and differentiated cells **(Figure 24)**. Also, miR-215 was reduced in both stem cells and daughter stem cells compared to differentiated cells. These results are consistent with recent findings that miR-215 regulates G1 and G2-M cell cycle arrest (395, 396). Stem cells need to proliferate to maintain the continuously self-renewing intestinal epithelium and hence components responsible for cell cycle arrest are generally repressed.

miR-17 and miR-92a are members of a well-known miRNA cluster miR-17-92 (397), and were highly expressed in both stem cells and daughter stem cells. In addition, miR-106a, a member of the miR-106a-363 cluster (a paralog of the miR-17-92 cluster) was also highly expressed in stem cells. Recent studies have reported similar findings wherein in the hematopoietic compartment, miR-17-92 is highly expressed in stem cells and early progenitors and decreased upon onset of myeloid and lymphoid differentiation (148, 322, 398) and during *in vitro* differentiation of acute leukemia blast cells (399, 400).

To determine stem cell specific miRNAs, miRNA profiles were compared in GFP^{high} and GFP^{low} cells. Interestingly, three miRNAs, miR-125a-5p, miR-190 and miR-191 exhibited significantly ($P < 0.05$) higher levels in the stem cells compared to the daughter cells and the differentiated cells **(Figure 26)**. These miRNAs were not differentially expressed in daughter stem cells compared with differentiated cells, suggesting that they might serve a specific role in intestinal stem cells. Additional studies are needed to elucidate the role of these miRNAs in colon cancer.

An interesting outcome of this study was the fact that effects of the chemoprotective diet on miR-21 expression was dependent on cell location within the crypt. Upon comparison of miRNA expression profiles in stem cells obtained from mice fed a corn oil + cellulose vs fish oil + pectin diet in the presence of carcinogen (i.e. CCA-GFP^{high} vs. FPA-GFP^{high}), miR-21 was significantly upregulated in the CCA group compared to FPA. However, diet did not influence miR-21 levels in daughter cells or differentiated cells. The fact that, diet and carcinogen in stem cells selectively increase miR-21 expression stem cells demonstrates for the first time that intestinal stem cells are uniquely sensitive to environmental factors. This is noteworthy because, miR-21, a well-known "oncomiR" upregulated in several cancers, including colon cancer, is downregulated by other bioactive dietary agents such as resveratrol, docosahexanoic acid (DHA), retinoic acid and curcumin in several cancers, along with the upregulation of one of its targets, e.g., PTEN, a well-known tumor suppressor (175, 198, 401-403) **(Figure 28).** With respect to cell location within the colonic crypte, miR-21 was significantly reduced (P <0.05) in stem cells compared to differentiated cells **(Figure 27)**. Recent studies examining embryonic stem cells have reported similar findings, wherein miR-21 expression increased following induction of mouse embryonic stem cell differentiation (404, 405). Additionally, REST (RE1- silencing transcription factor), a key gene that maintains the self-renewal and pluripotency of mouse embryonic stem cells, inhibits the biogenesis of miR-21 in embryonic stem cells. Moreover, using gain and loss of function studies targeting pre-miR-21, miR-21 was shown to be one of the

regulators of self-renewal in mouse ES cells. Also, bioinformatics analyses showed that miR-21 is predicted to target Nanog and Sox2, which are markers of self-renewal (405).

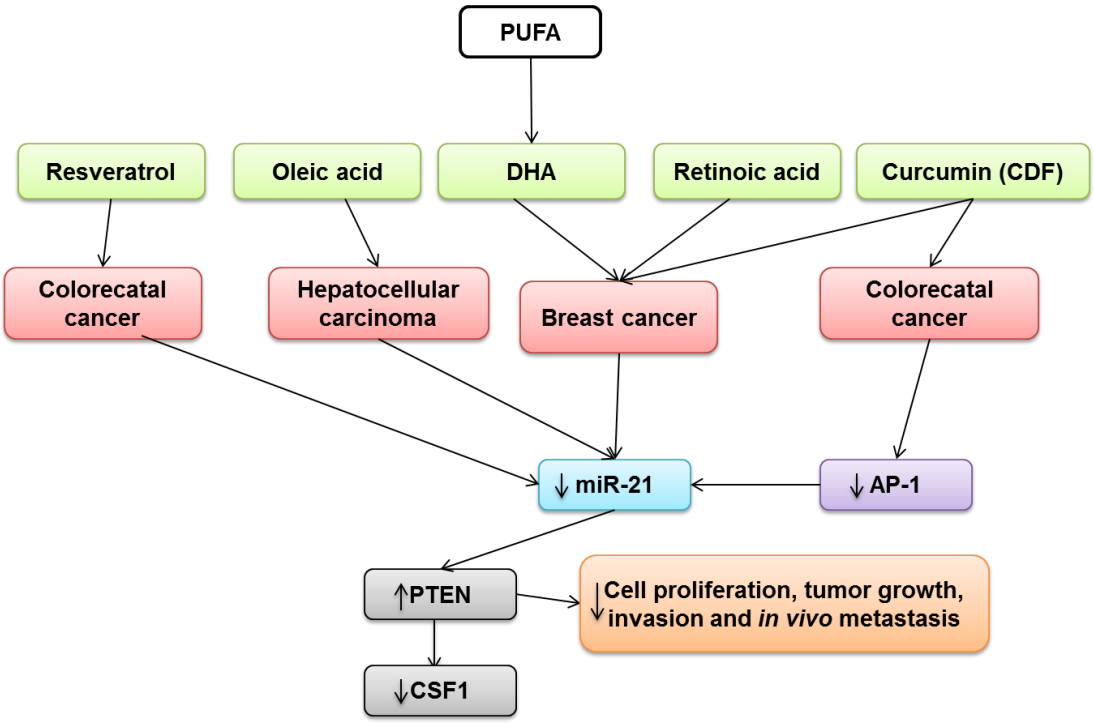

Figure 28. miR-21 is targeted by chemoprotective bioactive dietary agents. miR-21, a well-defined oncogene is downregulated by several dietary agents such as resveratrol, oleic acid, DHA, retinoic acid and curcumin in different cancer cell types, resulting in the upregulation of one of its targets-PTEN, a well-known tumor suppressor. Colorectal cancer cells treated with curcumin decreased AP-1, a transcription factor which reduced mature miR-21 formation. This is associated with decreased cell proliferation, tumor growth, invasion and *in vivo* metastasis and repression of its downstream target-CSF1. DHA, docosahexanoic acid; AP-1, activator protein 1; PTEN, phosphatase and tensin homolog; CSF, colony stimulating factor 1.

Another interesting outcome of the study was that dietary bioactive agents could differently modulate miRNAs based on their location in the colonic crypt. In, stem cells, miR-19b, miR-27b, miR-26b and miR-203 were upregulated by CCA compared to FPA. However, in differentiated cells, miR-19b and miR-18a were significantly downregulated (P <0.05) in CCA fed mice, which was also observed in our previous study in which scraped mucosa (containing heterogeneous population of cells) from the rat colon was used (151). The scrapped mucosa predominantly contains differentiated cells. Hence, these data suggest that stem cells respond uniquely to dietary agents.

In order to elucidate the role of miRNAs in stem cells, it is important to determine the function of these miRNAs by identifying their targets. This can be determined by performing global mRNA profiling by RNA sequencing from the same samples that were used to determine the miRNA changes. An integrative analysis can be performed by combining mRNA expression and miRNA expression with the help of miRNA target prediction.

In summary, our data indicate for the first time that select dietary cues can impact stem cell regulatory networks, in part, by modulating the steady-state levels of miRNAs such as miR-19b, miR-21, miR-26b and miR-203. To our knowledge, this is the first study to utilize $Lgr5^{+}$ reporter mice to determine the impact of diet and carcinogen on miRNA expression in intestinal stem cells.

6. SUMMARY AND CONCLUSIONS

6.1 Summary

In rats injected with carcinogen, administration of fish oil + pectin (FPA) vs control (corn oil + cellulose (CCA)) diet upregulated miR-19b, miR-26b and miR-203, while downregulating the mRNA and the protein expression of their respective targets – PTK2B, PDE4B and TCF4 in colonic mucosa. Upregulation of these miRNAs increased apoptosis and decreased cell proliferation. These data suggest that fish oil + pectin diet protects against carcinogen induced colon tumorigenesis, in part, by modulating the above mentioned miRNAs and their targets. Additionally, we demonstrated for the first time that miR-125a-5p, miR-190 and miR-191 are enriched in colon stem cells compared to differentiated cells. Moreover, FPA compared to CCA, specifically modulated the expression of miR-18a, miR-19b, miR-26b and miR-203 based on their location in the colonic crypt. Also, FPA decreased the expression of miR-21, an "oncomiR" specifically in the stem cells compared to CCA. The effect of fish oil + pectin combination on miRNA expression and their targets on mucosal physiology is summarized in **Figures 29** and **30**.

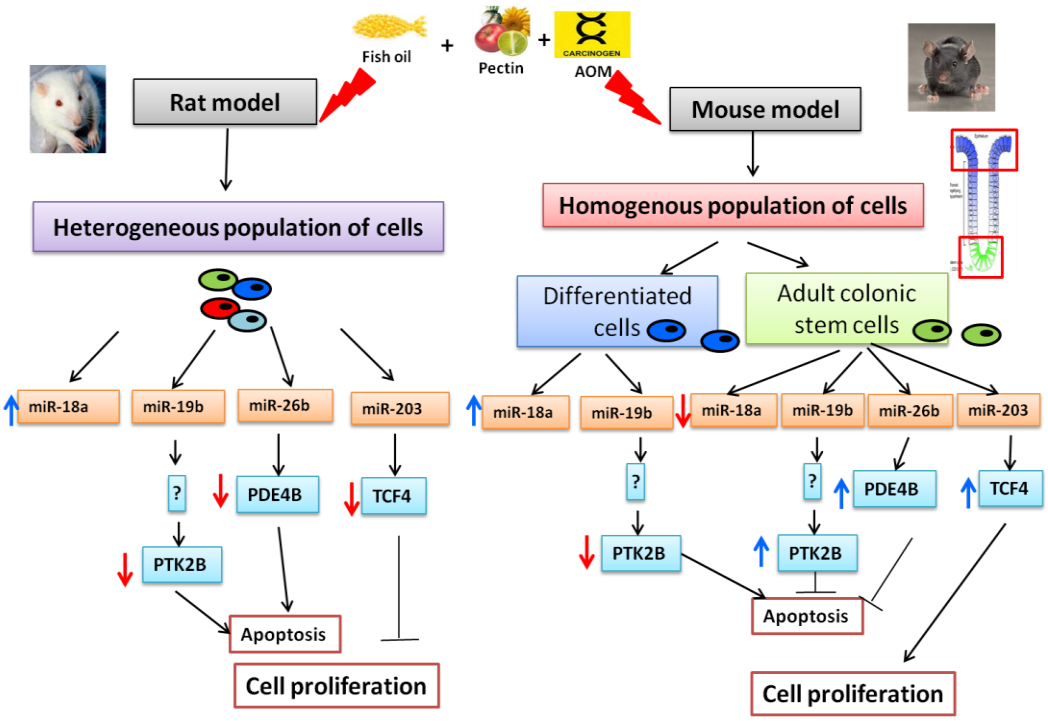

Figure 29. Fish oil and pectin combination alters expression of miRNAs in the colonic crypt. In AOM injected Sprague Dawely rats, the chemoprotective diet i.e., fish oil + pectin (FPA) feeding, increased the expression of miR-18a, miR-19b, miR-26b and miR-203 and decreased the expression of target genes, PTK2B, PDE4B and TCF4 in a mixed (heterogeneous) population of mucosal colonic cells. FPA as compared to CCA increased apoptosis and reduced cell proliferation. In AOM injected C57BL/6 mice, FPA also increased the expression of miR-18a and miR-19b in differentiated cells, while decreasing their levels in stem cells. Solid arrows show observations, while dashed arrows indicate the predicted associations. PTK2B, protein tyrosine kinase 2 beta; PDE4B, phosphodiesterase 4B; TCF4, transcription factor 4.

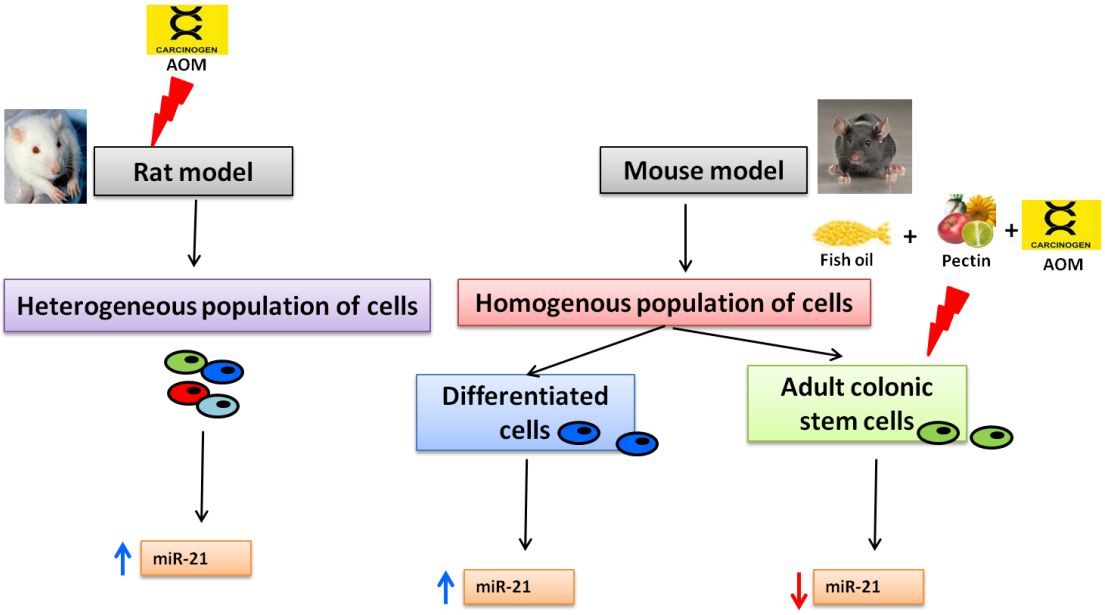

Figure 30. Expression of miR-21 in rodents. Expression of miR-21 was higher in a mixed (heterogeneous) population of mucosal colonic cells from AOM injected rats compared to saline (control) injected Sprague Dawley rats. In C57BL/6 mice, expression of miR-21 was also higher in differentiated cells compared to stem cells. In AOM injected mice, fish + pectin diet decreased the expression of miR-21 specifically in the stem cells as compared to corn oil cellulose diet.

6.2 Conclusions

miRNAs target critical genes known to regulate apoptosis, cell proliferation and differentiation (63-69, 73, 75-78, 406).

The role for n-3 PUFA in prevention and treatment of colon cancer is well documented, but the efficacy of n-3 PUFA is still a matter of contention. Despite cogent scientific evidence indicating the protective effect of fish oil and fiber, there is a lack of data regarding the molecular mechanisms by which these dietary agents protect against colon tumorigenesis, especially with respect to the role of miRNAs. Hence, we determined whether the synergistic effects of chemoprotective dietary n-3 PUFA and fermentable fiber are mediated by impacting intestinal miRNA signatures.

In the absence of comprehensive human data, the azoxymethane (AOM) chemical carcinogenesis model serves as one of the most definitive means of assessing human colon cancer risk (265, 281). Therefore, the first portion of the study was designed to determine the effects of cancer progression on miRNA expression in rats exposed to AOM. We observed that common global miRNA expression patterns (for example, miR-21) exist in human and rat colon tumors, confirming the utility of the model. In addition, *n*-3 PUFA-enriched chemoprotective diets suppressed the effects of AOM on let-7d, miR-15b, miR-107, miR-191 and miR-324-5p. We also demonstrated that BACE1 and PTEN are targets of miR-107 and miR-21, respectively **(Section 2)**.

To further elucidate the biological effects of diet and carcinogen on miRNAs, we integrated global miRNAs, total and polysomal gene expression datasets obtained from the above mentioned study. Four complementary computational approaches were

utilized, including gene set enrichment analysis (GSEA), coherent analysis, linear discriminant analysis (LDA) and pathway analysis in the context of three biological conditions: (a) tumor vs saline; (b) dietary fat effects in the presence of carcinogen (CA vs. FA); and (c) dietary fat x fiber interaction (CCA vs. FPA) in the presence of carcinogen **(Section 3)**. miR-206 was significantly enriched with respect to both total and polysomal mRNA targets. These data suggest that polysome tracking of transcripts and miRNAs play an important role in experimental colon carcinogenesis. Using coherent expression analysis of miRNAs and their targets in the above mentioned comparisons, we identified several miRNAs, e.g. miR-15b, miR-103, and miR-107 which were significantly altered in tumor vs saline comparisons along with their predicted targets. With respect to diet, miR-19b, miR-26b, miR-200c and miR-203 and their putative targets PTK2B, IGF1R, PDE4B and TCF4, respectively, were modulated. **(Section 3)**. These results demonstrate for the first time a novel role for fish oil/pectin feeding in protecting the colon from carcinogen–induced miRNAs dysregulation. Importantly, the data link dietary fish oil/pectin treatment with canonical pathways regulating intestinal stem cell fate.

In complementary experiments **(Section 4)**, we further elucidated the biological role of miR-19b, miR-26b and miR-203 in colon cancer. Using a series of loss and gain of function experiments along with luciferase reporter assays, we demonstrated that PDE4B and TCF4 are direct targets of miR-26b and miR-203, respectively. PTK2B was an indirect target of miR-19b. Also, phenotypic assays showed that these miRNAs

confer protection against colon cancer by increasing apoptosis and decreasing cell proliferation.

Our data demonstrate that chemoprotective diets are capable of modulating key intestinal stem cell regulators. For example, TCF4, a key component of the Wnt signaling pathway which is essential for intestinal stem cells, was suppressed by the fish oil + pectin diet following exposure to AOM **(Section 3).** As described in **Section 5**, we demonstrated that based on the relative expression of miR-125a-5p, miR-190b and miR-191, these miRNAs may be stem cell specific miRNAs. With respect to the effect of dietary fat x fiber in presence of carcinogen (CCA vs. FPA) on miRNA expression in intestinal stem cells, we noted that miR-19b, miR-18a and miR-27b were altered in a manner similar to our previous studies, which utilized colonic scraped mucosa to assess miRNA expression. We also identified miR-21, a well-known "oncomiR" in colon cancer, to be significantly reduced in stem cells compared to differentiated cells. Interestingly, miR-21 was selectively upregulated by corn oil - cellulose feeding in only stem cells, but not in daughter or differentiated cells. This suggests that stem cells selectively respond to environmental factors. In order to understand the mechanism of action of these miRNAs, integration of miRNAs with mRNA expression datasets should be carried out as described above.

The work described herein is noteworthy and elucidates mechanisms by which n-3 PUFA inhibit colon carcinogenesis. Understanding the effect of bioactive dietary factors on miRNA expression and function may provide insight on prevention strategies to reduce the burden on cancer. In colon cancer, cancer stem cells play an important role

in cancer recurrence. Hence, in order to develop therapies to target these cells, it is important to identify the regulatory mechanisms and signaling pathways that impact the colon stem cell niche. Results from our study and the published literature point to the ability of bioactive food components to modify the self-renewal capabilities of cancer stem cells by modifying molecular targets (407, 408).

6.3 Future directions

The research presented herein sets the groundwork for a number of future studies that could further explore the effects of omega-3 PUFA and fermentable fiber on intestinal miRNAs and their targets.

6.3.1 Validation of in vivo results

We determined that FPA treatment in mice increased the expression of miR-26b and miR-203, while decreasing the expression of their targets, PDE4B and TCF4, respectively. *In vitro* models are a powerful tool to determine the mechanism of action of dietary agents on modulation of miRNA expression (409). Hence, in order to determine how bioactive dietary agents modulate miRNA expression, the first step would be to recapitulate the results seen *in vivo* study **(Section 2 and 3)** by treating HCT116 cells with bioactive dietary agents such as DHA and EPA (main components of fish oil) and butyrate (fermented product of pectin) and assessing the mRNA and protein expression of miR-26b and miR-203 and their respective targets. HCT116 is a human colon cancer cell line, which has a mutation in serine at codon 45 of β-catenin. This is one of the amino acid residues typically mutated in several tumors (410, 411). This cell line also

has genetic mutations of K-RAS, MLH1, PIK3CA (412, 413) and the expression for dinucleotide-repeat (microsatellite instability) similar to human hereditary nonpolyposis colon cancers (414). Moreover, the HCT116 cell line has been utilized to probe the synergy between DHA and butyrate with respect to apoptosis (58). Future experiments should validate the *in vivo* results and also determine that HCT116 cell line can be utilized to evaluate the effects of nutritional bioactives on downstream mediators of PDE4B and TCF4.

6.3.2 Determining downstream mediators of PDE4B in the colon

In this study, FPA increased the expression of miR-26b while reducing the expression of its targets-PDE4B as compared to CCA. Moreover, we also demonstrated that induction of miR-26b induced apoptosis. Previous studies from the lab have demonstrated that fish oil + pectin combination enhances colonocyte apoptosis in colon cancer (58, 59, 247, 415). In order to determine how miR-26b induces apoptosis in colonic cells, it is necessary to probe the downstream mediators of one of its targets, PDE4B. In diffuse large B-cell lymphoma, it was shown that inhibition of PDE4B activated c-AMP induced apoptosis, which was associated with inhibition of the phosphatidylinositol 3- kinase (PI3K)/ AKT pathway (329). However, this mechanism of action has not been determined in colonic cells. Hence, one potential way to study this would be to treat HCT116 cells with rolipram, a PDE4B inhibitor (416) and measure the cellular cyclic-AMP levels. This will help to clarify the function of miR-26b in the intestine.

6.3.3 Determine methylation status of miR-203 promoter in colon cancer

We have demonstrated that FPA increased miR-203 levels and decreased the expression of its target gene TCF4. There is evidence suggesting that in cancer, due to the hypermethylation of miR-203 promoter, transcription of miR-203 is reduced (351, 417, 418). Therefore, it would be interesting to determine if bisulfite reactions indicate a differential methylation pattern at the mir-203 promoter locus. This would determine whether epigenetic modifications such as DNA methylation alterations are modulated by dietary n-3 PUFA and fiber in colonic cells and mediate miRNA expression.

6.3.4 Role of miR-203 in invasion and migration in colon cancer

miR-203 has been shown to inhibit tumor cell migration and invasiveness in prostate, breast and esophageal squamous cell cancers, partly by reducing the expression of several targets such as SNAI2 and LASP1 (351, 419-422). Moreover the target of miR-203, TCF4, was shown to promote invasion in breast cancer cells (422). However, the effect of miR-203 in invasion and migration is not yet known, hence, future studies should focus on determining the role of miR-203. One potential way to study this is to overexpress miR-203 in human colon cancer cells, followed by cell migration and wound healing assays. Also, tumorigenicity assays *in vivo* can be carried by transplanting HCT116 cells transfected with miR-203 mimics or inhibitors into xenograft nude mice. Tumor growth can be assessed which will indicate the protective effect of miR-203 in colon cancer. This would provide a further understanding of the role of miR-203.

6.3.5 Determining downstream mediators of miRNAs enriched in stem cells

We carried out a global miRNA profiling in mouse colonic stem cells, daughter stem cells and differentiated cells. It was shown for the first time that miRNAs are differentially expressed in the three cell lineages. Also, the chemoprotective diet (fish oil + pectin combination) in AOM injected mice (FPA) modulated expression of several miRNAs, such as miR-18a, miR-19b and miR-21 based on their location in the colonic crypt. There is evidence suggesting that miRNA-gene regulatory pathways establish stem cell identity (423, 424). However, to date the effect of these dietary agents on mRNA targets in stem cells during different stages (initiation, promotion and progression) of colon cancer development has not yet been elucidated. We hypothesized that FPA will suppress growth of colonocytes /crypts by regulating genes involved in stem cell signaling, in part through miRNA-mediated mechanisms. To elucidate the biological functions of these miRNAs, mRNA expression changes can be determined by performing global mRNA profiling by RNA sequencing from the same samples that were used to determine the miRNA changes. Hence, an integrative analysis can be performed by combining mRNA expression and miRNA expression with the help of miRNA target prediction. We predict that novel stem cell signaling networks and markers of intestinal stem cells will be identified (151, 375).

6.3.6 Conditional expression of miR-21 in mouse colon stem cells

One of the interesting observations from our study was the modulation of miR-21 expression in stem cells. Specifically, we observed that miR-21 expression was higher in the differentiated cells compared to stem cells. Interestingly, the expression of miR-21

was significantly reduced by FPA vs control (CCA) treatment. This is noteworthy, because recent evidence has shown that miR-21 and its targets are modulated by several dietary agents in cancers of other tissue types (175, 198, 401-403). Hence, to elucidate the biological function of miR-21 in colon stem cells and to understand how chemoprotective diets modulate its expression, it is necessary to identify the targets of miR-21 and show that they are inversely correlated with miR-21 expression. One of the ways to determine the biological role of miR-21 is to use a mouse model that conditionally expresses miR-21 (425). The expression of miR-21 in this mouse model is Cre/*loxP* dependent and tetracycline-dependent at a *Rosa*26 locus. Hence, on crossing this mouse with Lgr5-EGFP, we can generate a mouse model that specifically overexpresses miR-21 in LGR5 positive stem cells. Dietary intervention studies in this model system, such as feeding with fish oil + pectin combination followed by assessment of changes in the expression of miR-21 and its downstream targets will help to elucidate the role of miR-21 in intestinal stem cell biology.

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APPENDIX A

LIST OF SEQUENCES OF MIRNA MIMICS, INHIBITORS & THEIR CONTROLS

(1) miRNA inhibitors:

miR-19b: 5' CAGTTTTGCATGGATTTGCAC 3'
miR-26b: 5' ACCTATCCTGAATTACTTGA 3'
miR-203: 5' TAGTGGTCCTAAACATTTCA 3'
Scrambled control: 5' GTGTAACACGTCTATACGCCA 3'

(2) miRNA mimics:

miR-19b:5' UGUGCAAAUCCAUGCAAAACUGA 3'
miR-26b: 5' UUCAAGUAAUUCAGGAUAGGU 3'
miR-203: 5' GUGAAAUGUUUAGGACCACUAG 3'
Scrambled control: 5' UCACAACCUCCUAGAAAGAGUAGA 3'

(3) Morpholino sequence:

PDE4B target protector: 5' TCTGACTCCAAACTCAAGTAACTGA 3'

(4) Target 3' UTR sequences

PTK2B 3' UTR sequence:

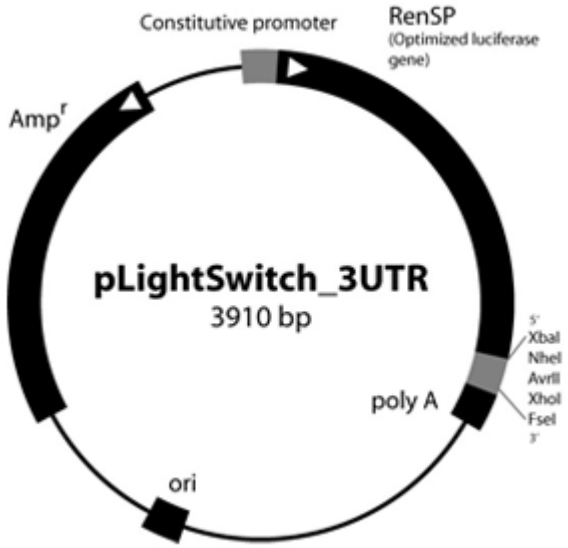
Gene accession: NM_004103.4
Insert size: 973bp

5'
AAGAACCTGCTCGACGCTGTGGACCAGGCCAAGGTTCTGGCCAATCT
GGCCCACCCACCTGCAGAGTGACGGAGGGTGGGGGCCACCTGCCTG
CGTCTTCCGCCCCTGCCTGCCATGTACCTCCCCTGCCTTGCTGTTGGTC
ATGTGGGTCTTCCAGGGGGAAGGCCAAGGGGAGTCACCTTCCCTTGC
CAC**TTTGCAC**GACGCCCTCTCCCCACCCCTACCCCTGGCTGTACTGCT
CAGGCTGCAGCTGGACAGAGGGGACTCTGGGCTATGGACACAGGGT
GACGGTGACAAAGATGGCTCAGAGGGGGACTGCTGCTGCCTGGCCA
CTGCTCCCTAAGCCAGCCTGGTCCATGCAGGGGGCTCCTGGGGGTGG
GGAGGTGTCACATGGTGCCCCTAGCTTTATATATGGACATGCAGGCCG
ATTTGGGAACCAAGCTATTCCTTTCCCTTCCTCTTCGGCCCTCAGATGT
CCCTTGATGCACAGAGAAGCTGGGGAGGAGCTTTGTTTTGGGGGTCA
GGCAGCCAGTGAGATGAGGGATGGGCCTGGCATTCTTGTACAGTGTAT
ATTGAAATTTATTTAATGTGAGTTTGGTCTGGACTGACAGCATGTGCCC

TCCTGAGGGAGGACCTGGGGCACAGTCCAGGAACAAGCTAATTGGG
AGTCCAGGCACAGGATGCTGTGTTGTCAACAAACCAAGCATCAGGGG
GAAGAAGCAGAGAGATGCGGCCAAGATAGGACCTTGGGCCAAATCC
GCTCTCTTCCTGCCCCTCTTTCTCTTTCTTCCTTTACTTTCCCTTGCTTT
TCCCTCTTTTCTTACTCCTCCTCTTTCTCTCCCCAACCCCCATTCTCATC
TGCACCCTTCTTTTCTCATGTGTTTGCATAAACATTCTTTTAACTTCTTT
CTATTTGACTTGTGGTTGAATTAAAATTGTCCCATTTGCTTTGCGGTTT
GTTTTGTTTGTTTGACC 3’

Note: The underlined and bolded area is the predicted binding site for miR-19b. For details on PTK2B and miR-19b binding, please refer to **Figure 13(A)**

Vector map:



Vector sequence: pLightSwitch_3UTR
Base pairs: 3910
RPL10 Promoter: 33-307
RenSP reporter gene (synthetic renilla luciferase; includes mODC PEST): 350-1408
Multiple cloning region 2 (UTR region): 1409-1446
SV40 late poly (A) region: 1455-1676
ColE1-derived plasmid replication origin: 2001
Synthetic Beta-lactamase (Ampr) coding region: 2792-3652
Synthetic poly (A) signal/transcriptional pause site: 3757-3910

5’
GGCCTAACTGGCCGGTACCTGAGCTCACGCGTGTACCCGGTCACCTCTCTGA
TCTGCGCATGTGCTGGGCTACGCGCGGGCGCAAGCGCCAAGAGCGGCTGCG
TCTATGGTCATGACGTCTGACAGAGCGTCCACCCGTCTTCGACAGGACTCTA
TGGTTCTTACGCGCGCAGACAGACCGCCTATATAAGCCATGCGCAGGCGGA
GGAGCGCCTCTTTCCCTTCGGTGTGGGGAGCAAGCGCAGTTGTCGTCTCTTG
CGGTGCCGTCGCTGGTTCTCACACCTTTTAGGTCTGTTCTCGTCTTCCCGAGA
TCTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGCTTCCAAGGT
GTACGACCCGGAGCAGCGCAAGAGGATGATCACCGGCCCTCAGTGGTGGGC
TCGGTGCAAGCAGATGAACGTGCTCGACTCCTTCATCAACTACTACGACAGC
GAGAAACATGCGGAGAACGCCGTGATCTTCCTCCACGGCAACGCCGCTTCCT
CCTACCTGTGGCGCCACGTCGTGCCCCACATCGAGCCCGTCGCCCGGTGCAT
CATCCCTGATCTGATCGGGATGGGGAAGAGCGGGAAGAGCGGCAACGGCAG
CTACCGCCTGCTCGACCACTACAAGTACCTCACCGCCTGGTTCGAGCTGCTG
AACCTCCCCAAGAAGATCATCTTTGTGGGCCACGACTGGGGCGCTTGTCTCG
CTTTTCACTACTCCTACGAGCACCAGGATAAGATCAAGGCTATCGTGCATGC
TGAGAGCGTCGTGGACGTGATCGAGTCCTGGGACGAGTGGCCCGATATCGA
GGAGGATATTGCTCTGATCAAGTCCGAGGAGGGCGAGAAGATGGTCCTGGA
GAATAACTTCTTCGTGGAGACTATGCTGCCTAGCAAGATCATGCGCAAGCTG
GAGCCCGAGGAGTTCGCTGCTTACCTGGAGCCCTTCAAGGAGAAGGGCGAG
GTCAGAAGACCAACCCTCAGCTGGCCTCGGGAGATCCCTCTGGTCAAGGGC
GGGAAGCCGGACGTGGTGCAGATCGTCCGGAACTACAACGCCTACCTGCGC
GCCAGCGACGACCTGCCTAAGATGTTCATCGAGTCCGACCCCGGCTTCTTCA
GCAACGCTATCGTGGAGGGCGCCAAGAAGTTCCCCAACACCGAGTTCGTGA
AGGTGAAGGGCCTCCACTTCTCCCAAGAGGACGCCCCTGATGAGATGGGGA
AGTACATCAAGAGCTTCGTCGAGCGCGTCCTCAAGAACGAGCAGAATTCTCA
CGGCTTCCCTCCCGAGGTGGAGGAGCAGGCCGCCGGCACCCTGCCCATGAG
CTGCGCCCAGGAGAGCGGCATGGATAGACACCCTGCTGCTTGCGCCAGCGC
CAGGATCAACGTCTAATCTAGAGCTAGCCCTAGGGATATCCTCGAGGGCCGG
CCGCTTCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACA
ACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTG
CTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTG
CATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGC
AAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACCGAT
GCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATG
ACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACA
GGTGCCGGCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTAT
CCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGG
CTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG
CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCC
TCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT

CTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGT
TCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCG
GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGC
AGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT
ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT
TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT
GGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAG
GATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAA
CGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC
ACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT
ATGAGTAAACTTGGTCTGACAGCGGCCGCAAATGCTAAACCACTGCAGTGGT
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTC
ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGC
TTACCATCTGGCCCCAGCGCTGCGATGATACCGCGAGAACCACGCTCACCGG
CTCCGGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAA
GTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCG
CTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCC
GGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT
GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGA
ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT
CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA
ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG
GCGACACGGAAATGTTGAATACTCATACTCGTCCTTTTTCAATATTATTGAA
GCATTTATCAGGGTTACTAGTACGTCTCTCAAGGATAAGTAAGTAATATTAA
GGTACGGGAGGTATTGGACAGGCCGCAATAAAATATCTTTATTTTCATTACA
TCTGTGTGTTGGTTTTTTGTGTGAATCGATAGTACTAACATACGCTCTCCATC
AAAACAAAACGAAACAAAACAAACTAGCAAAATAGGCTGTCCCCAGTGCAA
GTGCAGGTGCCAGAACATTTCTCT 3’

PDE4B 3' UTR sequence:

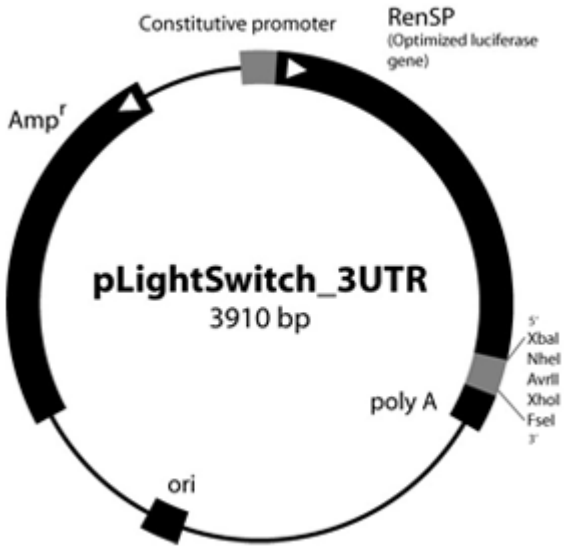
Gene accession: NM_001037339.1
Insert size: 1996

5'
AAAACAGAGATTCCCTGGGAGAGACTGACATAGACATTGCAACAGAA
GACAAGTCCCCCGTGGATACATAATCCCCCTCTCCCTGTGGAGATGAA
CATTCTATCCTTGATGAGCATGCCAGCTATGTGGTAGGGCCAGCCCACC
ATGGGGGCCAAGACCTGCACAGGACAAGGGCCACCTGGCCTTTCAGT
TACTTGAGTTTGGAGTCAGAAAGCAAGACCAGGAAGCAAATAGCAGC
TCAGGAAATCCCACGGTTGACTTGCCTTGATGGCAAGCTTGGTGGAGA
GGGCTGAAGCTGTTGCTGGGGGCCGATTCTGATCAAGACACATGGCTT
GAAAATGGAAGACACAAAACTGAGAGATCATTCTGCACTAAGTTTCGG
GAACTTATCCCCGACAGTGACTGAACTCACTGACTAATAACTTCATTTA
TGAATCTTCTCACTTGTCCCTTTGTCTGCCAACCTGTGTGCCTTTTTTGT
AAAACATTTTCATGTCTTTAAAATGCCTGTTGAATACCTGGAGTTTAGT
ATCAACTTCTACACAGATAAGCTTTCAAAGTTGACAAACTTTTTTGACT
CTTTCTGGAAAAGGGAAAGAAAATAGTCTTCCTTCTTTCTTGGGCAATA
TCCTTCACTTTACTACAGTTACTTTTGCAAACAGACAGAAAGGATACA
CTTCTAACCACATTTTACTTCCTTCCCCTGTTGTCCAGTCCAACTCCAC
AGTCACTCTTAAAACTTCTCTCTGTTTGCCTGCCTCCAACAGTACTTTT
AACTTTTTGCTGTAAACAGAATAAAATTGAACAAATTAGGGGGTAGAA
AGGAGCAGTGGTGTCGTTCACCGTGAGAGTCTGCATAGAACTCAGCA
GTGTGCCCTGCTGTGTCTTGGACCCTGCCCCCCACAGGAGTTGTACAG
TCCCTGGCCCTGTTCCCTACCTCCTCTCTTCACCCCGTTAGGCTGTTTT
CAATGTAATGCTGCCGTCCTTCTCTTGCACTGCCTTCTGCGCTAACACC
TCCATTCCTGTTTATAACCGTGTATTTATTACTTAATGTATATAATGTAATG
TTTTGTAAGTTATTAATTTATATATCTAACATTGCCTGCCAATGGTGGTGT
TAAATTTGTGTAGAAAACTCTGCCTAAGAGTTACGACTTTTTCTTGTAA
TGTTTTGTATTGTGTATTATATAACCCAAACGTCACTTAGTAGAGACATA
TGGCCCCCTTGGCAGAGAGGACAGGGGTGGGCTTTTGTTCAAAGGGT
CTGCCCTTTCCCTGCCTGAGTTGCTACTTCTGCACAACCCCTTTATGAA
CCAGTTTTGGAAACAATATTCTCACATTAGATACTAAATGGTTTATACTG
AGCTTTTACTTTTGTATAGCTTGATAGGGGCAGGGGGCAATGGGATGTA
GTTTTTACCCAGGTTCTATCCAAATCTATGTGGGCATGAGTTGGGTTATA
ACTGGATCCTACTATCATTGTGGCTTTGGTTCAAAAGGAAACACTACAT
TTGCTCACAGATGATTCTTCTGAATGCTCCCGAACTACTGACTTTGAAG
AGGTAGCCTCCTGCCTGCCATTAAGCAGGAATGTCATGTTCCAGTTCAT
TACAAAAGAAAACAATAAAACAATGTGAATTTTTATAATAAAATGTGA
ACTGATGTAGCAAATTACGCAAATGTGAAGCCTCTTCTGATAACACTTG
TTAGGCCTCTTACTGATGTCAGTTTCAGTTTGTAAAATATGTTTCATGCT
TTCAGTTCAGCATTGTGACTCAGTAATTACAGAAAATGGCACAAATGT

GCATGACCAATGTATGTCTATGAACACTGCATTGTTTCAGGTGGACATT
TTATCATTTTCAAATGTTTCTCACAATGTATGTTATAGTATTATTATTATAT
ATTGTGTTCAAATGCATTCTAAAGAGACTTTTATATGAGGTGAATAAAG
AAAAGCATGATTAGATTAGTCTGTAGGCCCACTTATTTCACA 3’

Note: The underlined and bolded area is the predicted binding site for miR-26b. For details on PDE4B and miR-26b binding, please refer to **Figure 15(A)**

Vector map:



Vector sequence: pLightSwitch_3UTR
Base pairs: 3910
RPL10 Promoter: 33-307
RenSP reporter gene (synthetic renilla luciferase; includes mODC PEST): 350-1408
Multiple cloning region 2 (UTR region): 1409-1446
SV40 late poly (A) region: 1455-1676
ColE1-derived plasmid replication origin: 2001
Synthetic Beta-lactamase (Ampr) coding region: 2792-3652
Synthetic poly (A) signal/transcriptional pause site: 3757-3910

5’
GGCCTAACTGGCCGGTACCTGAGCTCACGCGTGTACCCGGTCACCTCTCTGA

TCTGCGCATGTGCTGGGCTACGCGCGGGCGCAAGCGCCAAGAGCGGCTGCG
TCTATGGTCATGACGTCTGACAGAGCGTCCACCCGTCTTCGACAGGACTCTA
TGGTTCTTACGCGCGCAGACAGACCGCCTATATAAGCCATGCGCAGGCGGA
GGAGCGCCTCTTTCCCTTCGGTGTGGGGAGCAAGCGCAGTTGTCGTCTCTTG
CGGTGCCGTCGCTGGTTCTCACACCTTTTAGGTCTGTTCTCGTCTTCCCGAGA
TCTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGCTTCCAAGGT
GTACGACCCGGAGCAGCGCAAGAGGATGATCACCGGCCCTCAGTGGTGGGC
TCGGTGCAAGCAGATGAACGTGCTCGACTCCTTCATCAACTACTACGACAGC
GAGAAACATGCGGAGAACGCCGTGATCTTCCTCCACGGCAACGCCGCTTCCT
CCTACCTGTGGCGCCACGTCGTGCCCCACATCGAGCCCGTCGCCCGGTGCAT
CATCCCTGATCTGATCGGGATGGGGAAGAGCGGGAAGAGCGGCAACGGCAG
CTACCGCCTGCTCGACCACTACAAGTACCTCACCGCCTGGTTCGAGCTGCTG
AACCTCCCCAAGAAGATCATCTTTGTGGGCCACGACTGGGGCGCTTGTCTCG
CTTTTCACTACTCCTACGAGCACCAGGATAAGATCAAGGCTATCGTGCATGC
TGAGAGCGTCGTGGACGTGATCGAGTCCTGGGACGAGTGGCCCGATATCGA
GGAGGATATTGCTCTGATCAAGTCCGAGGAGGGCGAGAAGATGGTCCTGGA
GAATAACTTCTTCGTGGAGACTATGCTGCCTAGCAAGATCATGCGCAAGCTG
GAGCCCGAGGAGTTCGCTGCTTACCTGGAGCCCTTCAAGGAGAAGGGCGAG
GTCAGAAGACCAACCCTCAGCTGGCCTCGGGAGATCCCTCTGGTCAAGGGC
GGGAAGCCGGACGTGGTGCAGATCGTCCGGAACTACAACGCCTACCTGCGC
GCCAGCGACGACCTGCCTAAGATGTTCATCGAGTCCGACCCCGGCTTCTTCA
GCAACGCTATCGTGGAGGGCGCCAAGAAGTTCCCCAACACCGAGTTCGTGA
AGGTGAAGGGCCTCCACTTCTCCCAAGAGGACGCCCCTGATGAGATGGGGA
AGTACATCAAGAGCTTCGTCGAGCGCGTCCTCAAGAACGAGCAGAATTCTCA
CGGCTTCCCTCCCGAGGTGGAGGAGCAGGCCGCCGGCACCCTGCCCATGAG
CTGCGCCCAGGAGAGCGGCATGGATAGACACCCTGCTGCTTGCGCCAGCGC
CAGGATCAACGTCTAATCTAGAGCTAGCCCTAGGGATATCCTCGAGGGCCGG
CCGCTTCGAGCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACA
ACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTG
CTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTG
CATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGGAGGTTTTTTAAAGC
AAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACCGAT
GCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATG
ACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGACA
GGTGCCGGCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTC
GTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTAT
CCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG
CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGG
CTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG
CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCC
TCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT
CTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCA
GTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGT
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GTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGC
AGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT
ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT
TACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCT
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ACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT
ATGAGTAAACTTGGTCTGACAGCGGCCGCAAATGCTAAACCACTGCAGTGGT
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTC
ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGC
TTACCATCTGGCCCCAGCGCTGCGATGATACCGCGAGAACCACGCTCACCGG
CTCCGGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAA
GTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAA
GCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATCG
CTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCC
GGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG
CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT
GTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGA
ATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAAT
ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTT
CGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA
ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGG
GCGACACGGAAATGTTGAATACTCATACTCGTCCTTTTTCAATATTATTGAA
GCATTTATCAGGGTTACTAGTACGTCTCTCAAGGATAAGTAAGTAATATTAA
GGTACGGGAGGTATTGGACAGGCCGCAATAAAATATCTTTATTTTCATTACA
TCTGTGTGTTGGTTTTTTGTGTGAATCGATAGTACTAACATACGCTCTCCATC
AAAACAAAACGAAACAAAACAAACTAGCAAAATAGGCTGTCCCCAGTGCAA
GTGCAGGTGCCAGAACATTTCTCT 3’

Scrambled 3’ UTR plasmid (Switchgear genomics)

This is a scrambled sequence pLightSwitch 3’ UTR plasmid which is used as a negative control.

Insert size: 2538 bp

5’

TTTGCAAAAATTCAGTTCTCCAATAGTACATGCCGCATTTAAACTTGAA
CAAACAAACCCAATCTTCTGATATTTGATAATAGCGCCTTTTAAAAACC
ATAATCATTAGTTTTAAAGCTCTTTGGAACATGACGGATTTAAGCACAA
TATGGATATTTTCTTTTTTTTTTCAAATAGAGATATCCATTTGAAAAGAA
GATATTAATTCAGGATAGTAAAGCAGTATCTTCAGCTACAGTTAAGAAA
AACATGTATTTATACCTACAATATCTAAGAGTTTCTAAAGGTAATTTTCA
AAGTTATTTTCCTCATCTATGTGTTTATAGCACAGTAAAGTTCAGATAAC
TTCCTACTGAAAAAGTTTAACAGAAAAAGCTAAGGTTAAAATGTAGCT
TCAAAAACACCAGGAATACTAAACATAATTGTCTGATTTA
AAAAAGCAGCCATAATTGGGGGTCTAAAAACTAGGAGTGGTTGCACA
GAATAAATATACAAATCCTTAAAAATACAATCAGAAAACACAAACAGA
AATTTGCAAAATAATAAAATAGGATTTTTTTTTTGGCAAGATTAAGAAC
CAGACATTTCTAGGCAGTAAGTAAAGCATTATATCATTCAGGGATAAAT
AAAGCCCATAAATCAAATCACTCCCACAGAGGTTATGAACTGAAGGTC
TTAAATACCTAAATGTGTCTGCCATGGCACTCTCCTTTCTCATTTGTCAA
TAGCTTCCACAGTAATCCATGGATATTCAGACTACTGGCTTTCCCTAAG
GGTCAATTATTATGAGCATATTATTGCATAATGCACGTGTAAAAAATGTT
GTACCTGACTCTCTTCTTATCAATATTCTGATAAATGTTTTGACTTTCAG
GGAAAAATTTTAAAAAATGCCTTATATAGCAGTGTTTTTAGCTGTTTTC
CAATATAACCAAGCTAAAATGTACATGATTTTTGTAGCATCATCCTTTCA
AAGTCATTATGTGTGTAAAGAAAAGAATCAATACTTTTCAAGTATATGT
AAAATATATAAGCATGTTCAACTTTATCCAAACATTAAAAATGAGACCT
TTTTAATAGATAGACAAAATATATTGGATATATTAAAATTATGAAAACAA
AATAACTGTGCTTTTAATTACATCATAATTTTGTTTTATTATTAATAAGGT
AATATGGTAAATTCTAAGCAATTGTTCATAAATGATAACAATTTCATCTT
CCTATATTGTCCAGCAAATTTTCCCCCTCTAGTGTAAAGTGGTTCAGTG
TATCTATGCCTTCTGAATGGACTCTGTCTTT
CTTGTTGTGAATCACACTCAGAAGCTATACTGGTATTATAACTCTCGTAC
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CCATTGACTTGCATACTTTGAGGTAGTCTCCAAATAAATCCTGTTTTCAT
GTTCTAGGTAGATAAGGTCCTCTACCCTAAACCACTTGATAACCTTCAT
TCTGCTTTAGGCCTGGTGAACCTGATAACATAACCCCTGAGTTACTGTG
CTTAGCCTTGAGCTCCCTTTAAATTATCCAACCAATCCTCTCAGAGGAA
ACCCATCTCTATGATGCCTTAGACCCCAATAGTCTTTTGCCCACAGGTA
CCCATCTCTCCCTCTTGCTTCCCACATACTCGTTAAGCTCCTTTATCCTA
GATATCCCCCACCTCCCTTCCTGTGGGCCTTATGACA
CAGCCTGTCCTCTTCTCTCAGATATTTGTGTGTAATAAACCTAATCTTTC

TGCCATTCTGGCTTGAGTACTTCTCATTATGCTATACTTTACTACGCTGA
CCTTAACTTACAAACTTCACTTAAAAAGTCACCAGTGAAAAGTTCCAA
AGTCCTAGATATCTGGGCACTGGGATTGATGGAATTTTTTGTCCTTGAA
ATACAGCTTGGAATACAATTTTTACTTTCTTTCCCATTGGCTCATACTAT
GACTTGAGGGGAAACTAACCTATATTCTGAAAGGTTTCTGAAATTTTCT
ATTTGGTTGTTACAAGAGTTGCCTAACTTAAGAAAAATAATAAATCAAA
TTTGGGTGCTGCCAAGCAAAAACCCCAATATATATGCCACTCAACAGTT
GAAAAGATAACTGCCATCAGAGGTCAGAAAGCAACAGATTGGTGTTAT
TGAGGAAGCCCAACGAGAACTTTCCAACAGACTTGCCTTATTTTGCAC
TAGCAACTTTTTCCCAGCTGGTGAGAAAAGCTGAGATAGACTGCTGAA
TAATGAGACACATTTAGCCATTGCCCCAATTGACACAGTTAATCACAAG
ATATGTGAATAAAGTTTTTCCCTCACACCAACCAGGCTGCCTATCCCAG
ATAGTTGAATGAGGCCACTTGGGACCATCCGGCCCCAGCTGAACCAGC
CCACATCAGAAAACAGGCTCTGACAACGTGTGGAGCTATGAGAATCAA
TTCATAATTTTTTTAAGCCAGCAAAACAAAAATGTAAACCACCTGTGTT
TGGCTGGAACCATGTAGAAGC 3’

Note: The vector map and the sequence are similar to PTK2B and PDE4B plasmids.

TCF4 3' UTR

Gene accession: NM_003199.2
Insert size: 2857bp

5'
GCGATCGCGAATTCCGTACCTGGAGAAACATAATCTGCACCTATCCAT
ATTAATTGGGTTGTATCCCCATTAAAAAAGAAAAAAAGGGAATGTGG
CCTTTTTAGTGTGTTTTTTATTGTTGTTGTTTTGTAATTATCAAACCCA
GGTAAGATATTGGTATCCTGCACTGGATTTTCAAATGAAGTTCAGCAG
AAGACAGTTAAGATTAAAGTACTATACAAAAATTTCAAAAGGGTCCA
TACTACGCTATCTGTATGACGACACTTAGGCTGGGGATCTCTTTCAGA
AACTCGGACTTTAAAAGCAACTTGGAGCAGTTGATCCACCTCCACATT
CAAGTAATTTATGAATATGCAGAATAGGGATCTGTTCATCTAGAAATT
TTTACCATTTGTCTTCTGTGTAGCTGCAAGGAACACTAATGTTTATAC
AACTGTCAGTCCACCCAGTGGTGCAACTGGTTCTGATTCAGTCTTCCG
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TGATCTTGATTTTGATGAGGGGTTGGGGAGTGGGGAGGGAGTCGAAC
CAAGACTTGGAGTTAAGAGGATTTTCATCTTTTGCATCCAACAGGCAG
AATATGATCTGTGTCCAAAAGTGAACTTGAGTCAGGAATGAATCAAT
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CAAAAAAGTCAAGATGGAATATGATGAATTCCAACACAATGGGGCAC
CAAGGCCTTTAGGCCTCTCTTTTTATTTTGCTTTGGTTTTGTTTGTTTTT
CTTTAGAGACATGCTCTTTCTCATGGGACTTGAAGTGGACTCATCTTT
GTGCAGTGCTGGTTTTGCCATACT**CATTTCAA**GTATTATAGACATATG
TAATGGTGAAAATATATGAACTGTGGCCTTTTTCATTCTTGTTACTTGT
GATGCAATTAAGTGAAGATAAGAAAAAAAAAAAAAAAGCAGAGATT
TACCATGTATCAGTGCCTGGCTTTTTGTTATAAAGCTTTGTTTGTCTAG
TGCTCTTTTGCTATAAAATAGACTGTAGTACACCCTAGTAGGAAAAAA
AAAAAACTAAATTTAAAAATAAAAAATATATTTGGCTTATTTTTCGCA
GGAGCAATCCTTTTATACCATGAATATTACAAAAAAATTGTCAGATTC
TGAATATTTCTTCTTTGTAGATTTTTGGAATCATTATGAGTAAAAGTTT
GTTACTTTATTTTACTATTTAAAAGATGTTATTTTACCATGTGTTACCA
AGATGAAACTGTATGGGTAGCTTTTTTGTTTGTTTTTTGTTTTGTTTTT
GTTTTTGTTTTTGTTTTTAGTTGTAGGTCGCAGCGGGGAAATTTTTTGC
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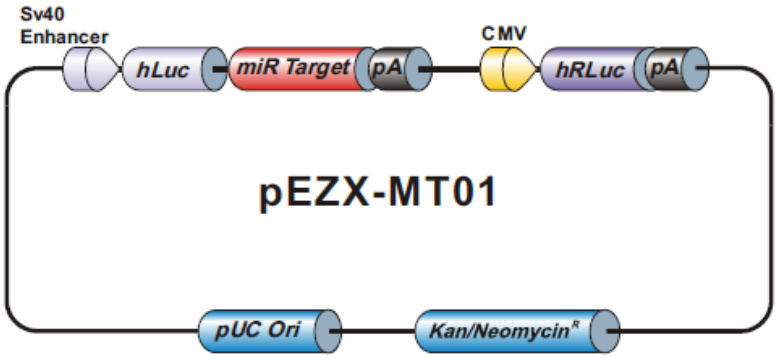
AAAAAAAAAAGGGAAAA**CATTTCAA**AAAAAAAAAAAAAGATAAAC
AGTTACACCTTGTTTTCAATGTGTGGCTGAGTGCCTCGATTTTTTCATG
TTTTTGGTGTATTTCTGATTTGTAGAAGTGTCCAAACAGGTTGTGTGCT
GGAGTTCCTTCAAGACAAAAACAAACCCAGCTTGGTCAAGGCCATTA
CCTGTTTCCCATCTGTAGTTATTCGATGAAGTCATGTACATGACCGTT
CTGTAGCAATAAATGTGCCATTTTTATAAACTGTTTCTGACACTTGTTT
CATTTCATTTTGCATTGTCCATATAGCTATGATTCTCTTCTGTAAGTAA
AACGCATCTATATTTCATTTTCCAAGTGTTGGAGGTATTGACAGCTTA

ACAAACAAAACATACAAAAAAAATCACAAAAACAAATTGAAAAGCA
AAGCACATGATTGATCAAGGAAGAGATGCCCTTAATGAAAATGGAAC
GGGATGCATGCAAAACAAAAAGAAAACTGTCTAGAGGATTAACTAAT
TGAAGGAATATAATTAATGTGTGTGTAACACTGAAGCTATGCATTTGA
AGAGCTCTGAACTGCACCAGTGTTTTCGGTTGTGCTGCAGGTTGCTAA
GTCAAGTCAGCCTTAACCTTTTGCACCAGTTGGTCGGCTGTTTGGCAG
AACATTCTCAGATCTTTTCAGTCAAAAATCTAAGATGATTTATTTTGT
ATCACTTTGTTAAAAGCTGAATATTGTTAACTACAGTTAATATTAACA
CTGTATTTATACTTTCTCAAACTACATCCGCCCCACCACTTCTGGTTGC
CTCTGTTGACTATTAATCCAGATGTAAACAACCAGATGTTTTTTTCTA
ACTTGTACAAACTGACGTGTGTCAACTATCATGGAAGGAAAAAAATG
TACAGATTAAAATTATTCAGTGTTATGTACTGTAAGTTAATATTTTTGT
AGAATGGACATCAATCTACTTTGCAAAATTTGGAGGCTATTTCAACAT
TGCACTGTAGAAATGTAAAGTAATGTATGCAATGTAAAGGAAAGCCC
GCGGTAGCTGAGCGCTTCATAACAGAATGTTCTAATCAAGTACGTGG
TATTTGGGGATGTCTCCAATATTGCTCTTGTATTCTTTCTAATTGGGTT
TAGTGACTAGTTGAAGGAAAATGTTATAACGCCATTTGGTTCACATGT
GAAGTGCCCTCCATAGCCAAATGTTGGGATTTTTTTTTTTTTCGTTTTT
GGTTGGACTGTTTGCAGATATTTAAATTTTATGAAATTTCCAAAGATT
TTGGTTGATAACCCCCTTTTACCTTCTAAATGATTTGAGATGTTCTTAT
GTTCTTACTGTGTGTTTTAAATATATATAAAAGAGCCACAAGCATTTA
GTCTTTTAGTATTCTCGAGACTAGT 3’

Note: The underlined and bolded areas are the predicted binding sites for miR-203. For details on TCF4 and miR-203 binding, please refer to **Figure 17(A)**

Vector map:



Information about the vector:

Whole Plasmid Size: 10232 bp
Antibiotic: Kanamycin
Stable Selection Marker : Neomycin

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APPENDIX B

HCT116 CELL CULTURE

Purpose: To grow HCT116 p53$^{+/+}$ cells.

Day 1:

Protocol # 1: Preparation of Complete McCoy's 5A modified media for HCT116 cells

Preparation of Media:
Purpose: To prepare complete McCoy's 5A media for HCT116 cell culture p53+/+ and p53-/- cells
Reagents: (supplier, catalog number)

McCoy's 5A (Gibco 16600-082) w/o HEPES

10% FBS (Hyclone AJA9530) (55ml to obtain a final concentration of 10%)

1% Glutamax (Gibco 3505-061)(5.5ml to obtain a final concentration of 1%)

Materials: Take the glass tube which should be autoclaved and unopened. After you open and use, then close it back and put it in the drawer in cell culture room.

Procedure
(1)Thaw 5.5 ml of Glutamax (horizontal freezer behind Laurie's general desk) and 56 ml FBS (1 tube has 27.5 ml of FBS, hence take 2 tubes*) at 4°C overnight.
(2) Add Glutamax, FBS into 500 ml of McCoy's 5A modified medium without HEPES..
(3) Gently tilt the bottle to mix (upside down)
(4) Label as sterile, complete (after adding FBS and glutamax, the media is now called complete), your initials and the date and store at 4°C.
Final concentration: 10% FBS, 1% Glutamax

* When there are only 3 conical tubes in the freezer, then aliquot a bottle and put back some tubes in the horizontal freezer at the back behind the Laurie's general desk in the Styrofoam rack)

Protocol # 2, Preparation of HCT-116 Cell Culture

Purpose: To start HCT 116 culture by growing cells in separate T-75 flasks.

Preparation
-Turn on the UV in the hood for at least15 min prior to keep it sterile for culture.

Procedure

***** After making media, aliquot PBS and then take out the vial (which has the HCT116 cells)

1. Warm complete McCoy's 5A media to room temperature.
2. Aliquot 9 ml PBS (w/o Ca^{2+} and Mg^{2+}) in a 15 ml conical tube (to spin down frozen cells).
3. Take a vial of p53+/+ cells from the liquid nitrogen storage system (located behind Jennifer's desk-white nitrogen tank-rack 5, box 4) thaw rapidly at 37^{o}C with gentle agitation.
4. Add 1 ml of PBS (w/o Ca^{2+} and Mg^{2+}) in the thawed cells and then into the PBS in the conical tube. (Slowly introduce cells to new environment by adding a few drops of PBS to cryovial before transferring to tube of PBS).
5. Add 9ml of the PBS and wash cells in 10 mL PBS (w/o Ca^{2+} and Mg^{2+}) by (pipetting up and down)
6. Centrifuge at 150 x g for 5 min at room temperature. Remove the supernatant by attaching the glass pipette to the vacuum and sucking the supernatant out).
7. Add 13 ml of the McCoy's medium in T-75 flask.
8. Resuspend the cell pellet in 3 ml McCoy's 5A complete medium.
9. Add resuspended cells (3ml) into a T-75 flask with 13 ml McCoy's
10. Gently rotate the flask to distribute the cells evenly
11. Label the flask - specify the cell type, passage number, date and initials
12. Incubate at 37^{o}C; under 5% CO_2 atmospheric pressure
13. Check daily until 70-90% confluent, usually 3 days.
14. If cells are not passaged, re-feed cells.
15. Repeat the same procedure for p53-/- cells. Take care not to culture both wild type and knock out cells at the same time.

[Feeding cells

Check of cell confluence every day.

Feed cells every 48 h (maximum 72 h).

Procedure

1. Aspirate old media from the flask using sterile technique and replenish with fresh complete McCoy's 5A media.

2. When culture reaches 70-90% confluence, trypsinize the cells and passage them.

Day 2:

Protocol # 3, HCT-116 Cell Culture: Seeding, Passing or Freezing

Purpose: To pass and to seed or freeze p53+/+ and p53-/- cell culture.

Preparation

- Use sterile hood conditions for the procedure.
-Warm complete McCoy's 5A media and trypsin-EDTA (Gibco, #25300-054) to room temperature.

Procedure
For any 70% confluent cell culture in a T-75 flask:

1. Remove all media.
2. Wash cells once with PBS (w/o Ca^{2+} and Mg^{2+}), aspirate gently without disturbing the cell monolayer.
3. Add 5 ml Trypsin/b EDTA, incubate 3 min at 37^{o}C in CO2 incubator until > 90% cells detach. Tap the flask from the side and bottom.
4. Add 5 ml complete medium to trypsinized cells which are then pipetted from the flask into a 50 ml conical tube.
5. Spin cells down at 150 x *g*, 5 min.
6. Vacuum aspirate supernatant (media with trypsin), taking care not to disturb the pellet.
7. Resuspend pellet in complete McCoy's 5A media.
8. Incubate flasks in a humidified, 37^{o}C, 5% CO_2 incubator. Look at cells before incubating.
9. Repeat the same for p53-/- cells.

A. <u>Procedure to count cells and seed cells (similar to YAMC cells):</u>

1. Take Hemacytometer and the coverslip and spray it with alcohol.
2. Wipe it with kim-wipe
3. Put coverslip on the Hemacytometer (place the Hemacytometer in the hood on a flat surface).
4. Take 10 ul cells and put it them in the notch in the Hemacytometer with the coverslip.
5. Open Nikon microscope and place the Hemacytometer.
6. Count the no of cells in at least 4 squares on one side of Hemacytometer.

<u>Calculations</u>: (say need 25,000 cells/ml and you have to add 1.5 ml per well for a 6 well plate)

- Total no. of cells in all the counted squares = 67
- Number of squares counted = 4

- Cell density (cells count per ml) = $\frac{67 \times 10{,}000}{4}$ = **167500 cells/ml**

- Now, for a one 6 well plate , need 25,000 cells in 1.5ml. Hence the required cell density = 16666.66 cells/ml

- **ViCi=VfCf**

  Vi= $\frac{16666.66\ \textbf{cells/ml} \times 1.5\text{ml}}{167500\ \text{cells/ml}}$ =0.149 **ml**

- Therefore, 1.5ml- **0.149 ml** = **z ml** media. Therefore, add 1.359 ml media and 0.149 ml of cell suspension. Then dispense **1.5 ml** into the well.

- Seed cell according to desired density.

B. Procedure for cell freezing (to store cells in liquid nitrogen)

Freezing Medium: Cell Culture Freezing Medium with DMSO (Specialty Media S-002-D).

1. Label cryovials with cell type, passage number, date and initials.
2. Defrost freezing medium, place cryovials and freezing medium both on ice
3. To collect cells, follow Steps 1 - 6 of Procedure: *Passage Cells*
4. Resuspend pellet from T-175 flask in freezing medium to give 5 x 10^6 cells / ml
5. Keep vials in Mr. Frosty (Nalgene, #5100-0001) at –80˚C for a day (not more than 48 h).
6. Transfer vials into liquid nitrogen for storage
7. Enter the rack, box # and vial position into cell culture log book

APPENDIX C

TRANSFECTION OF MIRNA INHIBITORS-24 WELL

Purpose: Transfection of miRNA inhibitors (anti-miR-19b, miR-26b and miR-203) in HCT116 cell line using Lipofectamine2000 transfection reagent-24 well format

Objective: To determine if the miRNA knockdown is successful by checking out the miRNA expression utilizing Real Time PCR after transfection. The Real Time PCR protocol that can be used is "Protocols for RTPCR and Real time PCR of miRNA and gene" by MSS.

Material required:
(1) Pipette and pipette tips
(2) DPBS (Gibco-Cat #14190)
(3) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(4) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(5) Sterile 0.6ml RNase free tubes
(6) Ice bucket
(8) Lyophilized fluorescent miRNA inhibitors (Exiqon: 5' FITC labeled miR-19b inhibitor-Cat # 410119-04; 5' FITC labeled miR-26b inhibitor-Cat #411928-04; 5' FITC labeled miR-203 inhibitor-Cat #410129-04)
(9) 24 well plate (Costar –Cat # 3524)
(10) Glass pipettes
(11) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(12) RNase free water

Resuspension of lyophilized miRNA inhibitors *(under sterile hood):*
1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.
2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nmole microRNA Inhibitor vial which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.
8. Store at -20^0C

Procedure:
Day 1:
(1) Seed $2x10^5$ cells in 24 well tissue culture plate in 500 µl of complete medium

Day 2:

Note: The volumes mentioned are for one well in a 24-well plate.

(2) For each well, use one 0.6 ml sterile RNase free tube.

(3) Add 47.5 µl of Opti-mem medium and 2.5 µl of Lipofectamine in the tube. Finger-flick to mix and spin down.

(4) Incubate for 5 min at RT.

(5) Dilute the required amount of miRNA inhibitor (from the stock solution –made above) in 50 µl of opti-mem medium. The concentration of inhibitor is 20 µM in the stock solution. See MSS's dilution of mimcs and inhibitors page for calculations)

(6) Add the 50 ul of the diluted miRNA inhibitor *from step 5* into the 0.6 ml tube containing *50 µL from step 3* diluted Lipofectamine solution.

(7) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.

(8) During this 20 min incubation time, take out the 24-well plate in which the cells were seeded the day earlier.

(9) Using a glass pipette, suck out the old media and add 400 µl of complete McCoy's 5A median in each well.

(10) After 20 minutes, add the total 100 µl solution drop-wise from each 0.6 ml tube (from step 6) to the corresponding well in the 24-well plate.

(11) Gently swirl the plate around and put the plate back in the incubator for 24 hours.

(12)After 6-8 hours of the transfection, take the plate out and look under the microscope to see whether the transfection has successfully happened by observing the fluorescent cells.

(13)Based on the end point of this experiment, several options are possible. One of them is below:

For RNA isolation from transfected cells:

(14) After 24 hours, take out the plate and suck off the old media. Add PBS to the wells and swirl the plate. Suck off the PBS.

(15)Repeat Step 14 twice.

(16)After the last PBS wash, take the plate and put it on ice. Add 500 ul of miRVana lysis buffer (from miRVana miRNA isolation kit). Scrape off the cells and transfer the lysate to a 2 ml RNase free tube. Pipette up and down. Vortex the tube and carry it forward for miRNA isolation utilizing "MSS's miRNA isolation-using miRVana kit" protocol

APPENDIX D

TRANSFECTION OF MIRNA INHIBITORS-100MM PLATE

Purpose: Transfection of miRNA inhibitors (anti-miR-19b, miR-26b and miR-203) in HCT116 cell line using Lipofectamine2000 transfection reagent-100 mm plate format

Objective: To determine if the protein expression of the predicted targets of the miRNAs are changed by inhibition of miRNA, HCT116 cells are transfected with miRNA inhibitors and total cell lysates are obtained.

Material required:
(1)Pipette and pipette tips
(2) DPBS (Gibco-Cat #14190)
(3) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(4) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(5) Sterile 0.6 ml RNAse free tubes
(6) Ice bucket
(8) Lyophilized fluorescent miRNA inhibitors (Exiqon: 5' FITC labeled miR-19b inhibitor-Cat # 410119-04; 5' FITC labeled miR-26b inhibitor-Cat #411928-04; 5' FITC labeled miR-203 inhibitor-Cat #410129-04)
(9) 100mm tissue culture plate (BD falcon –Ref 353803)
(10) Glass pipettes
(11) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(12) RNase free water

Resuspension of lyophiolized miRNA inhibitors (under sterile hood):
1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.
2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nmole microRNA Inhibitor vial which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.
8. Store at -20^0C

Procedure:
Day 1:

(1) Seed $1x10^6$ cells in 100mm tissue culture plate in 7000 µl of McCoy's 5A complete medium

Day 2:

Note: The volumes mentioned are for one 100mm plate.

(2) For each well, use one 15 ml sterile conical tube.
(3) Add 1485 µl of Opti-mem medium and 15 µl of Lipofectamine in the tube. Finger-flick to mix and spin down.
(4) Incubate for 5 min at RT.
(5) Dilute the miRNA inhibitor in 1000 µl of Opti-mem medium from the stock solution (Refer to MSS's dilution of inhibitor and mimics protocol).
(6) Add the diluted miRNA inhibitor into the 15 ml conical tube containing diluted lipofectamine solution (from step 3).
(7) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.
(8) During this 20 min incubation time, take out the 100 mm plate in which the cells were seeded the day earlier.
(9) Using a glass pipette, suck out the old media and add 4500 µl of complete McCoy's 5A median in each well.
(10) After 20 minutes, add the total 2500 µl solution from each 15ml tube to the corresponding 100 mm plate.
(11) Gently swirl the plate around and put the plate back in the incubator for 24 hours.
(12) After 6-8 hours, of the transfection, take the plate out and look under the microscope to see whether the transfection has successfully happened by observing the fluorescent cells.
(13) Based on the end point of this experiment, several options are possible. One of them is below:

For total cell lysate from transfected cells:

(14) After 24 and 48 hrs of transfection, take out the plate and suck off the old media. Add PBS to the wells and swirl the plate. Suck off the PBS.
(15) Repeat Step 14 twice.
(16) After the last PBS wash, take the plate and put it on ice.
(17) Add 200 µl of lysis buffer and use a scraper to scrape the cells from the plate and follow "MSS- Total cell lysate protocol."

APPENDIX E

TRANSFECTION OF MIRNA MIMICS -24 WELL

Purpose: Transfection of miRNA mimics (miR-19b, miR-26b and miR-203) in HCT116 cell line using Lipofectamine2000 transfection reagent-24 well format

Objective: To determine whether the miRNA mimics are successfully transfected, miRNA expression will be determined by utilizing Real time PCR. The Real time PCR protocol that can be used is "Protocols for RTPCR and Real time PCR of miRNA and gene" by MSS.

Material required:
(1) Pipette and pipette tips
(2) DPBS (Gibco-Cat #14190)
(3) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(4) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(5) Sterile 0.6 ml RNAse free tubes
(6) Ice bucket
(8) Lyophilized miRNA mimics (Thermoscientific -Dharmacon: miRIDIAN mimic-miR-19b-Cat # C-300489-03; miR-26 mimic-Cat # C-300501-07; miR-203 mimic-Cat #C-300562-03; control mimic-Cat # CN-0010000-01-05)
(9) 24 well plate (Costar –Cat # 3524)
(10) Glass pipettes
(11) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(12) RNase free water

Resuspension of lyophiolized miRNA mimics (under sterile hood):
1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.
2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nmole microRNA Inhibitor vial which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.
8. Store at -20^0C

Procedure:
Day 1:

(1) Seed $2x10^5$ cells in 24 well tissue culture plate in 500 µl of complete medium

Day 2:

Note: The volumes mentioned are for one well in a 24-well plate.

(2) For each well, use one 0.6 ml sterile RNase free tube.
(3) Add 47.5 µl of Opti-mem medium and 2.5 ul of Lipofectamine in the tube. Finger-flick to mix and spin down.
(4) Incubate for 5 min at RT.
(5) Dilute the required amount of miRNA mimics (from the stock solution –made above) (Also,refer to MSS's dilution of inhibitor and mimics protocol) in 50 µl of Opti-mem medium. The concentration of inhibitor is 20 µM in the stock solution.
(6) Add the 50 µl of diluted miRNA mimic into the 0.6 ml tube containing (50 µl from step 3) diluted lipofectamine solution.
(7) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.
(8) During this 20 min incubation time, take out the 24-well plate in which the cells were seeded the day earlier.
(9) Using a glass pipette, suck out the old media and add 400 µl of complete McCoy's 5A median in each well.
(10) After 20 minutes, add the total 100 µl solution from each 0.6 ml tube to the corresponding well in the 24-well plate.
(11) Gently swirl the plate around and put the plate back in the incubator for 24 hours.
(12) Based on the end point of this experiment, several options are possible. One of them is below:

For RNA isolation from transfected cells:

(13) After 24 hours, take out the plate and suck off the old media. Add PBS to the wells and swirl the plate. Suck off the PBS.
(14) Repeat Step 14 twice.
(15) After the last PBS wash, take the plate and put it on ice. Add 500 µl of miRVana lysis buffer (from miRvana miRNA isolation kit). Scrape off the cells and transfer the lysate in a 2 ml RNase free tube. Pipette up and down. Vortex the tube and carry it forward for miRNA isolation utilizing "MSS's miRNA isolation-using miRVana kit" protocol

APPENDIX F

TRANSFECTION OF MIRNA MIMCS -100MM PLATE

Purpose: Transfection of miRNA mimics (miR-19b, miR-26b and miR-203) in HCT116 cell line using Lipofectamine2000 transfection reagent- for 100mm dish

Objective: To determine if the miRNA mimics are successfully transfected, miRNA expression will be determined by utilizing Real Time PCR. The Real Time PCR protocol that can be used is "Protocols for RTPCR and Real time PCR of miRNA and gene" by MSS.

Material required:
(1)Pipette and pipette tips
(2) DPBS (Gibco-Cat #14190)
(3) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(4) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(5) Sterile 15 ml conical tubes (BD Falcon)
(6) Ice bucket
(8) Lyophilized miRNA mimics (Thermoscientific -Dharmacon: miRIDIAN mimic-miR-19b-Cat # C-300489-03; miR-26 mimic-Cat # C-300501-07; miR-203 mimic-Cat #C-300562-03; control mimic-Cat # CN-0010000-01-05)
(9) 100 mm tissue culture plate (BD falcon –Ref 353803)
(10) Glass pipettes
(11) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(12) RNase free water

Resuspension of lyophiolized miRNA mimics (under sterile hood):
1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.
2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nanomole microRNA Inhibitor vial which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.
8. Store at -20^0C

Procedure:
<u>Day 1:</u>

(1) Seed $1x10^6$ cells in 100 mm tissue culture plate in 7000 µl of McCoy's 5A complete medium

Day 2:

Note: The volumes mentioned are for one 100mm plate.

(2) For each well, use one 15 ml sterile conical tube.
(3) Add 1485 µl of Opti-mem medium and 15 µl of Lipofectamine in the tube. Finger-flick to mix and spin down.
(4) Incubate for 5 min at RT.
(5) Dilute the miRNA mimic in 1000 µl l of Opti-mem medium from the stock solution (made above in step 3 of resuspension protocol) (Also, refer to MSS's dilution of inhibitor and mimics protocol).
(6) Add the diluted miRNA mimic into the 15 ml conical tube containing diluted lipofectamine solution (from step 3).
(7) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.
(8) During this 20 min incubation time, take out the 100 mm plate in which the cells were seeded the day earlier.
(9) Using a glass pipette, suck out the old media and add 4500 µl of complete McCoy's 5A median in each well.
(10) After 20 minutes, add the total 2500 µl solution from each conical tube to the corresponding 100 mm plate.
(11) Gently swirl the plate around and put the plate back in the incubator for 24 hours.
(12) Based on the end point of this experiment, several options are possible. One of them is below:

For total cell lysate from transfected cells:

(13) After 24 and 48 hours of transfection, take out the plate and suck off the old media. Add PBS to the wells and swirl the plate. Suck off the PBS.
(14) Repeat Step 14 twice.
(15) After the last PBS wash, take the plate and put it on ice.
(16) Add 200 µl of lysis buffer and use a scrapper to scrap the cells from the plate and follow "MSS- Total cell lysate protocol."

APPENDIX G

DUAL –GLO LUCIFERASE ASSAY

Purpose: To determine the luciferase activity after transfecting HCT116 cells with 3'UTR plasmids (containing Firefly and Renilla luciferase genes)
Objective: HCT116 cell line is co-transfected with 3' UTR TCF4 and miRNA mimic (as per MSS:" Co-transfection/Transfection of miRNA mimics/inhibitors in HCT116 cell line using lipofectamine 2000" protocol). 24 hours after the transfection, to determine the luciferase activities, Promega Dual-glo luciferase assay is carried out.
Materials:

(1) Multichannel pipette (100-300 µl)
(2) Multichannel 300 µl pipette tips
(3) Pipette Basin (13681500)
(4) Foil
(5) Luminometer. (Molecular devices: SpectraMaxL-located at back of Rm 323)
(6) 96 well plate which has the cells growing (details from MSS:" Co-trasnfection/Transfection of miRNA mimics/inhibitors in HCT116 cell line using lipofectamine 2000" protocol)
(7) Dual-Glo Luciferase Assay system (Promega: E2920)
(8) 15 ml conical tube

Notes:
1. Assay reagents are stable at room temperature for several hours. Freezing the reagent can reduce the loss of activity of the Dual-Glo® Luciferase Reagent. **Do not** thaw the reconstituted reagent at temperatures above 25°C. Mix well after thawing. To thaw the reagent, place the reagent in a 25^0C temperature water bath *(just thaw on room temp bench).* Prepare only the amount of Dual-Glo® Stop & Glo® Reagent required. For best results; prepare the Dual-Glo® Stop & Glo® Reagent immediately before use.

2. Dual-Glo® Luciferase Reagent Stability: Liquid reagent has approximately a 10% loss of firefly RLU after 8 hours at room temperature and after 48 hours at 4°C. Frozen reagent has approximately a 10% loss of firefly RLU after 1 week at –20°C and after 6 months at –70°C. Do not store the reagent at –20°C for longer than 1 week. The reagent can be exposed to 5 freeze-thaw cycles with approximately a 15% loss in firefly RLU. Holding or storing the reconstituted reagent may cause *Renilla* RLU to rise.

3. Approximate stability of Dual-Glo® Stop & Glo® Reagent after reconstitution: 8.1% loss after 8 hours at room temperature, 8.5% loss after 24 hours at 4°C. Hence, always prepare the Dual-Glo® Stop & Glo® Reagent immediately before use.

4. The temperature optimum for the activity of both luciferases is approximately room temperature (20–25°C), so it is important that the reagents be equilibrated to room

temperature before beginning measurements. To avoid the need to temperature equilibrate reagents before use, store the Dual-Glo® Luciferase Buffer and the Dual-Glo® Stop & Glo® Buffer at room temperature. If reagents are colder than room temperature, place them in a room temperature water bath to equilibrate before use.

5. To achieve maximum reproducibility, equilibrate cells in media to room temperature before performing luciferase measurements

Procedure:

Reagent preparation:

(1) Transfer the contents of one bottle of Dual-Glo Luciferase Buffer to one bottle of Dual-Glo luciferase substrate to create the Dual-Glo Luciferase reagent. Mix by inversion until the substrate is thoroughly dissolved.

(2) Calculate the amount of Dual-Glo Stop & Glo Reagent (100 µl per well) needed to perform the experiment. Dilute the Dual-Glo Stop & Glo substrate 1:100 into an appropriate volume of Dual Glo Stop and Glo buffer in a 15 ml conical tube.
Eg: If 6 ml of Dual Glo Stop & Glo Reagent is needed, dilute 60 µl of Dual-Glo Stop & Glo substrate into 6 ml of Dual-Glo Stop & Glo buffer.

Assay Procedure:

(1) Remove 96 well plates containing mammalian cells from the incubator. Make certain that the plates are compatible with the type of luminometer being used (i.e white tissue culture treated 96 well plates).

(2) **Measuring firefly luciferase activity:** Add 100 µl of Dual-Glo® Luciferase Reagent equal to the culture medium volume (i.e.100 µl) to each well and mix. Do not remove the cell culture medium from the wells.

(3) Wait at least 10 minutes, and then measure the firefly luminescence at 470 nm. Optimal results will be generated if the luminescence is measured within 2 hours of addition of Dual-Glo® Luciferase Reagent.

(4) **Measuring *Renilla* luciferase activity:** Add 100 ul of Dual-Glo® Stop & Glo® Reagent to each well and mix.
Note: *Dual-Glo® Stop & Glo® Reagent should be added to plate wells within 4 hours of addition of Dual-Glo® Luciferase Reagent.*

(5) Wait at least 10 minutes, and then measure luminescence *at 540 nm. Renilla* luminescence should be measured in the same plate order as the firefly luminescence was measured.

(6) Optimal results will be generated if the luminescence is measured within 2 hours of addition of Dual-Glo® Stop & Glo® Reagent.

(7) Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter

APPENDIX H

LIGHTSWITCH LUCIFERASE ASSAY (SWITCHGEAR GENOMICS)

Purpose: To determine the luciferase activity after transfecting cells with plasmids w/wo miRNA mimics.

Note: This assay is to be carried out after transfecting cells with plasmids from Switchgear Genomics Company.

Materials required:

(1) 3' UTR PDE4B plasmid (Switch gear genomics: S810648)
(2) 3'UTR pTK2B plasmid (Switchgear genomics: S806790)
Control 3'UTR (Switchgear genomics: S806790)
(3) Light switch Luciferase assay reagent kit (Switchgear genomics-LS010)
(4) Multichannel pipette (100-300 µl)
(5) Multichannel 300 µl pipette tips
(6) Pipette Basin (Fisherbrand:13681500)
(7) Foil
(8) Luminometer. (Molecular devices: SpectraMaxL-located at back of Rm 323)
(9) 96 well white plate (Costar-3912)-optional
(10) 96 well plate which has the cells growing (details from MSS:" Cotransfection /Transfection of miRNA mimics/inhibitors in HCT116 cell line using Lipofectamine 2000" protocol)

Procedure:

(1) Thaw the Assay buffer for at least one hour before the assay is carried out. If there is shortage of time, thaw it in a 37C water bath
(2) For reconstitution of 100X substrate, add 100 µl of substrate solvent to tube of lyophilized Assay substrate. Dissolve completely, Protect from light and minimize time at room temperature. The 100x substrate can be stored at -20C and protected from light for 2-3 weeks.
(3) For preparation of Assay solution (for e.g.: for a full 96 well plate), take the thawed assay buffer and add 100 µl of reconstituted 100x substrate just prior to use. Prepare assay solution (buffer+substarte mix) fresh for each use and use within 2-3 hours. To assay fewer wells, make up only what you need and store remaining substrate and buffer separately at -20^0C. For better results, avoid additional freeze thaw cycles. To re-thaw re-frozen buffer, incubate in a warm 37^0C water bath for at least 1 hour and mix well to ensure that all the components go back in the solution.

(4) Take out the 96 well plate from the incubator. Do not wash the cells or remove the cell culture medium. The solutions in the next step will be directly added to

(5) Pour the assay solution (buffer + substrate) in a pipette basin and using a multichannel pipette, pipette 100ul directly to each sample well, which already has 100 µl of cell culture medium.

(6) If the cells were grown in a plate that is not compatible with the luminometer, then transfer the entire volume of samples to a white 96 well plate (i.e. total 200 µl). This can be done since the assay solution has lysis buffer which will lyse the cells.

(7) Cover the plate with foil and incubate for 30 minutes at room temperature.

(8) Read each well for 2 seconds in a plate luminometer at 470 nm and then second time with 570 nm.

APPENDIX I

DILUTION OF MIRNA INHIBITORS, MIRNA MIMICS AND PLASMIDS

Purpose: To dilute miRNA inhibitors, mimics and plasmids.

I. miRNA inhibitors and miRNA mimics:

---Concentration of Stock solution: 20 µM
---Total volume of stock solution= 250 µl

For one well of 24 well plate:
Required amount: 30 nM in 600 µl.
Volume of diluted miRNA= 50 µl.
Therefore, take 0.9 µl of 20 µM stock solution and add 49.1 µl of Opti-mem medium.
Add this 50 µl to 550 µl, so the final concentration is 30 nM in 600 µl.

For one well of 96 well plate:
Required amount: 30 nM in 100 ul.
Volume of diluted miRNA = 12.5 ul.
Therefore, take 0.15 ul of 20 uM stock solution and add 12.35 ul of Opti-mem medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 30 nM in 100 µl.

For one 100mm plate:
Required amount: 30nM in 7000 µl.
Volume of diluted miRNA = 1000 µl.
Therefore, take 10.5 µl of 20 uM stock solution and add 989.5 µl of Opti-mem medium.
Add this 1000 µl to 6000 µl, so the final concentration is 30 nM in 7000 µl.

II. 3' UTR plasmid:

For one well of 96 well plate:

PDE4B plasmid:
Original concentration: 1136.3 ng/ µl
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.08 µl of original concentration and add 12.42 µl of optimum medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.

Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 0.8 µl of original concentration and add 124.2 µl of optimum medium.

PTK2B plasmid:
Original concentration: 933.11 ng/ µl
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.10 µl of original concentration and add 12.40 µl of optimum medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.
Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 1.0 µl of original concentration and add 124.0 µl of optimum medium.

Scrambled 3' UTR (Switchgear genomics)
Original concentration:1146.85 ng/ul
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.09 µl of original concentration and add 12.41ul of optimum medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.
Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 0.9 µl of original concentration and add 124.1 µl of optimum medium.

TCF4 plasmid
Original concentration: 852.70 ng/ul
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.12 µl of original concentration and add 12.38 µl of optimum medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.
Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 1.2 µl of original concentration and add 123.8 µl of optimum medium.

Mutant TCF4 plasmid
Original concentration:235 ng/ul
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.42 µl of original concentration and add 12.08 µl of optimum medium.
Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.
Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 4.2 µl of original concentration and add 120.8 µl of optimum medium.

Scrambled 3'UTR (Genecopiea)
Original concentration: 1903.2 ng/ul
Need 100 ng in 100 µl
Volume of dilution: 12.5 µl
Therefore, take 0.05 µl of original concentration and add 12.45 µl of optimum medium. Add this 12.5 µl to 87.5 µl, so the final concentration is 100 ng in 100 µl.
Since the volume to be taken is small, you can scale up the dilution. i.e If you are going to add this to 10 wells, then take 0.5 µl of original concentration and add 124.5 µl of optimum medium.

APPENDIX J

CO-TRASNFECTION OT MIRNA MIMCS, PLASMIDS AND TARGET

PROTECTORS

Purpose: To cotransfect 3' UTR plasmids miRNA mimics and target protectors in HCT116 cells to determine if the mRNA is a direct target of miRNA. (i.e. Co-transfection of morpholinos (target protectors), miR-26b mimic and 3'UTR plasmids in HCT116 cell line using Endoporter reagent)

Materials:

(1) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(2) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(3) Sterile 0.6 ml RNase free tubes
(4) Ice bucket
(5) Lyophilized miRNA mimics (miR-26 mimic-Cat # C-300501-07; control mimic-Cat # CN-0010000-01-05)
(6) 3' UTR PDE4B plasmid (Switch gear genomics: S810648)
(7) Firefly normalization plasmid - Cypridina TK control construct (Switchgear genomics: SN0322S)
(8) Control 3'UTR (Switchgear genomics: S806790)
(9) 3' UTR PDE4B Target protector (TP) (Gene tools)(stored at RT) (Sequence: TCTGACTCCAAACTCAAGTAACTGA)
(10) Random control target protector 25-N, 100 nmol (Gene tools) (stored at RT)
(11) Endo-Porter Aqueous, 1 ml (Gene Tools) (stored at 4C)
(12) Glass pipettes
(13) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(14) 96-well white tissue culture treated plate (BD Falcon- Cat # 353296)

Notes:

(1) Make sure to do the experiment in triplicates.
(2) Make table as shown on page 212 to prevent mistakes and confusion while transfecting.

Resuspension of lyophiolized miRNA mimics: (under sterile hood):

1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.

2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nmole microRNA Inhibitor vials which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.

8. Store at -20^0C

Resuspension of lyophilized target protectors (under sterile hood):

1, The target protector is sent as a sterile freeze dried morpholino oligo that is custom made. 300 nanomoles is provided.

2. Carefully open the glass vial and add 0.3 ml of sterile water to give 0.3 ml of a 1mM oligo stock solution.

3. The target protector control vial contains 100 nanomoles of freeze dried standard control morpholino oligo. Add 0.1 ml of sterile water to give 1 mM oligo stock solution.

4. Wrap Parafilm tightly around the vial closure to prevent evaporation. Keep it in a dark box at RT.

Procedure:

Day 1:

(1) Seed 2.5x 10^5 cells in 96 well plate in 100 µl of complete medium.

Day 2:

Note: The volumes mentioned are for one well in a 96-well plate.

Look at table on page 212 to determine the volume of reagents to be added in each well

(2) For each well, use a 0.6 ml sterile RNase free tube.(i.e. if transfection will be carried out in 10 wells, take 10 tubes).
(3) Add 24.45 µl of Opti-mem medium and 0.55 µl of Lipofectamine in the tube. Finger-flick to mix and spin down.
(4) Incubate for 5 min at RT.
(5) Dilute the miRNA mimic in 12.5 µl of opti-mem medium (see MSS's"Dilution of miRNA inhibitors/mimics or plasmids" protocol).
(6) Dilute the PDE4B plasmids in 6.5 µl of opti-mem medium (see MSS's "Dilution of miRNA inhibitors and mimics" protocol)
(7) For control wells, dilute the scrambled control plasmid in 6.5 µl of opti-mem medium
(8) Also dilute normalization plasmid (called the transfection control) in 6.5 ul of optimum medium. This is added in every well where PDE4B plasmid is added.
(9) Add the diluted miRNA mimic **(step 5)** and diluted plasmids **(Step 6)** into the 0.6 ml tube containing diluted lipofectamine solution **(Step 3).**
(10) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.
(11) During this 20 minute incubation time, take two 0.6 µl sterile RNAse free tubes.

(12) In one tube, dilute morpholinos (target protector) in 6.5 µl of sterile RNAse free water. Similarly, also dilute the target protector control in 6.5 µl of sterile RNAse free water.
(13) In the other tube, add 19.5 µl of Opti-mem medium and 1.8 µl of Endoporter reagent (For one well, need 7.1 µl. For three wells, need 21.3 µl).
(14) Now, take 7.1 µl from **Step 13** and add it in the tube made in **Step 12** containing the diluted morpholino. Mix well by pipetting up and down. The final solution in this tube should be 13.6 µl (i.e 6.5 µl of diluted morpholino+7.1 µl of endoporter solution)
(15) Take out the 96 well plate in which the cells were seeded the previous day.
(16) Using a glass pipette, suck out the old media and appropriate amount of complete McCoy's 5A median in each well based on the table on 4th page of this protocol.
(The total volume should be 100 µl. We have 25 µl of lipofectamine solution +12.5 µl of miRNA mimic+12.5 µl of PDE4B 3' UTR+13.6 µl of morpholino. These add up to 62.5 µl and 36.4 µl remain). See the table n page 4 for more clear details.
(17) Add the total 50 µl solution (from **step 9**) containing the plasmid-miRNA-lipofectamine complex from each 0.6 ml tube to the corresponding well in the 96 well plate
(18) Gently swirl the plate around.
(19) Add the total 13.6 µl from **step 14**.
(20) Gently swirl the plate around.
(21) Put the plate back in the incubator for 24 hours.
(22) Carry out luciferase activity using either MSS's "Lightswitch luciferase assay (used with Switchgear genomics plasmids)

- The table below shows what would be added for each combination that is tested.

Combinations	Amount of media (optimum medium) (µl)	Lipofectamine solution 200+ opti-mem (µl)	PDE 4B plasmid (µl)	miR-26b mimic (µl)	Normalization plasmid (µl)	PDE4B Target protector (with Endoporter sol.) (µl)	Target protector control (with Eendoporter sol.) (µl)	Scrambled plasmid (µl)	Scrambled mimic (µl)
PDE4B plasmid +miR-26b+ normalization plasmid	49.5	25	6.5	12.5	6.5	-	-	-	-
3' UTR scrambled plasmid + miR-26b+ normalization plasmid	49.5	25	-	12.5.5	6.5	-	-	6.5	-
PDE4B plasmid +scrambled mimic+ normalization plasmid	49.5	25	6.5	-	6.5	-	-	-	12.5
3' UTR scrambled plasmid +scrambled mimic+ normalization plasmid	49.5	25	-	-	6.5	-	-	6.5	12.5
PDE4B plasmid +Target protector +normalization plasmid	48.4	25	6.5	-	6.5	13.6	-	-	-
PDE4B plasmid +Target protector control + Normalization plasmid	48.4	25	6.5	-	6.5		13.6		
PDE4B plasmid +Target protector+ miR-26b mimic	35.9	25	6.5	12.5	6.5	13.6	-	-	-
PDE4B plasmid+ Target protector + scrambled control mimic	35.9	25	6.5	-	6.5	13.6	-	-	12.5

APPENDIX K

CO-TRASNFECTION OT MIRNA MIMCS AND PLASMIDS

Purpose: To cotransfect 3' UTR plasmids with miRNA mimics in HCT116 cells to determine if the mRNA is a direct target of miRNA (i.e. Cotransfection of 3' UTR plasmids with miRNA mimics in HCT116 cells using Lipofectamine 2000 transfection reagent

Materials:

(1) Lipofectamine 2000 transfection reagent (Invitrogen-Cat # 52887 for 1.5 ml)
(2) Opti-mem-I medium (reduced serum)-(Gibco-Cat # 11058)
(3) Sterile 0.6 ml RNase free tubes
(4) Ice bucket
(5) Lyophilized miRNA mimics (Thermoscientific -Dharmacon: miRIDIAN mimic-miR-19b-Cat # C-300489-03; miR-26 mimic-Cat # C-300501-07; miR-203 mimic-Cat #C-300562-03; control mimic-Cat # CN-0010000-01-05)
(6) 3' UTR TCF4 plasmid (Genecopoeia: HmiT017951-MT01) (This plasmid had both Renilla and Firefly luciferase genes)
(7) 3'UTR control plasmid (Genecopoeia: CmiT000001-MT01)
(8) 3' UTR PDE4B plasmid (Switchgear Genomics: S810648)
(9) 3'UTR pTK2B plasmid (Switchgear Genomics: S806790)
(10) Control 3'UTR (Switchgear Genomics: S806790)
(11) Glass pipettes
(12) McCoy's 5A modified complete medium (instructions for making up the complete medium are in HCT116 cell culture protocol) (Gibco-Cat # 16600-108)
(13) 96-well white tissue culture treated plate (BD Falcon- Cat # 353296)

Resuspension of lyophiolized miRNA mimics: (under sterile hood)

1. Briefly centrifuge the screw cap tube at low speed (maximum 4000 x g) to make sure that all material is collected at the bottom of the tube before removing the cap in step 2.
2. Remove screw cap carefully.
3. Add nuclease-free, sterile water using a pipette with a sterile filter tip to achieve the desired concentration. Adding 250 µl water to 5 nmole microRNA Inhibitor vials which will make a 20 µM solution.
4. Let the tube stand for a few minutes at ambient temperature.
5. Gently pipette up and down 5 times to resuspend.
6. Repeat steps 4 and 5.
7. Aliquot the inhibitor in several tubes to limit the number of thaw-freeze cycles.
8. Store at -20^0C

Procedure:

Day 1:

(1) Seed 2.5x 10^5 cells in 96 well plate in 100ul of complete medium.

Day 2:

Note: The volumes mentioned are for one well in a 96-well plate.

(2) For each well, use one 0.6 ml sterile RNase free tube.
(3) Add 24.45 µl of Opti-mem medium and 0.55 µl of Lipofectamine in the tube. Finger-flick to mix and spin down.
(4) Incubate for 5 min at RT.
(5) Dilute the miRNA mimic in 12. 5 µl of opti-mem medium (see MSS's"Dilution of miRNA inhibitors/mimics or plasmids" protocol)
(6) Dilute the plasmids in 12.5 µl of Opti-mem medium (see MSS's "Dilution of miRNA inhibitors/mimics or plasmids" protocol)
(7) Add the diluted miRNA mimic and diluted plasmids into the 0.6 ml tube containing diluted lipofectamine solution.
(8) Finger flick the tube and spin down. Incubate for 20 min at RT for the complex formation to occur.
(9) During this 20 minute incubation time, take out the 96 well plate in which the cells were seeded the previous day.
(10) Using a glass pipette, suck out the old media and add 50 µl of complete McCoy's 5A media in each well.
(11) After 20 minutes, add the total 50 µl solution containing the DNA-miRNA-lipofectamine complex from each 0.6 ml tube to the corresponding well in the 96 well plate
(12) Gently swirl the plate around and put the plate back in the incubator for 24 hours.
(13) Carry out luciferase activity using either MSS's "Lightswitch luciferase assay (used with Switchgear genomics plasmids)" or Dual-glo luciferase assay (used with Genecopeia plasmids)" protocols based on the type of plasmid used

APPENDIX L

ISOLATION OF MIRNA

Purpose: To isolate microRNA from tissues, adherent cell lines or single cells.

Materials:
(1) mirVana miRNA isolation kit (Ambion, Cat #1560/1561)
(2) Acid-phenol chloroform (part of miRNA isolation kit)
(3) RNase-free 2 ml / 0.6 ml polypropylene microfuge tubes
(4) pipettors
(5) RNase free tips
(6) 100% ethanol
(7) microcentrifuge.
(8) optional–vacuum manifold.
(9) cell scraper

Note: **USE RNAse FREE TIPS!!!**
Prepare the Wash Solutions:
Once ethanol has been added, cap the Wash Solution bottles tightly to prevent evaporation.

_____(1) Add 21 ml 100% ethanol to miRNA Wash Solution 1. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

____(2) Add 40 ml 100% ethanol to Wash Solution 2/3. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

***NOTE** : A precipitate may form in the Wash Solution 2/3 bottle over the next several days as excess EDTA falls out of solution. Simply leave these crystals in the bottle when removing Wash Solution for use.*

Sample amount
Samples of 10^2–10^7 cultured eukaryotic cells can be processed per prep. However, if the initial lysate volume exceeds 300 µl, the samples will need to be processed in multiple loads due to limitations of filter capacity. Note: one nearly confluent T-175 flask will contain approximately 0.8 x 10^7 cells. This is not a problem as I use flasks which have around 60-65% confluency.

Procedure:

For ***Adherent cells:***

1. Aspirate the culture medium, and rinse 2-3 times with PBS to remove the medium. Place the flask on ice.

2. Disrupt samples in 300–600 µl Lysis/Binding Buffer

Remove the PBS wash, and add 300–600 µl Lysis/Binding Solution for 10^2–10^7cells. low end of the range (~300 µl) for small numbers of cells (hundreds), and use closer to 600 µl when isolating RNA from larger numbers of cells (thousands–millions). For adherent cells lysed directly in the culture plate, collect the lysate with a **cell scraper**, and pipet it into a **2 ml tube**.

3. Vortex or pipet vigorously to completely lyse the cells and to obtain a homogenous lysate.

RECORD STARTING VOLUME OF SAMPLE. Sample can be stored at -80°C for later isolation.

4. Add 1/10 volume of miRNA Homogenate Additive to the cell lysate and mix well by vortexing or inverting the tube several times. (For example, if the lysate volume is 300 µl, add 30 µl miRNA Homogenate Additive.)

5. Leave the mixture on ice for 10 min.

6. Add a starting volume of Acid-Phenol:Chloroform that is equal to the lysate volume before addition of the miRNA Homogenate Additive. (For example, if the original lysate volume was 300 µl, add 300 µl Acid-Phenol:Chloroform.)

IMPORTANT: *Be sure to withdraw from the bottom phase in the bottle of Acid-Phenol:Chloroform, because the upper phase consists of an aqueous buffer.*

7. Vortex for 30–60 sec to mix.

8. Centrifuge for 5 min at 10,000 x g at room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation. Do not place tubes on ice

9. Carefully remove the aqueous (upper) phase without disturbing the interphase, and transfer to a fresh tube. **Note the volume removed**.

10. **Pre-heat elution solution from kit to 95°C (100 ul per sample)** for use in eluting the RNA from the filter at the end of the procedure.

11. Add 1.25 volumes of room temperature 100% ethanol to the aqueous phase. (e.g. if 300 µl was recovered in step E.3, add 375 µl ethanol). **Mix thoroughly**.

12. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied.

13. Pipette the lysate (from the previous step) onto the Filter Cartridge (700 µl at a time). For sample volumes greater than 700 µl, apply the mixture in successive applications to the same filter.

14. Centrifuge for ~15 sec at 10,000 x g (typically 10,000 rpm). (Spinning harder than this may damage the filters). Repeat application until all sample has been applied, discarding flow through.

15. Apply 700 µl miRNA Wash Solution 1 to the Filter.

16. Centrifuge for ~5–10 sec at 10,000 x g . Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

17. Wash the filter with 500 µl Wash Solution 2/3. Centrifuge for 5-10 sec at 10,000xg. Discard flow through.

18. Repeat this step. Discard flow through.

19. Replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.

20. Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit).

21. Apply 100 µl of pre-heated (95°C) Elution Solution to the center of the filter, and close the cap.

22. Spin for ~20–30 sec at 10,000xg to recover the RNA.

23. Collect the eluent (which contains the RNA). (About 80ul of eluent will be recovered).

DNase Treatment:

24. To eluted RNA, add 8 µl (0.1 vol) 10x DNase I buffer (Ambion DNA free kit) and 2ul DNase. Mix gently and incubate at 37°C for 20-30 minutes.

25. Add 8 µl DNase inactivation reagent (after resuspending it). Incubate for 2 min, flicking a few times during incubation.

26. Centrifuge tube for 1 min, 10,000 x g. Remove supernatant to new tube, leaving reagent behind.

RNA quantitation: by Nano Drop (no dilution necessary)
RNA quality assessment: by Agilent Bioanalyzer on a Nano chip.
(Note: if you won't be doing the Nano Drop and Agilent the same day, save a 5 µl aliquot in a separate tube so you don't have to thaw your entire sample.

APPENDIX M

WESTERN BLOTTING

Purpose: To measure the amount of total proteins.

Preparation:

-Thaw samples on ice
-Label 0.6 mL eppy tubes
-Turn heating block on and set temperature to 98°C
-Prepare the western template sheet
- Cut PVDF membrane and filter papers ready

Procedure:

Sample preparation

1. Thaw the samples on ice while you turn on the heating block and set the temperature to 98°C (takes about 15 min).
2. Dye used for the sample dilution is 5X Pyronin. Use 1X of the dye based on the total volume of sample required (usually 25 mL total).
3. With the aid of western template sheet make the necessary dilution (if required) and add the calculated amount of dye and water to the samples and standard.
4. Quick spin.
5. Boil the samples for 5-10 min depending on the volume of the samples (25 mL volume boil for 10 min). Do not boil the marker.
6. Quick- spin of the samples on the tabletop.

Gel unit set up

7. Take the pre-made gel (usually 4-20%) and carefully rip off and discard the white tape and the comb. Mark the lanes on the plate.
8. Attach the gel to the gel rack align 3rd with the lower gasket and clamp the unit. (Note that the red clip should have the broad end facing you, broad ends face outside on all 4 clips). Either run a gel on each side or attach the white space holder on the empty side.
9. Pour running buffer to fill the stand and the trough up to the top mark.
10. Use gel-loading tips (or 10 mL XL tips) and load the complete sample volume.
11. Close the unit with the lid and check the leads and make sure black-to-black and red-to-red.
12. In the cold room run the gel at 125 V for as long as needed. Check after 10 minutes to make sure it is running.
13. After about 1 h check every 15 min.
14. Stop the gel when it has run as far as needed.

Gel transfer

15. Crack open the plate with a scalpel between the markings on the plate all around by keeping the large side of the gel down.
16. Cut the gel just above the bottom.
17. Carefully separate the gel from the plate and cut the gel at lane one to identify the side (left end).
18. Take the gel transfer unit in a staining tray and pour transfer buffer into it and the trough. Allow gel to equilibrate in transfer buffer for 15-30 min. Wet membrane with methanol then equilibrate in transfer buffer for ~5 min.
19. Take the cassette and lay it open.
20. Put a sponge on the black side of the cassette and place a filter paper on top of it.
21. Pour transfer buffer to keep it wet.
22. Take the gel plate out of the running trough and transfer the running buffer into the bottle for reuse.
23. Place the gel on the filter paper with lane one on the right (protein side facing the membrane.
24. Cut the right hand top corner of the membrane to identify the side.
25. Place the membrane on the gel and place the other filter paper on the membrane. Now, use a roller on the filter paper to eliminate any air bubbles in between.
26. Place the sponge and close the white side of the cassette and clip it.
27. Place the cassette in the transfer unit with the hinges facing the top and black side facing back.
28. Put a stir bar into the transfer trough.
29. Fill the trough with transfer buffer just enough to cover the hinges of the cassette. Check the terminals black to black correspond.
30. Place it on the cold room stir plate.
31. Connect black-to-black and red-to-red and set current to 400 milliamps and let it transfer for at least 90 min.

Blocking

32. At the end of 90 min- make 4% nonfat dry milk/ PBS/ Tween in a 50 mL tube (to 30 mL of PBS –Tween and add 1.2 gm of pre-weighted milk powder). Mix gently by inverting. If 5% BSA is required, add 1.5 g IgG free BSA to 30 mL PBS-T.
33. Pour the milk into a dish and keep ready to transfer the membrane into it.
34. After the transfer is complete- open the gel unit and transfer the transfer buffer into the bottle.
35. Use a pair of forceps to take the membrane and place the membrane into the milk dish (with the side facing gel-protein side now facing top)
36. Place it on the shaker for 1 hr at room temperature.

Primary antibody

37. Take a dish with 1.2 gm dry milk powder and 30 mL PBS- Tween. Mix and pour into a new dish.
38. Transfer the membrane from the blocking buffer into the dish with fresh milk.

39. Now, add the appropriate volume of the primary antibody (based on the dilution and add it into the dish).
40. Close the lid of the dish and shake it gently on the cold room shaker overnight.

Washing
41. The next day take the membrane and give a quick wash with PBS –Tween.
42. Then replace the membrane in fresh PBS- Tween in the dish and keep on the shaker at room temp for 10 min. Let it shake vigorously.
43. Repeat the wash 2 times at 5-10 min interval.

Secondary antibody
44. Make 30 mL milk/ PBS/ Tween and pour into the dish after the second wash.
45. Add the required volume of secondary antibody based on the dilution.
46. Set on shaker for 1 hr at room temperature.
47. Repeat washing with PBS –Tween 3 times.
48. While the 1 wash of 2° antibody is going on turn on the imager and set focus.

Developing
49. Cut an acetate sheet into 2 halves and remove the black sheet.
50. Mix equal parts of chemiluminescent super signal reagent A and reagent B in an ependroff tube. Mix gently by inversion.
51. Transfer the membrane between the layers of the acetate sheet and evenly disperse the developing solution across on the top of the membrane.
52. Slowly close the top layer so that the solution gets evenly distributed on the membrane.
53. Expose for 5 minutes and then transfer the membrane on to the clean acetate sheet .
54. Transfer it into the BioRad imager for imaging immediately.

Imaging
55. Turn on switch and make sure the lever on the hood is at chemiluminescence.
56. Select QuantityOne on program on desktop.
57. Select scanner – click on chemidoc.xrs.
58. Step 1- option is chemiluminescences.
59. Step 2 – live focus. Focus with a printed sheet and set the iris as you need for brightness. Zoom and focus, as you need for clarification.
60. Freeze. Put the gel in the imager and zoom and freeze again. Close the door.
61. Click on live acquire.
62. Set the Starting exposure time, Total exposure time, and Number of exposures as needed.

Formulas for the buffers used in this assay:

Pyronin 5X Sample Buffer:

40% glycerol (Vol.)

25% β-mercaptoethanol (Wt)

12% SDS (Wt)

0.31 M Tris Base, pH: 6.8

25 mM EDTA

0.1% pyronin Y (Wt)

==> 1.5 g Tris base in 10 mL H_2O. Adjust with Conc. HCl to pH 6.8

Add 4.8 g SDS. Take to 15 mL with H_2O.

Heat gently to dissolve or leave at RT overnight.

Add 8.9 mL β-mercaptoethanol (stock=1.2g/mL)

Add 16 mL glycerol

0.37 g Na_4EDTA

0.04 g or less pyronin

Let stir at RT to dissolve.

Aliquot small amount in epi-tube, keep at RT for current use.

Store remaining buffer at -20°C.

Running Buffer: (Tricine SDS running buffer)

==> Dilute the 10X Tricine SDS running buffer (Novex, cat# LC1675) to 1X using H_2O.

Store at 4°C. Can be re-used.

Transfer Buffer: (Tris-Glycine transfer buffer)

1X Tris-Glycine

14% methanol

==> For 1 L working buffer:

100 mL of 10X Tris-Glycine solution (Fisher, cat# BP1306-1)

140 mL of methanol

760 mL of H_2O.

Store at 4°C. Can be re-used.

PBS/Tween:

0.1%(wt)Tween-20 in 1X PBS

==> (1) 10X PBS/Tween:

Dissolve 1 bottle of 10X Dulbecco's Phosphate-Buffered Saline (Gibco, cat#21600-069) in 1 L of H_2O.

Add 10 g of Tween-20 (Fisher, cat#BP337-500) (weight the PBS/Tween on the scale while adding the Tween-20)

(2) 1X PBS/Tween:

Dilute the 10X PBS/Tween to 1X PBS/Tween using H_2O.

APPENDIX N

SINGLE CELL ISOLTION FROM COLON FOR FACS SORTING

Purpose: To isolate single cells from a mouse colon for flow sorting

Materials:

(1) Weigh boats
(2) Surgical tools
(3) Ca^{2+}/Mg^{2+} free HBSS (Mediatech #21-021-CV)
(4) Advanced DMEM/F12 (ADF) (Invitrogen #12634-010)
(5) Na_4EDTA (Sigma, #ED4SS)
(6) gavage needle (Popper & Sons, # 7922)
(7) disposable gavage needle (Soloman Scientific # FTP-20-38)
(8) DNase (Sigma #D5025, 20 U/µL stock in PBS)
(9) TrypLE Express Trypsin (Invitrogen # 12605-010)
(10) 40 µm cell strainer (BD #352340)
(11) 50 ml and 15 ml conical tubes
(12) Polypropylene flow collection tubes
(13) EDTA (Ambion #9260G)

Note:*Use only Ca^{2+}/Mg^{2+} free HBSS (Mediatech #21-021-CV) in this protocol.

Procedure:

The day before:

*Inject Tamoxifen the day before isolation.

1. Prechill PBS in cell culture fridge. Prechill HBSS in Lab fridge. Put a bucket of dd-H_2O inside the 37°C incubator.
2. **Precoat 9 polypropylene flow collection tubes with ADF+, keep at 4°C fridge.**
3. Prepare ADF+ (Advanced DMEM/F12) (Invitrogen #12634-010) plus 1% glutamax, 1% P/S, 1% HEPES (50 ml/tissue). Keep cold.
   375 ml ADF + 3.75 mL 200 mM Glutamax + 3.75 mL Pen/Strep + 3.75 mL 1M HEPE S
4. Calculate amount of EDTA needed, have loosely tied string, gavage needle with 5 mL syringe ready, and label all tubes/vials
5. Get 2 ice buckets, one needs to be low, easy to work in the hood.

The day of isolation:

(When aspirating, always attached the tip of glass pipet to a 200 µL pipet tip, this will reduce the suction force and avoid aspirating the pellet)

In the lab:

- Make FRESH 20 mM EDTA/HBSS solution use:

0.378 g Na_4EDTA (Sigma, #ED4SS) in 45 ml HBSS, pH to 7.4. (Need ~ 45 ml/colon).
(~ 2 drops of 10 M HCl + 2 drops of 1 M HCl)

6. Warm up EDTA/HBSS solutions in 350 mL conical tubes, at 37°C water bath (in cell culture room).
7. Thaw FBS (need ~5ml per colon)
8. Prepare 100ml cold HBSS for each animal.
9. Have the gavage needle (Popper & Sons, # 7922) connected to 5 mL syringe, fill with ~ 5 mL of cold HBSS.
10. Remove colon from mouse and place in a medium weight boat containing cold HBSS.
11. Lay the tissue on one hand; trim the excess fat using a forcep.
12. Squeeze out the fecal pellets. Put them in the pre-labeled and pre-weighed cryovials. Weigh them and write down their weights. Quick freeze the feces in liquid nitrogen.
13. Then use gavage needle, perfuse colon with 5 ml cold HBSS.
14. If you want to fix some tissue for IHC later), Cut a small piece of colon from end and put it in the cassette. Put the cassette in 4% PFA.Fix for 4 hours on ice, followed by ethanol ashes as per our PFA fixation protocol.
15. Evert colon on disposable gavage needle (Soloman Scientific # FTP-20-38), then put the tissue attached with the gavage needle into the 30 mL cold HBSS (in 50 mL conical tube). Keep on ice.

In the cell culture room:

- Move the samples and all the materials (100 mm petric dish, forceps, EDTA, FBS, 40 μM cell strainer, trypsin/DNase) to cell culture room.

- Cells are fragile, keep cells on ice, except during the EDTA and Trypsin incubations.

16. Vortex colon (in the conical tube with cold HBSS) at max speed **6 x,** 5 sec each, to remove remaining debris. (To get a good vortex, you can only vortex 1 tube at a time). (Make sure the tissue is untangled between/after the vortexing) Vortex strongly to get rid of feces.
17. Transfer the colon to the pre-warmed **20 mM** EDTA/HBSS. Incubate at 37°C water bath.
18. Put thawed FBS on ice.
19. After **30 min** incubation, transfer the suspended tissue to the tube containing ~ 30 mL cold HBSS and vortex to release crypts. Need fairly vigorous vortexing so that colon whips around tube; setting at 10 for **8 x**, 5 sec each. (Make sure the

tissue is untangled between each vortex)**.** (Take 5 µL aliquot on petri dish to check under microscope to see whether there are some crypts)

20. Remove tissue and discard. Add FBS to the tube containing crypts to make 10% FBS/HBSS (e.g. 3 mL FBS to 30 mL of HBSS) and spin down the crypts at **800 rpm, 3 min**. If all the crypts are not pelleted, the re-spin again.
21. Aspirate solution and resuspend crypts gently (~ 3x) with **~ 13 mL cold ADF+** and transfer to 15 ml conical tube. Gently invert to mix, then keep on ice (First resuspend in ~ 8 mL, then with ~ 5 mL, so the tube contains ~ 13 mL)
22. Spin at **600 rpm, 2 min** (helps prevent pelleting of single cells). Aspirate and resuspend in 10 ml of 1X cold PBS. Take a 5 µL aliquot to count. (just add the drop of cells on petri dish, then count the cell# in the whole area of this small drop.) If all the cells are not pelleted, the re-spin.
23. Spin at **700 rpm, 3 min** to remove FBS.
24. Repeat the PBS wash 1 more time, 10 mL cold PBS, **700 rpm 3 min**.
25. Prepare trypsin/DNase (dissociation) solution: Use **50 µL DNase/10 mL trypsin**, need 5 - 10 mL/sample.
    (DNase: Sigma #D5025, 20 U/µL stock in PBS, aliquot in LD's -20° Freezer; TrypLE Express Trypsin: Invitrogen # 12605-010, in LD's small refrigerator)
26. Aspirate supernatant, resuspend pellet in **~X mL** trypsin/DNase solution*****. Pipet up and down **~15X**, gently (i.e. not lots of air bubbles, no squirting directly against side of tube). More crypts use more solution. Incubate in 37°C (water bath) incubator for 15 min.

    (*Use ~ 5 mL for 50K crypts, adjust the volume accordingly).

27. Prepare ADF+/EDTA: 30 mL ADF+ containing 5 mM EDTA per sample (eg. 300 µL of 0.5 M EDTA (Ambion #9260G, aliquot in YY's drawer or LD's extra shelf at RT) in 30 mL ADF+, keep on ice.
28. Take samples out of incubator. Pipet up and down gently **10 X**. *really break the pellet* (Take 5 µL aliquot onto petri dish to check under microscope to see whether cells are starting to dislodge from crypts). Continue incubation for another 15 min at 37°C. If the crypts are not broken completely, incubate the samples for another 15 minutes (third time)
29. Prepare 40 µm cell strainer (BD #352340) on top of clean 50 mL conical tube for each sample
30. Take samples out of incubator, keep on ice. Pipet up and down and take 5 µL aliquot onto petri dish to check under microscope to see whether cells are all dislodged from crypts. Make sure there are lots of single cells.
31. Pre-wet 40 µm cell strainer with 2 ml ADF+/EDTA. Adding the equal volume of ADF+/EDTA to the sample, **Make sure to resuspend the single cells** very well then filter the cell suspension through cell strainer to remove large clumps. Add 8 mL of ADF+/EDTA to the cell strainer, to rinse through remaining samples. (Save cell strainer until you know cells passed through). *(The volume is not critical, you just want to dilute the sample and exclude the clumps. But you want*

to record the total pass thru volume for use in the following cell counting calculation)
Take 10 µL aliquot for hemocytometer count:
10 µL cells + 9 µL PBS + 1 µL trypan Blue (2 fold dilution)

32. Spin down, 1000 rpm, 5 min.
33. Prepare *ADF+ with additional 4 mM MgCl2. (250 x diluted from 1 M bottle) (Need ~ 2 x 10^6 cells/mL).

**We want to keep cells happy during the sorting, so we use the complete medium (ADF+) containing all energy source for stem cells. The ADF contains ~ 0.7 mM Mg^{2+}, and ~1 mM Ca^{2+} it is recommend to have at least 1 mM (optimal 5 mM) Mg2 + for DNase to fully function, so I ad additional 4 mM MgCl2)*

34. Aspirate supernatant, wash cells one more time with ~ 10 mL ADF+ (No EDTA, to avoid residue of EDTA in the next step), count the cell # again. Then 1000 rpm, 5 min.
35. Resuspend cells in desired vol of ADF + 4 mM MgCl2 + DNase (200 U/mL, = 10 µL our DNase stock/mL; to attain up to ~4 x 10^6 cells/ml, pipet 10X. (We try to use ~ **2 x 10^6 cells/mL** to avoid clumping during sorting)
*Our DNase stock is [20 U/µL]. The EDTA stock is 500 mM EDTA.
Transport to FACS Aria for sorting, collect high and low GFP+ and negative cells.
Make sure to take the instruction sheet for flow sorting using the Aria flow cytometer.

Take to Med School: ADF+, flow runs, Flow form, Extra flow tubes, Pipets and tips, ice, coated flow tubes for each collected fractions, ADF+ medium to collect into sharpie, gloves, waste basket, lab coat and CD.

After flow, spin down negative sample and resuspend in 600 ul of miRVana lysis buffer from miRVana miRNA isolation kit. Store the tubes at -80C.The GFP high and low cells are sorted directly in the lysis buffer. Hence store them directly at -80C.

APPENDIX O

MIRNA PROFILING USING LOW DENSITY ARRAYS

Purpose: To carry out miRNA profiling from RNA isolated from Fluorescence Activated cell sorted (FACS) sample from mouse colon.

Materials:

(1) Megaplex RT primers Rodent Pool A (Applied Biosystems Part # 4399970)
(2) TaqMan microRNA Reverse Transcriptase Kit components (Applied Biosystems Part # 4366596)
(3) $MgCl_2$ (supplied with Megaplex RT primers)
(4) 96-well Micro Amp Optical plate (Applied Biosystems Part # 4346906)
(5) MicroAmp Clear Adhesive Film (specific for RTPCR)
(6) Megaplex preamp primers Rodent Pool A (10X) (Applied Biosystems Part # 4399203)
(7) TaqMan PreAmp Master Mix (2X) (Part # 4384266)
(8) Pipttes and tips
(9) RNase free water
(10) IX TE (Ambion, Cat # 9849
(11) 0.2 ml RNase free tube tube (Genemate, Cat # C-3328-2)
(12) TaqMan Rodent MicroRNA Array A (Applied Biosystems Part # 4398979)
(13) TaqMan Universal PCR Master Mix (Applied Biosystems Part # 4440040)

Note: Use RNase free tubes in the entire protocol.

I. RT-PCR (using Megaplex pool)

1. Thaw the following on ice:
2. Megaplex RT primers Rodent Pool A (Applied Biosystems Part # 4399970)
3. TaqMan microRNA Reverse Transcriptase Kit components (Applied Biosystems Part # 4366596). Leave RT enzyme in freezer till ready to use.
4. $MgCl_2$ (supplied with Megaplex RT primers)
5. Total RNA samples (2 ng total RNA diluted in 3 ul)
6. Combine the following in a 0.65 ml RNase free microcentrifuge tube.

If you have to carry out a minus RT reaction, dispense the individual contents directly in the well of 96 well plate.

RT reaction mix components	Volume for 1 sample (ul)
Megaplex RT primers Rodent Pool A (10X)	0.80
dNTPs with dTTP (100 mM)	0.20
MultiScribe Reverse Transcriptase (50 U/ul)	1.50
10X RT buffer	0.80
MgCl2 (25 mM)	0.90
RNase inhibitor (20 U/ul)	0.10
Nuclease free water	0.20
Total	4.50

7. Invert the tube 6 times to mix and then centrifuge briefly
8. In 96- well Micro Amp Optical plate (Applied Biosystems Part # 4346906) pipette **4.5 ul** of the RT reaction mix into each well.
9. Add **3 ul** of total RNA **into** each well containing RT reaction mix. Pipette up and down to mix.
10. Seal the plate using MicroAmp Clear Adhesive Film. Do not use the PCR film. Spin briefly.
11. Incubate the plate on ice for 5 min.
12. Set up the run method using the following conditions:
    - Ramp Speed or mode: 7900HT (real time machine) using Std ramp speed (select std in mode, delete 1st and 2nd steps).
    - Reaction volume: 7.5 ul
    - Thermal-cycling conditions:

Stage	Temp	Time
Cycle (40 cycles)	16°C	2 min
	42°C	1 min
	50°C	1 sec

Hold	85°C	5min
Hold	4°C	∞ (99:59)

13. Load plate and run with foam compression pad (brown side up).
14. Label 0.2 ml tubes with the sample name
15. Take out the 96 well plate and take out the entire volume from each well into labeled tubes.

II. Pre-Amplification Reaction

16. Thaw the Megaplex preamp primers Rodent Pool A (10X) (Applied Biosystems Part # 4399203) and TaqMan PreAmp Master Mix (2X) (Part # 4384266) on ice and mix by inverting the tube six times. Spin briefly.
17. Mix the TaqMan PreAmp Master Mix (2X) by swirling the bottle.
18. Combine the following in a 0.65 ml microcentrifuge tube:

PreAmp Reaction mix components	**Volume for 1 sample (ul)**
TaqMan Pre-Amp Master Mix (2X)	12.5
Megaplex PreAmp Primers (10X)	2.5
Nuclease free water	7.5
Total	22.5

19. Invert the tube 6 times to mix and centrifuge the tubes briefly.
20. In a new 96 well MicroAmp Optical Reaction Plate (which was used to run the RT plate), pipette **2.5 ul** of each RT product from the 0.2 ml tubes (from above RT step) into its corresponding well. You can use the old PCR plate if there
21. Dispense **22.5 ul** of PreAmp reaction mix into each well of the 96 well plate containing the RT product. Pipet to mix
22. Seal the plate using MicroAmp Clear Adhesive Film. Spin briefly.
23. Incubate the plate on ice for 5 min.
24. Set up the run method using the following conditions:
    - Ramp speed or mode: **7900HT (real time machine)** using **Std** ramp speed.
    - Reaction volume (ul): 25
    - Thermal-cycling parameters:

Stage	Temp	Time
Hold	95°C	10 min
Hold	55°C	2 min
Hold	72°C	2 min
Cycle (12cycles)	95°C	15 sec
	60°C	4 min
Hold	99.9°C	10 min
Hold	4°C	∞

25. Load, then run the plate.

Dilute the reaction:

26. Remove the 96-well plate from the thermal cycler
27. Briefly centrifuge the plate
28. Add **75 ul** of 0.1X TE (dilute from IX TE (Ambion, Cat # 9849) (pH 8.0) to each well. Mix by pipetting up and down. Take out the entire 100ul from the 96 well plate then transfer to a 0.2 ml RNase free tube (Genemate, Cat # C-3328-2) (labeled according to the sample).
29. Take out 10 ul into another 0.2 ml RNase free tube to avoid freeze thaw cycles.
30. Store both the tubes at -20°C if not containing the Real time reaction immediately.
31. Proceed directly to "III. Real-Time PCR reactions".

III. Real time PCR reactions

Prepare TaqMan microRNA array:

32. Take out TaqMan Rodent MicroRNA Array A (Applied Biosystems Part # 4398979) cards from fridge (**30 mins before loading sample in cards)** and let them sit at room temperature. Carefully remove it from its packaging.
33. Take the diluted PreAmp product and thaw on ice. Mix by inverting the tubes six times, then micro centrifuge the tube (tiny microcentrifuge on lab bench).
34. Mix the TaqMan Universal PCR Master Mix (Applied Biosystems Part # 4440040) by swirling the bottle.
36. Combine the following in a 1.5 ml RNase-free microfuge tubes:

Component	Volume for One Array (ul)
TaqMan Universal PCR Master Mix, no AmpErase UNG, 2X	450
Diluted PreAmp product	9
Nuclease-free water	441
Total	900

37. Invert the tube 6 times to mix, vortex for 2 seconds and microcentrifuge for 20 seconds at RT or 4°C. Leave at room temp covered in foil for 30 minutes. (These 30 minutes should be in parallel with the time the cards sit at RT <u>Load and run the microRNA array:</u>
38. Dispense 100 ul of PCR reaction mix from the 1.5 ml tube (having 900 ul of reaction mix) from earlier step into each fill port of the TaqMan MicroRNA array (total 8 ports). Each array card will be for one sample.
39. Make sure the sample is loaded into the bigger port (called the fill port). Do not allow the tip to be in contact and damage the coated foil beneath the fill port. Also, make sure there are no bubbles in the reservoir.
40. Centrifuge based on the following instructions:

- Open centrifuge *(Sorvall, located next to Real Time pcr machine in Room 323)* and take out the bucket with the empty spot for the card. Place the bucket with the label facing you. Place the TaqMan array in the buckets, making sure that the fill reservoirs project upwards out of the array holder. The reaction wells face the same direction as "This Side Out" label.
- Power on centrifuge and using the front panel controls, set the bucket type to 15679.
-

Parameter	EasySet (touchpad)
Up Ramp rate	9
Down Ramp rate	9
Rotational speed	1200rpm (331xg)
Centrifugation time	2x1min

- Press the start button and centrifuge for total of 2 consecutive 1-min spins.
- After centrifugation, take out the array from the bucket.

Look at the TaqMan arrays to determine whether filling is complete.

41. Seal the TaqMan array based on the following instructions:

    - Place the sealer on a sturdy lab bench.
    - Make sure the sealer is turned at the front end (i.e. step 1 and 2 is at the farthest end)
    - Put the array card gently on the sealer with the foil side up ensuring that the front end of the array (see Manufacturer's instructions for more specific details). The TaqMan Array's fill reservoir end should be the end closest to the arrows etched in the base of the sealer.
    - Push the cartridge across the base of the sealer in the direction of arrows. Use a slow deliberate motion to push the cartridge across the length of the card until the carriage reaches the mechanical stops. Avoid moving the cartridge rapidly across the card.
    - Take out the array card and then move the carriage (handle) back into the starting position.

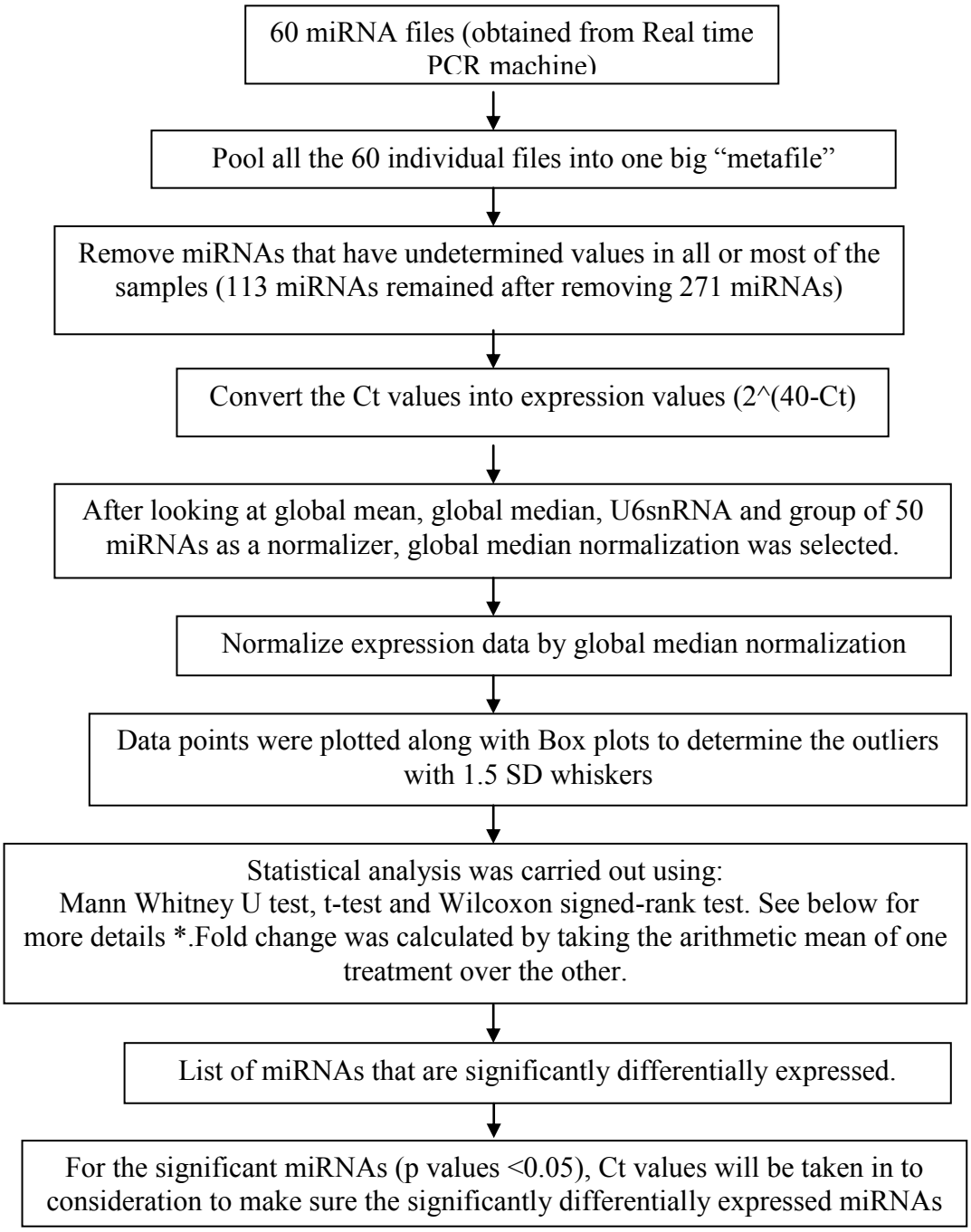
42. Using scissors, trim the fill reservoirs from the Taq Man Array.
43. Import the SDS setup file (SDS.txt) located on the **information CD (that comes with the array card) (refer to bulletin protocol):**

    - Start the SDS v2.2 or later software.
    - In the main menu, select File ⟶ New
    - In the new document dialog box, select the following from the drop-down menu:
        - Relative Quantification (ΔΔCt)
        - 384-well TaqMan Low Density Array
    - In the main menu, select File ⟶ Import to open the new document.
    - In the Open dialog box, navigate to the Setup.txt file specific for the array being run and click Import.
    - (Optional) Save as an SDS 7900 Template (.sdt) file.

44. Load and Run the array using the 384 well TaqMan Low Density Array default thermal-cycling conditions.

APPENDIX P

MIRNA DATA ANALYSIS PIPELINE



APPENDIX Q

FIGURES

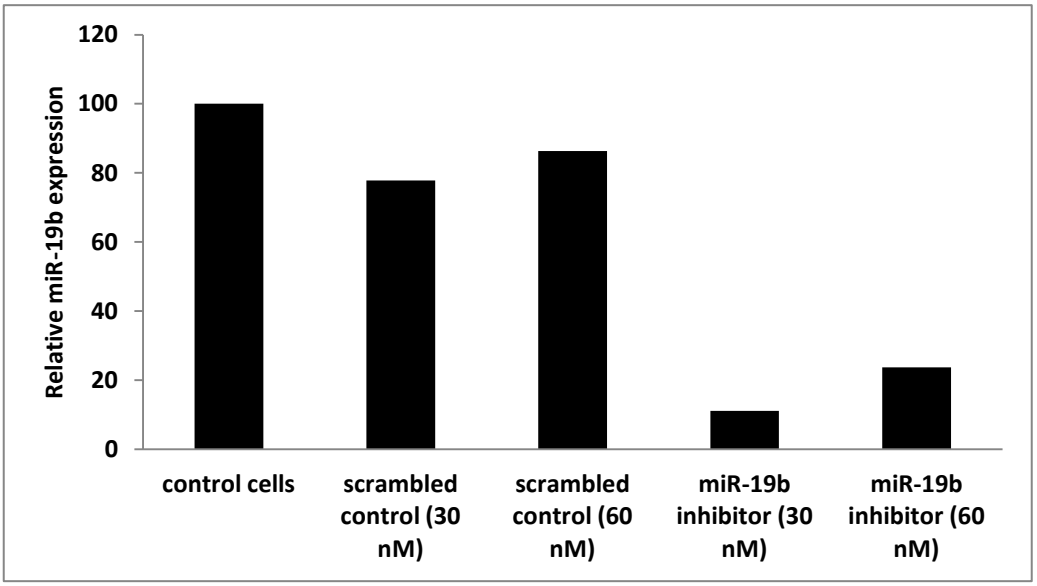


Figure Q1. Dose-dependent knockdown of miR-19b. Knockdown of miR-19b was carried out by transfecting HCT116 cells with either 30 nM or 60 nM miR-19b inhibitor or scrambled inhibitor. miR-19b expression was measured 24 h following transfection by qRT-PCR. (n=2). The values were normalized to control.

Based on this finding, 30 nM miR-19b inhibitor and scrambled control were selected for further experiments.

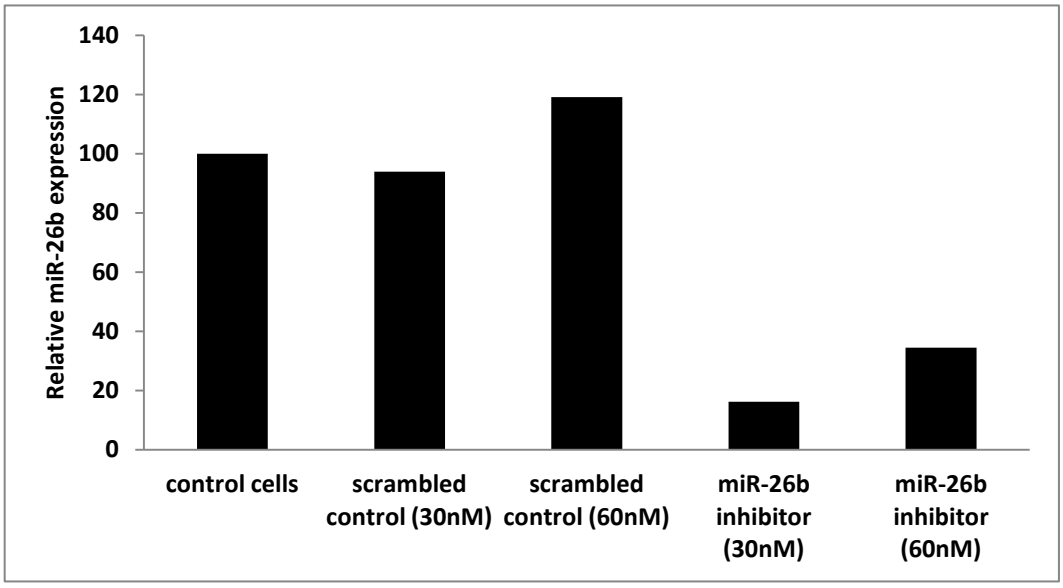


Figure Q2. Dose-dependent knockdown of miR-26b. Knockdown of miR-26b was carried out by transfecting HCT116 cells with either 30 nM or 60 nM miR-26b inhibitor or scrambled inhibitor. miR-26b expression was measured 24 h following transfection by qRT-PCR. (n=2). The values were normalized to control.

Based on this finding, 30 nM miR-26b inhibitor and scrambled control were selected for further experiments.

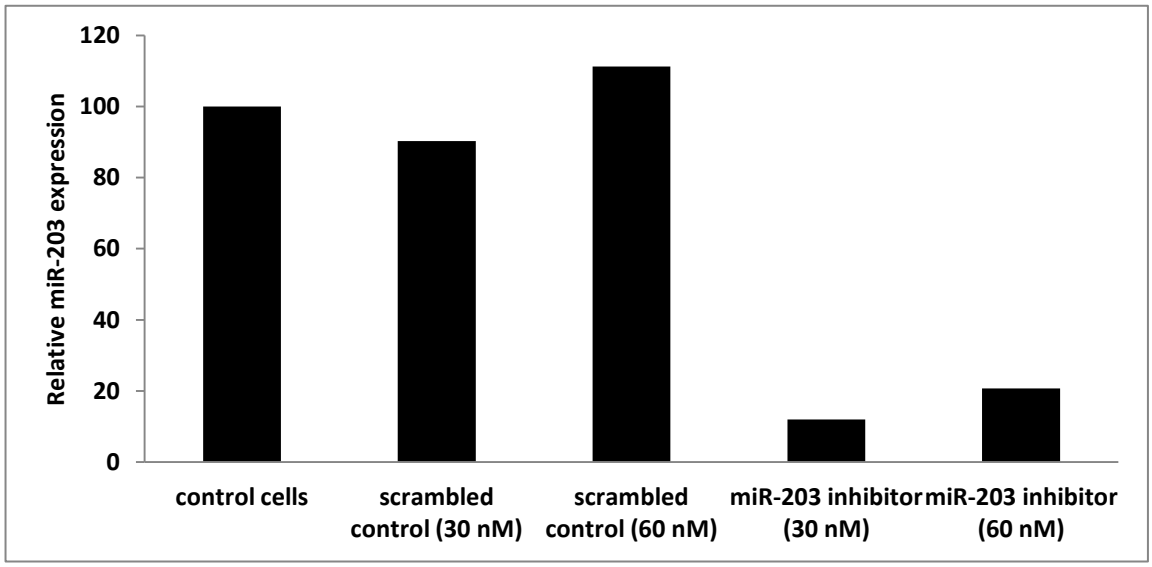


Figure Q3. Dose-dependent knockdown of miR-203. Knockdown of miR-203 was carried out by transfecting HCT116 cells with either 30 nM or 60 nM miR-203 inhibitor or scrambled inhibitor. miR-203 expression was measured 24 h following transfection by qRT-PCR. (n=2). The values were normalized to control.

Based on this finding, 30 nM miR-203 inhibitor and scrambled control were selected for further experiments.

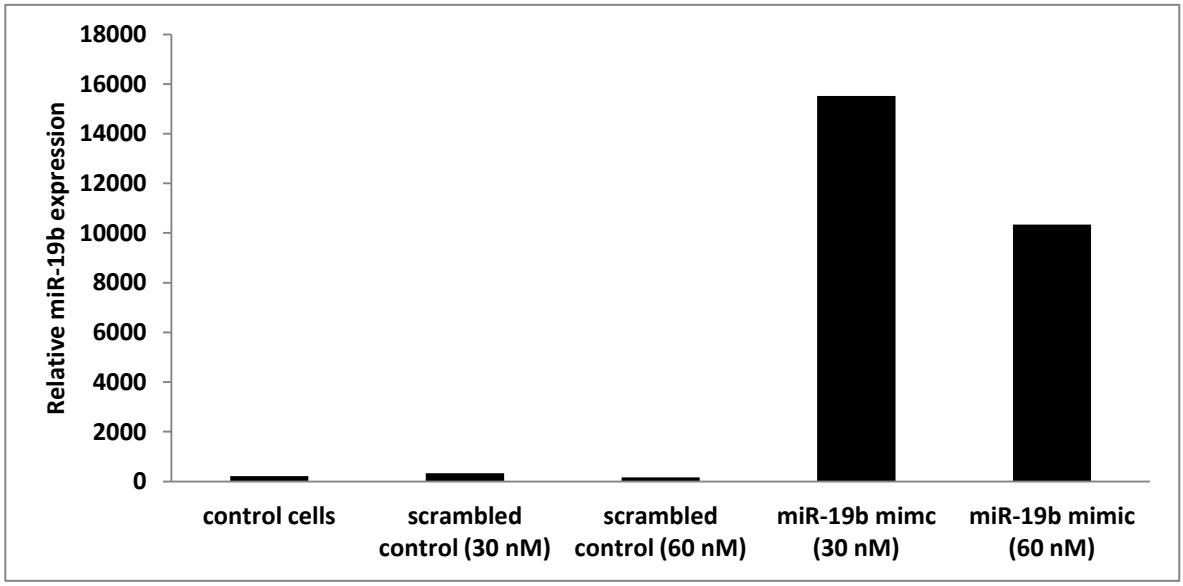


Figure Q4. Dose-dependent overexpression of miR-19b. Overexpression of miR-19b was carried out by transfecting HCT116 cells with either 30 nM, 60 nM or 100 nM miR-19b mimics or scrambled mimic. miR-19b expression was measured 24 h following transfection by qRT-PCR. (n=2).

Based on this finding, 30 nM miR-19b mimic and scrambled control were selected for further experiments.

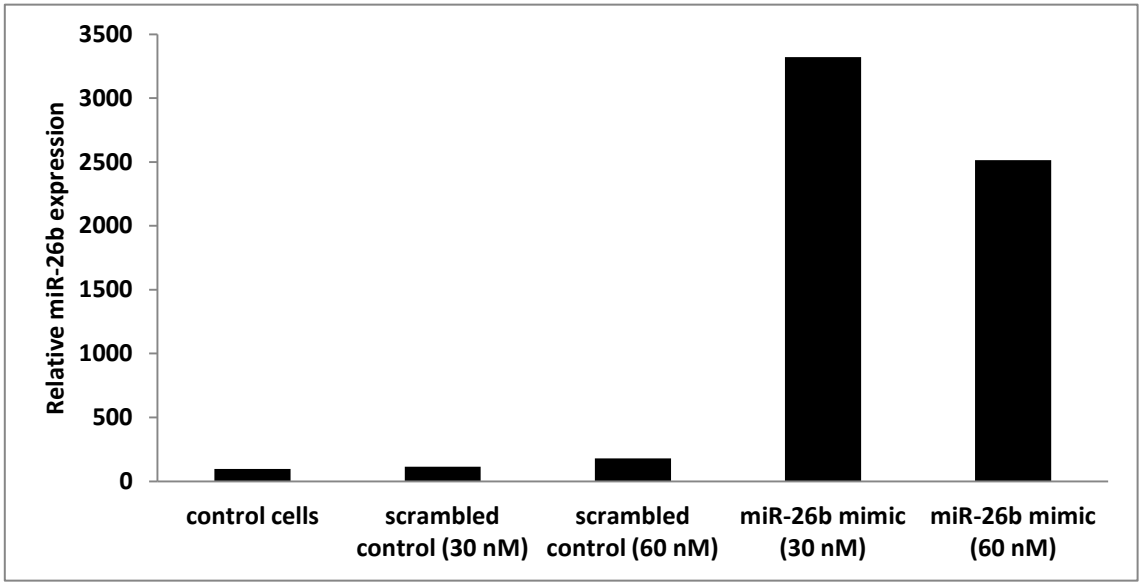


Figure Q5. Dose-dependent overexpression of miR-26b. Overexpression of miR-26b was carried out by transfecting HCT116 cells with wither 30 nM, 60 nM or 100 nM miR-26b mimics or scrambled mimic. miR-26b expression was measured 24 h later by qRT-PCR. (n=2)

Based on this finding, 30 nM miR-26b mimic and scrambled control were used for further experiments.

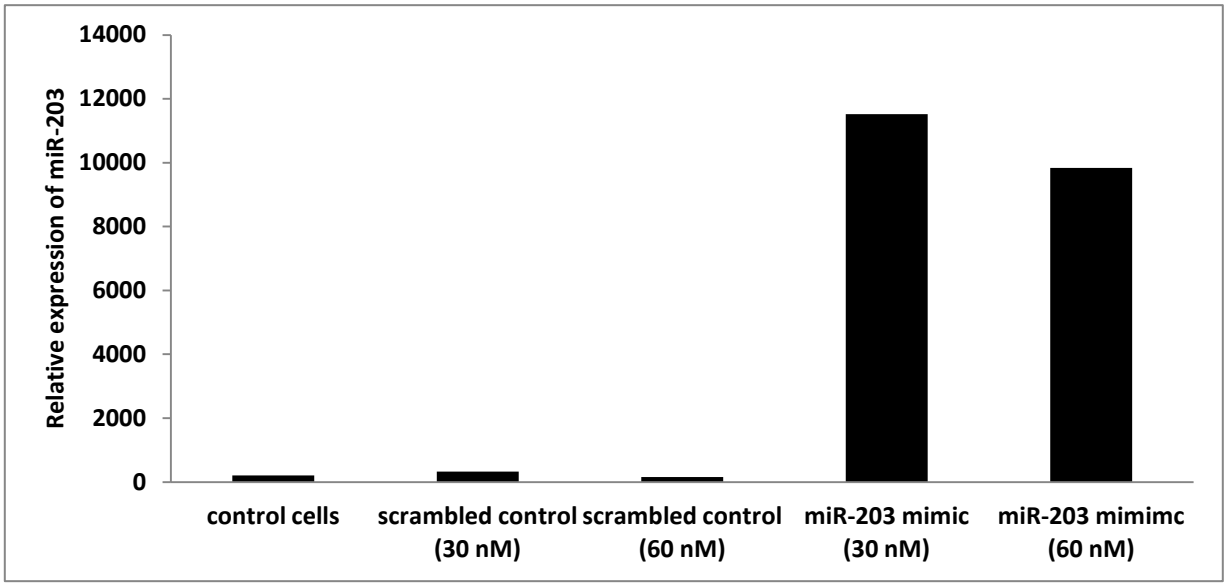


Figure Q6. Dose-dependent overexpression of miR-203. Overexpression of miR-203 was carried out by transfecting HCT116 cells with either 30 nM, 60 nM or 100 nM miR-203 mimics or scrambled mimic. miR-203 expression was measured 24 h later by qRT-PCR. (n=2)

Based on this finding, 30 nM miR-203 mimic and scrambled control were used for further experiments.

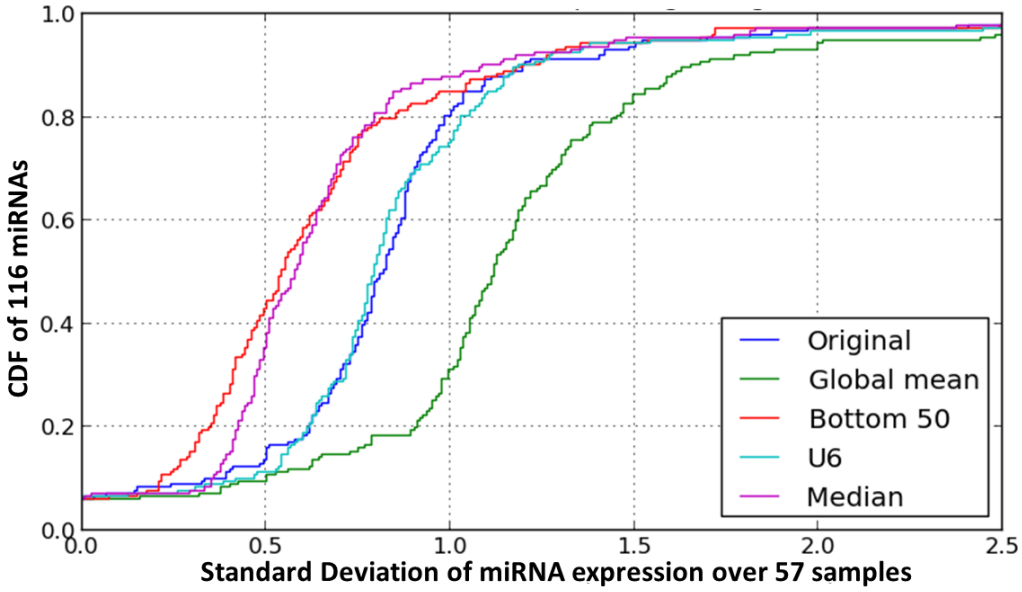


Figure NQ7. Cumulative distribution function plot of normalized miRNA data. The plot describes the cumulative distribution of relative miRNA expression levels pooled from 57 samples after normalization. The purple line corresponds to the original (raw) data, green line corresponds to global mean normalization, blue line corresponds to normalization using U6snRNA and the red line corresponds to 50 miRNAs whose expression was not altered in the 57 samples.

Note: Based on the variance shown by the different colored lines, global median normalization was determined to be fit for the miRNA expression data. It had the least variance. Bottom 50 was not selected since it is not a well-defined method to normalize miRNA profiling data.