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Expression of miRNA in different regions of the colon: An investigation in Zucker(fa/fa) rats

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by

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ABSTRACT

The role of obesity in regulation of microRNA (miRNA) expression in distal and proximal colon was assessed: 1) isolation and quantification methods for miRNAs were established in rat liver tissue; 2) miRNA expression patterns were compared using miRNA PCR arrays in lean proximal and distal colonic tissue; and 3) the influence of obesity on miRNA expression in these colonic regions was investigated by screening cancer miRNA coding genes in colonic mucosal samples from Zucker obese and lean rats. Up-regulation of 20 miRNAs was observed in obese liver tissue. Colonic mucosal miRNA expression patterns and abundance differed for distal and proximal colonic regions in both lean and obese tissue. Obesity exerted a profound region-specific effect on miRNA expression patterns and levels indicating biologically distinct tissue in distal and proximal colon. Obesity markedly affects miRNA gene regulation, and miRNA is a key molecular player in the genesis of colon cancer.

DEDICATION

Dedicated to my parents and grandparents

ACKNOWLEDGEMENTS

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CHAPTER1

INTRODUCTION

Overview

Development of colon cancer is a complex multistep process involving sequential clonal selection and propagation of transformed cells. It is recognized that there are multiple players which orchestrate the important events in the life of a transformed cells. These players include host's physiological state and interaction of the transformed cells with the normal surrounding cells of the host organ as well other organ systems. The complex orchestrated events allow the transformed cells to either complete their life cycle and emerge as tumor or lead to premature death.

It is recognized now that the term "colon cancer" is normally used to represent multiple cancer sub-types based on the mutational spectra of the cancers and their morphology. Ample evidence exist to support the concept that colon is not a single organ and that proximal (right sided colon) and distal colon (left sided colon) are embryologically and physiologically different. Subsequently, the tumors appearing in these regions are also biologically different. A majority of these evidences are from epidemiological and clinical studies. A limited exploration has been conducted in animal models.

Our ability to dissect the process of colon carcinogenesis at the molecular level has added a number of new molecular players, miRNA being prominent among them. miRNAs or miRNAs are known as the master regulators of genes involved in normal functioning of the cell as well as pathogenesis of cancer. Hence the study of their functioning and regulation in normal versus tumor cell is important for identification of new molecular targets in cancer preventive therapies.

Obesity is linked to heightened risk of colon cancer. In our laboratory we have used the Zucker obese (Zk-Ob) rats as a model to study the link between obesity, metabolic syndrome-X and colon cancer. In this model, tumors mainly appear in the

distal colonic regions however, a number of tumors also appear in the proximal colonic regions. Now it is known that several miRNAs are associated with inflammation and associated diseases like colitis, rheumatoid arthritis, atherosclerosis, allergic airway inflammation. In our laboratory, we are interested to develop animal model of carcinogenesis focusing on the biology of the tumors. The purpose of this research was to establish the method of miRNA isolation and to quantify their expression levels in rat colon under obese conditions. We used the Zucker obese (Fa/fa) and lean (fa/fa) rats as model system.

Hypothesis

The hypothesis which provides the basis of this dissertation is that obese state will influence the composition and expression levels of miRNAs in the colons of Zucker rats. During this investigation it was of interest to further determine if obesity known to heighten the risk of colon cancer will influence miRNA expression in distal versus proximal colon. This hypothesis is based on the fact that the clinical tumor samples from proximal colon exhibit different mutational spectra than those from distal colon. We have recently published that in Zucker obese rats, the molecular feature of the tumors appearing in proximal colon also differ from those that appear in the distal regions. We speculated that the ability of proximal and distal colonic regions to respond to DNA damage and repairs as well as sustain the growth of transformed cells with specific mutations depends on the biology of the host tissue.

Specific aims of research

Aim 1: To establish the method of isolation and quantification of miRNAs in rat tissue (liver).

Aim 2: To determine if obesity influences miRNA expression level and pattern in distal and proximal regions of the colon.

To meet the specific aim 1 we used liver tissue from Zucker obese and lean rats. . To meet the aims 2 we assessed the expression levels of miRNA in lean rats and compared the expression pattern in the distal and proximal colonic regions. This study was followed by the assessment of miRNAs in obese rat colonic tissues by the regions. To put this dissertation in proper perspective a background information on colonic structure and anatomical differences between the distal and proximal colonic regions, development of colon cancer, link between obesity and colon cancer and role of miRNAs structure and function are provided. For brevity the key information is provided with specific references.

CHAPTER 2

REVIEW OF LITERATURE

Colon

The large intestine or the colon is a muscular tubular organ about 1.5 meters in length which represents the terminal part of the gastro-intestinal tract. The colon consists of the caecum (with an attached appendix), ascending, transverse, descending and sigmoid components ending with the rectum. Primary functions of the colon involve processing of waste material, reabsorption of water and nutrients, and mucous production.(Arthur F. Dalley 1999).

Histologically, the colon has four distinct layers: innermost mucosa, sub mucosa, muscularis externa and outermost serosa. (Arthur F. Dalley 1999) (Figure1.1). The innermost mucosal layer consists of invaginations known as crypts lined with colonic epithelium. Unlike the small intestine, colonic crypts have no villi for nutrient absorption. Crypts are supported by the lamina propria consisting of connective tissue, blood vessels, and immune cells including lymphocytes and macrophages. (Arthur F. Dalley 1999) The mucosal layer is the most common origin for colon cancers.
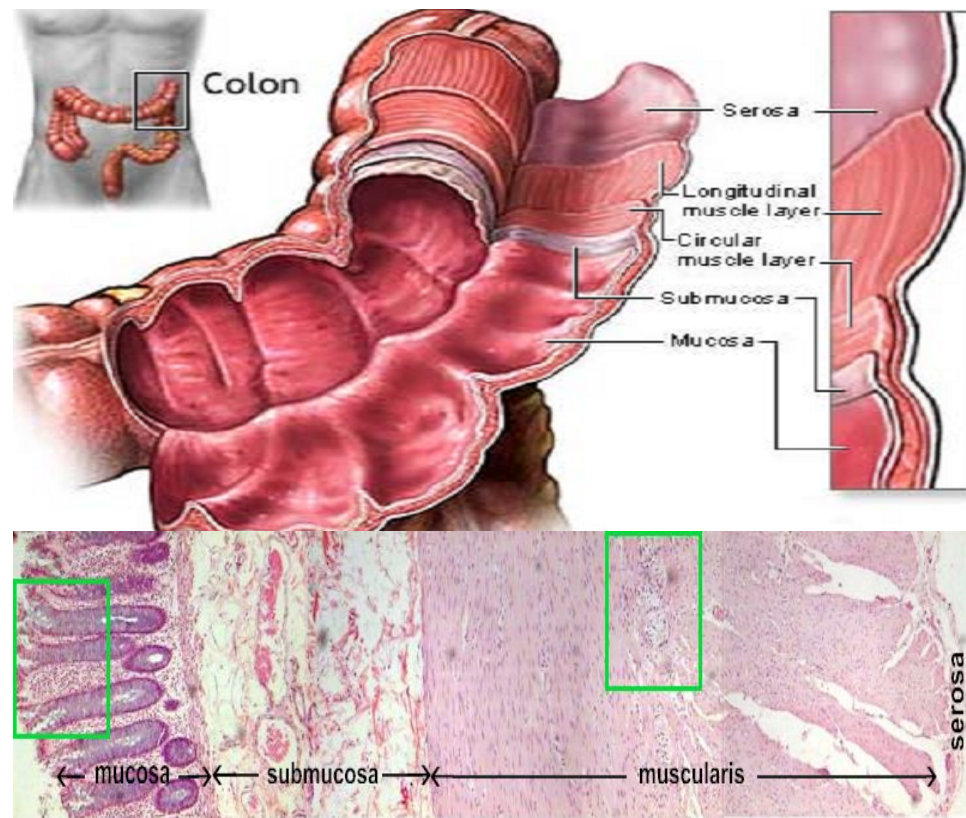
Figure 1.1 Structure of colon. The colon or the large intestine is a hollow, muscular and tubular organ with four layers: the innermost mucosa, sub mucosa, muscularis externa and outermost serosa. The innermost mucosal epithelium has invaginations called 'colonic crypts' which are the site of origin of colon cancer. (adapted and modified from Yi-Ben Chen, updated 2010)

Right and left side of colon

Embryologically, the colon develops partly from the midgut (ascending colon to proximal transverse colon) and partly from the hind gut (distal transverse colon to sigmoid colon). (Figure1.2) The ascending colon which lies vertically on the right side of the splenic flexure, is also referred to as the 'proximal' colon. The transverse colon again takes a right-angled turn just below the spleen (splenic flexure) and

becomes the descending (left) colon, which lies vertically in the most lateral left part of the abdominal cavity.
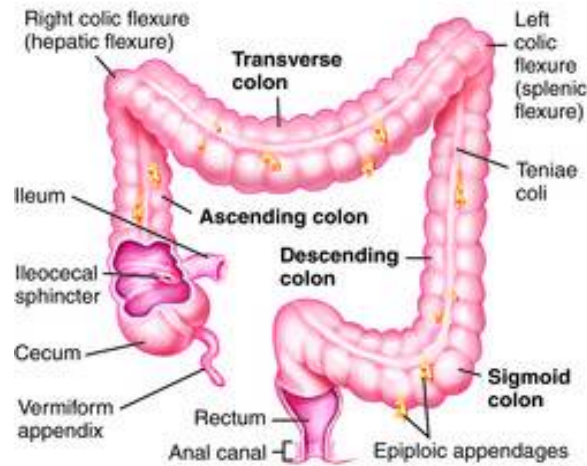
Figure 1.2 Colon anatomy. Colon has been divided into three parts depending on its location with respect to splenic flexure. On right side of the splenic flexure is the ascending colon also known as 'proximal' colon and on the left side of the splenic flexure is the descending colon also known as the 'distal' colon. Transverse colon runs horizontal.

Colonic epithelium is a highly diverse and dynamic system involving constant cell renewal. (Ding, Ko et al. 1998). Continuously proliferating stem cells situated at the base are responsible for replenishing the entire epithelium every 3-8 days.(Cotran R.S. 1999) This cell regeneration occurs within the basal two-third portion of the crypt. As the cells migrate upwards they differentiate into specialized cell types including absorptive, mucous secreting, endocrine, and anti-bacterial protein secreting Paneth cells. (Radtke and Clevers 2005; Schneikert and Behrens 2007) (Figure 1.3)
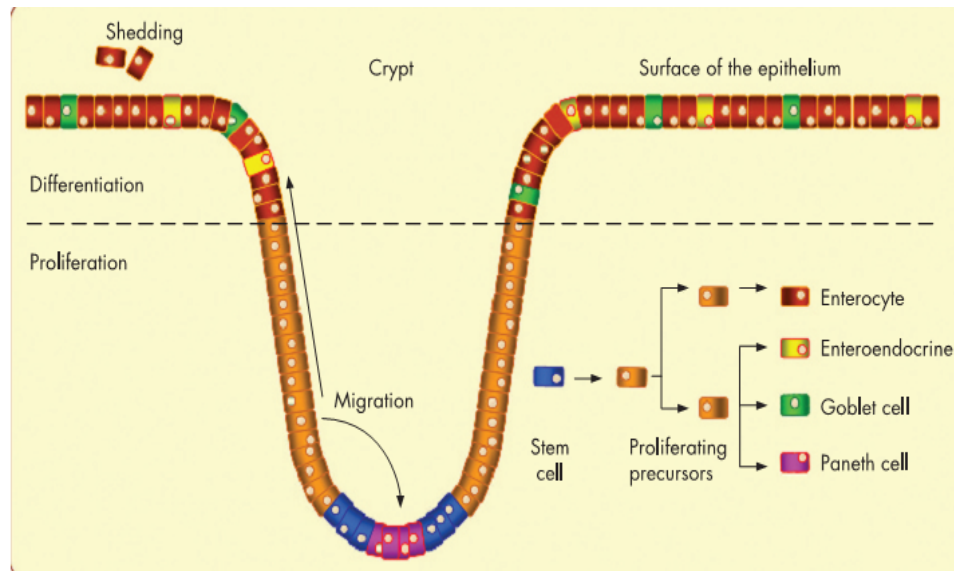
Figure 1.3 Differentiation of colonic epithelium. The colonic epithelium has invaginations called crypts which have a population of stem cells at the base that give rise to actively dividing precursors. These precursor cells migrate to the top of the colon and and differentiate as enterocytes (absorptive cells), entero endocrine (hormone secreting cells), goblet cells (mucus secreting cells) and paneth cells (antimicrobial toxin secreting cells) (adapted and modified from Jean Schneikert, Jurgen Behrens, Gut 2007)

Colorectal Cancer

Colorectal cancer (CRC) is the 3rd most common malignancy and 4th most common cause of cancer related mortality worldwide. (Tenesa and Dunlop 2009) It happens to be the 2nd leading causes of cancer related deaths in the United States and other developed countries, despite considerable advances in diagnosis and treatment techniques.(Jemal, Murray et al. 2005; Jemal, Siegel et al. 2009). It alone accounts for 11% of worldwide cancer cases, preceded only by prostate, lung and breast cancer. In addition, it is estimated that 50% of the population will develop an adenomatous polyp by the age of 70. (Jemal, Murray et al. 2005)

Approximately, 20% of CRC cases have a familial basis, (Rustgi 2007) where some are associated with well-defined syndromes, such as hereditary non polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). However, majority of the CRC cases have been linked to environmental factors and food borne mutagens, specifically intestinal microflora including pathogens and chronic intestinal inflammation, which predisposes tumor formation.

CRC is multistep, multi factorial disease that involves the clonal selection and clonal propagation of initiated colonic epithelial cells, which progress from normal to precancerous to malignant states over a span of 5-40 years.(Harris 1991; Roncucci L. 1991.; Lei Cheng 2003 December) . The sequential events of colon carcinogenesis concerning genetic and phenotypical changes are very well characterized by the adenoma-carcinoma model first described by Fearon and Vogelstein (1990). According to this model, CRC is caused by accumulation of mutations in the classical oncogenes and tumor suppressors genes, which correspond to a sequential transition of single pre-neoplastic cell to aberrant crypt foci (ACF) and then to adenoma and metastatic carcinoma. (Figure 1.4) The earliest molecular alteration in this pathway is mutation in the adenomatous polyposis coli (APC) tumor suppressor gene which is a component of the Wnt signaling pathway. This is followed by mutation in the KRAS and TP53, in the larger adenomas and invasive carcinoma. (Fearon and Vogelstein 1990) This pathway is referred to as the 'APC pathway' or the 'chromosomal instability (CIN) pathway' because the colorectal tumors arising by this pathway are characterized by gross chromosomes abnormalities including deletions, insertions, and loss of heterozygosity. Another molecular pathway proposed is the DNA mismatch repair pathway.(Aaltonen, Peltomaki et al. 1993) The key element of this pathway is mutation in the DNA repair enzymes e.g MLH1, MSH2, which results in accumulation of mutations in the microsatellite regions of the genome. (H Iino 2000 July) This results in microsatellite instability in the tumors arising from this pathway and hence it is also called microsatellite instability (MSI) pathway.
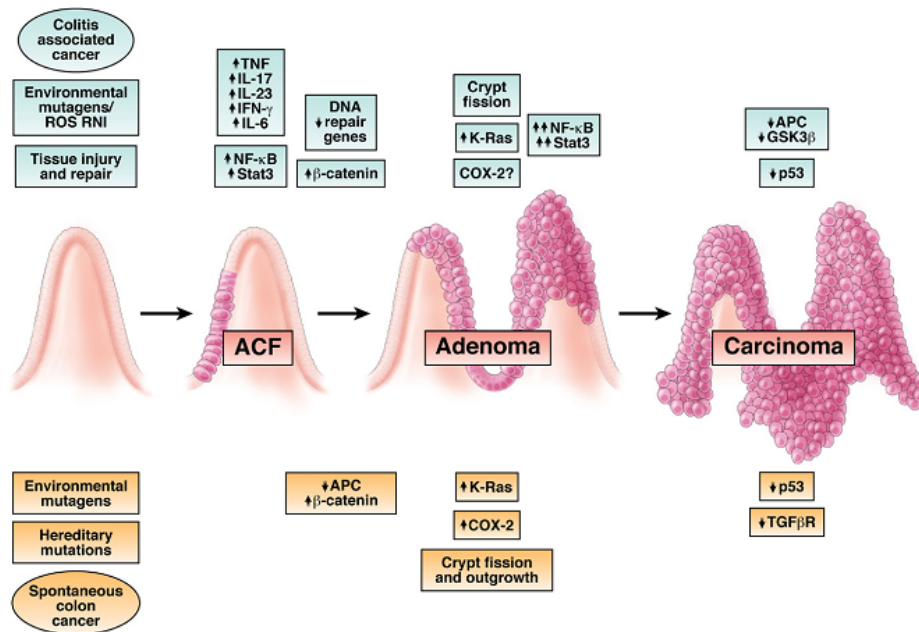
Figure 1.4 Fearon–Vogelstein model of colon carcinogenesis. The sequential events of colon carcinogenesis concerning genetic and phenotypical changes are very well characterized by the adenoma-carcinoma model first described by Fearon and Vogelstein (1990)

Evidences indicating that adenomas might not be the only CRC precursor began to emerge in 1990. Longacre and Fenoglio - Preiser studied a group of polyps that showed mixed histology of both hyperplastic and adenomatous polyps.(Longacre TA 1990) These lesions had glands with serrated appearance and showed nuclear features of adenomas, hence they were called serrated adenomas. The adenoma carcinoma pathway for serrated polyps is different from the traditional CIN or MSI pathway. For e.g- APC and TP53 mutations are rare, whereas alterations in microsatellite sequence and hypermethylation of CpG islands are more common. There are two overlapping pathways for development of serrated lesions. The first one is more common in the right sided colon (proximal) and is characterized by high

level of MSI and CpG island methylation and BRAF mutation.(Spring, Zhao et al. 2006) The second one is more common in left sided colon (distal) and shows microsatellite stability (MSS) or low grade of MSI (MSI-L) and also show mutations in KRAS. (Jass, Whitehall et al. 2002)

Obesity associated inflammation and colon cancer

Obesity is a chronic inflammation state and a rising epidemic in North America, with both environmental and genetic etiological factors.(Formiguera and Cantón 2004) The connection between obesity and risk of colon cancer is well established and has a great deal of supporting evidence from genetic, pharmacological and epidemiological data. (Rapp, Schroeder et al. 2005). While the biochemical and molecular links between the two physiological disorders are still under review, the interconnected roles of insulin resistance , adipose tissue and inflammation are well documented in literature. (Figure 1.5) Key mediators of inflammation-induced cancer include nuclear factor kappa B (NFκB), reactive oxygen and nitrogen species (RONS), inflammatory cytokines, chemokines, adhesion molecules and specific miRNAs. Chronic inflammation predisposes cells for oncogenic transformation by inducing genomic instability, increasing cell proliferation and angiogenesis, altering the genomic epigenetic state.

Physiological parameters linking obesity and cancer

Obesity or chronic inflammation state is often encountered with high levels of glucose, triglycerides, cholesterol in the blood plasma due to inactivity of the insulin hormone or its receptor. Insulin resistance implies a cells inability to respond to normal levels of insulin, thus requiring abnormally high amounts of insulin hormone for glucose metabolism. This condition further leads to irregular triglycerides, fatty acids and glucose levels, all metabolic aberrations that also correlate with an increased risk of colorectal cancer.(Gunter and Leitzmann 2006). There are convincing evidences that suggest the connection between obesity and colon cancer risk in three possible ways. (reviewed by Gunter and Leitzmann 2006) The first being the fact that insulin hormone is a growth factor which is known to promote cell

growth and proliferation. Hence, the exposure of high levels of insulin to parts of the body that are not accustomed to it e.g- colon, can interfere with the normal cell signaling and promote cell proliferation. (Gunter and Leitzmann 2006) This has been experimentally proved by Kiunga et al 2004, who reported the presence of high levels of insulin and insulin receptor in colon tumor cells compared to normal mucosa. Secondly, an insulin resistant state also promotes high levels of circulating insulin-like growth factor (IGF), which can also promote colonocyte division and block apoptosis by receptor binding. (Giovannucci 2001) Increased IGF-1-receptor (IGF-IR) also stimulates cell division in intestinal epithelial cells (Ma J. 1999) and several epidemiological studies have shown positive correlation between IGF and IGF-IR levels and colon cancer risk.(Komninou, Ayonote et al. 2003) Finally, the high circulating plasma glucose and lipids associated with insulin resistance serve as a reservoir for reactive oxygen species (ROS) generation, known contributors to carcinogenesis. High visceral abdominal fat found in obese individuals is especially a critical source for ROS. (Furukawa, Fujita et al. 2004; Frezza, Wachtel et al. 2006)

Along with oxidative stress, increased amount of fat tissue promotes a pro-inflammatory environment, also strongly correlates with increased cancer risk. Cytokines/adipokines such as tumor necrosis factor-α (TNF-α) are readily secreted by adipocytes and further enhance inflammation, insulin resistance, impaired glucose metabolism and ROS production. (Furukawa, Fujita et al. 2004; Sonnenberg G.E. 2004.)

Leptin, which is also known as the hormone of satiety, has been implicated in obesity associated colon cancer. Though its role is not very clear, it has been shown to accelerate HT-29 cell proliferation and promote ACF formation in vivo.(Liu Z 2001.) Interestingly, F344 rats administered with continuous high dose of leptin have shown reduction in the number of ACF compared to controls. (Aparicio, Guilmeau et al. 2004) Leptin secreted from adipose tissue has also been shown to enhance insulin resistance. (Matsuzawa 2006)

Metabolic Syndrome X

Many of the physiological abnormalities associated with obesity or insulin resistance are collectively referred to as Metabolic syndrome, or syndrome X. It is a term given by Reavan in 1988, to the physiological disorders associated with obesity, specifically abdominal/visceral obesity. Although there are contradicting views regarding the inclusion of insulin resistance and dyslipidaemia (including hypercholesterolemia and hypertriglycerolemia) in this syndrome, hyperglycemia and hypotension have been unanimously included in this category.(Shaw, Hall et al. 2005; Sorrentino 2005). These symptoms or abnormalities have been associated with diabetes, atherosclerosis and cancer.

Oncogenic mechanisms associated with inflammation

Prolonged inflammation can induce DNA mutations and contribute to genomic instability in more than one ways. (Perwez Hussain and Harris 2007; Colotta, Allavena et al. 2009) Free radicals (RONS), produced during inflammation can reduce the expression and enzymatic activity of the critical DNA repair enzymes eg. mut S homolog 2 and 6. It can further cause increased expression of DNA methyltransferases that can cause global hypermethylation of the genome. The downstream effect of this includes silencing of DNA mismatch repair enzymes (eg- hMLH1 and hSMH2) and several tumor suppressor genes (eg- APC, CDKN 2, BRCA1,Rb and MDM2) by promoter methylation. (Fleisher, Esteller et al. 2000; Das and Singal 2004)

Molecular mediators common to inflammation and cancer

Inflammation can induce cancer by both extrinsic and intrinsic pathways. In extrinsic pathway, chronic inflammation itself is the driving force, whereas in intrinsic pathway, the genetic alteration of oncogenes and tumor suppressors as well as various inflammatory mediators play an important role in carcinogenesis. The inflammatory mediators include the cytokines, chemokines RONS, COX-2 and

NFκB. For eg- a dominant mutation in Ras proto-oncogene, can induce inflammatory response through induction of pro-inflammatory cytokines interleukins (ILs) IL1, IL6 and IL11 and the chemokine IL8, which aid in Ras mediated oncogenesis. (Bos 1989) Ras also induces IL-8 that mediates tumorigenesis and angiogenesis. (Sparmann and Bar-Sagi 2004) Genetic mutations stimulate the tumor cells to produce inflammatory cytokines and free radicals, which creates a feedback loop where the tumor cells recruit inflammatory cells that can produce additional cytokines and free radicals to the site of tumor that can aid tumorigenesis. (Figure 1.5)

Some of the key players are described in detailed in the following sections:

Nuclear Factor Kappa B

Nuclear factor kappa B or NFκB as it is commonly called is a transcription factor and key mediator of inflammation induced carcinogenesis. (Shen and Tergaonkar 2009) Under normal condition, NFκB is bound to its negative regulator also called the inhibitor of kappa B (IκB) and stays inactive in the cytoplasm. Following an inflammation stimulus, IκB kinase phosphorylates IκB and targets it for proteosomal degradation. Activated NFκB translocates to the nucleus where it drives the transcription of its target genes, many of which are inflammation related eg- cytokines and chemokines, nitric oxide synthase (NOS) , cyclooxygenase (Cox-2) and tumor necrosis factor (TNF)α.(Naugler and Karin 2008)

NFκB creates a protumorigenic environment by turning on the expression of following set of genes:
1.) Cell cycle related genes eg c-Jun-N-terminal kinase that increase cell proliferation
2.) Vascular endothelial growth factor (VEGF) and angiopoetin that stimulate angiogenesis
3.) Bcl2, Bcl-xL, and cFLIP that makes cell resistant to apoptosis and necrosis.
4.) Pro-inflammatory cytokines, chemokines and adhesion molecules.

Inflammatory bowel disease (IBD) which is associated with colitis associated cancer (CAC), show persistant activation of NFκB in the myeloid and epithelial cells of colonic mucosa. (Rogler and Andus 1998; Chung 2000)

Reactive oxygen and nitrogen species (RONS)

These are highly reactive free radicals produced by inflammatory cells, collectively known as the reactive oxygen and nitrogen species (RONS). They have the ability to induce DNA strand breaks, mutations and aberrant cross linking, causing genomic instability. Chronic inflammation results in elevated levels of RONS that causes oxidative and nitrosative stress that contributes to tumorigenesis. (Hussain, Trivers et al. 2004; Hofseth 2008) In colitis associated cancer (CAC), chronic inflammation causes oxidative damage to DNA, leading to the p53 mutations observed in tumor cells.(Choi, Yoon et al. 2002; Kraus and Arber 2009). ROS can also directly oxidize and inactivate mismatch repair enzymes at protein level. (Choi, Yoon et al. 2002; Hussain, Hofseth et al. 2003; Kraus and Arber 2009). Nitrogen oxide (NO) is a reactive free radical produced in large amounts by the inducible nitric oxide synthase 2 (NOS2) under conditions of chronic inflammation. In its pro-tumorigenic role, NO induces DNA strand breaks, promotes angiogenesis by induction of VEGF and increases cell proliferation and invasion. (Hussain, Trivers et al. 2004). In its anti-tumorigenic role, NO can cause cytotoxic cell death of malignant cells and modulate the immune system to eradicate the cancerous cells. There is a negative loop between NO and tumor suppressor protein 53 (TP53), where NO causes the stabilization and accumulation of TP53 which induces apoptosis, cell cycle arrest in the malignant cells and represses levels of NO. (Forrester, Ambs et al. 1996; Ambs, Ogunfusika et al. 1998).

Cytokines and chemokines

Cytokines are signaling molecules that are key mediators of inflammation or an immune response. Upon binding to their receptor, they trigger a signaling pathway

that leads to cell proliferation, apoptosis, angiogenesis and cellular senescence. They broadly fall under two categories 1.) Pro-tumorigenic and 2.) Anti-tumorigenic. The pro-tumorigenic cytokines include IL1, IL6, IL15, IL17, IL23 and tumor necrosis factor α (TNFα) , whereas the anti-tumorigenic cytokines include IL4, IL10, IL13, transforming growth factor (TGFβ) and interferon (IFN α). In case of CRC and CAC, the role of IL-6, IL-1 and TNF-α in tumorigenesis has been well studied. (Tang, Katuri et al. 2005; Popivanova, Kitamura et al. 2008; Wang, Liu et al. 2009). IL-6 is a potent stimulator of colon cancer cell proliferation and tumor growth. (Becker, Fantini et al. 2005) It has been implicated in pro-tumorigenic activity for many cancers and has been found to be required for CAC in mouse models. (Grivennikov, Karin et al. 2009) TNFs are produced during early inflammation; they trigger production of other cytokines, chemokines and endothelial adhesion molecules.(Balkwill 2009; Li, Vincent et al. 2009).TNF-α expression increases during colon carcinogenesis which further confirms its role in inflammation, angiogenesis and tumor promotion. (Popivanova, Kitamura et al. 2008). One of the key functions of TNF-α is to activate the pro-inflammatory transcription factor NFκB. The anti inflammatory cytokines like IL-10 and TGF-β , have a general role in tumor suppression. IL-10 exerts its anti-tumorigenic effect by down-regulating NFκB, which results in low levels of pro-inflammatory cytokines eg- IL-6, IL-12 and TNF-α. (Zhang 2008 December) TGF-β is another anti-inflammatory cytokine that suppresses tumor growth by inhibiting proliferation, promoting apoptosis, stimulating the release of anti-inflammatory cytokines and suppressing the expression of pro-tumorigenic cytokines.(Yang and Moses 2008) Mutation in TGF-β pathway within epithelial cells predisposes to or facilitates colonic tumor development and growth.

Chemokines are a class of cytokines that recruit leukocytes at the site of inflammation. They are released by the inflammatory cells after the initial stimulation by the cytokines. Tumors generally show high levels of chemokines that recruit leukocytes to the site of tumor, creating a pro-inflammatory and pro-tumorigenic environment.

Chemokines and adhesion molecules

Early feature of inflammation is the release of chemokines like Monocyte chemoattractant protein (MCP). These factors increase the expression of interstitial and vascular cellular adhesion molecules like Interstitial Cellular Adhesion Molecules (ICAM) and E-selectin that attract monocytes and immune cells. Chemokines like MCP also induce the proliferation and pro-inflammatory gene activation producing cytokines like IL-1α, IL-6, IL-18 etc. Other factors that stimulate gene expression of pro-inflammatory cytokines in obese state are RONS, oxidized lipids, free fatty acids.
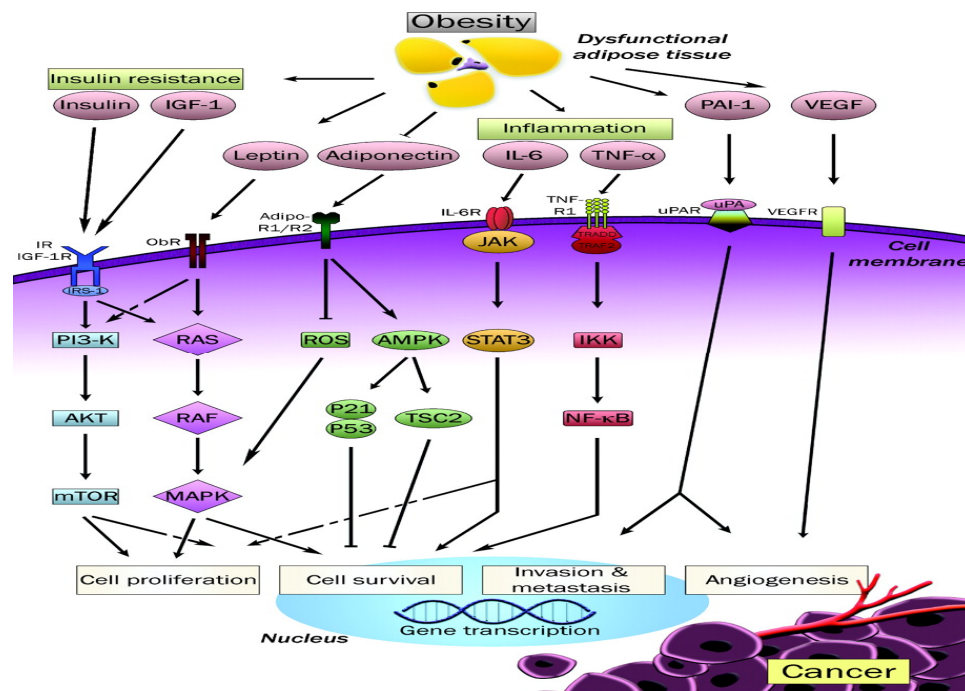
Figure 1.5 Schematic diagram showing pathways linking obesity to cancer. Insulin resistance and insulin like growth factor (IGF) are one of the several links between obesity and cancer. High levels of circulating insulin up-regulates growth hormone (GH) that induces hepatic production of IGF-1. IGF-1can inhibit apoptosis and stimulate cell proliferation through several downstream signaling networks ,

including the phosphatidylinsitol3-kinase (PI3-K)-AKT system and the Ras/Raf/mitogen activated protein kinase (MAPK) systems, respectively. Leptin, also secreted by adipocytes has mitogenic effects and anti apoptotic effects through MAPK and PI-3-Kinase pathways. Adiponectin exerts anti-carcinogenic effect through AMP activated protein kinase (AMPK) through receptors AdipoR1 and AdipoR2. Adipopectin regulates growth arrest and apoptosis through p53 and p21. IL-6 and TNF-α are the two major inflammatory cytokines that are elevated in obesity. They exert pro-tumorigenic effect by increasing cell survival and cell proliferation through activation of NF-κB

Animal Model

Preclinical or animal models provide a system to study the biological process of a disease in a physiological state relevant to human beings. They provide valuable preliminary data upon which human clinical trials can be based. However, they should always be evaluated with regards to how relevant they are to human conditions, and how predictive they can be for the disease process. (Green and Hudson 2005)

With regards to colon carcinogenesis, molecular and pathological similarities to the human condition should be observed in order to have an effective model.(Reddy 2004) Currently the APC $^{min+/-}$ mouse and azoxymethane rat model are the two main animal models used to study the effect of dietary supplements on colon cancer. Zucker obese rats, traditionally used to study obesity and related metabolic disorders, offer a novel way of studying the progression of colon cancer in an altered physiological state.

Zucker-Obese Animal Model

Zucker obese (Zk-Ob) rats are an excellent model of human obesity, and provide an ideal opportunity to study colon carcinogenesis in an altered physiological state. The Zucker or 'fatty' rat was developed in the laboratory of Zucker and Zucker in 1961, by spontaneous mutation in a single recessive gene (Fa allele is designated as

normal, while fa is the fatty mutation). Zk-Ob rats inherit obesity as an autosomal Mendelian recessive trait, fa/fa homozygous for nonfunctional leptin receptors, in comparison to their lean (Fa/fa or Fa/Fa) counterparts.(A. 1977.) Leptin is a peptide hormone produced by adipocytes that regulates body weight and fat metabolism by sending signals to the hypothalamus to suppress apetite.(Arthur F. Dalley 1999) In Zk-Ob rats, obesity is associated with metabolic dysfunction of fat metabolism characterized by latescence and increased adipose tissue formation. (Zucker T.F. 1962.) Genetic association of Fa gene to obesity is confirmed as Zk-Ob rats on low fat or energy restricted diet also exhibit these symptoms. Interestingly, female Zucker rats are sterile, which has implications for breeding programs.

Average weights of these animals at 40 weeks is 800g and 625g for Zk-Ob, and 480g and 295g for Zucker lean (Zk-Ln), males and females respectively.(Zucker T.F. 1962.) Zk-Ob rats exhibit hyperphagia, hypertriglyceridemia, hypercholesterolemia, hyperinsulinaemia and mild hyperglycemia at about six weeks of age. Pathological findings at death often include hydronephrosis and polycystic kidneys, as well as fatty livers.(Zucker T.F. 1962.) It has been shown that Zk-Ob rats are more sensitive to chemically induced colon carcinogenesis in comparison to their lean counterparts.(Raju and Bird 2003) Since there is little difference in ACF number between Fa/fa and Fa/Fa animals, the recessive gene linked to leptin receptor deficiency is not solely responsible to higher susceptibility of colon cancer.(Weber, Stein et al. 2000) Use of this animal model helps understanding carcinogenesis in relation to obesity and associated metabolic disorders, providing valuable information for treatment and prevention strategies.

Micro-RNA

Brief introduction

Micro RNAs (mi RNAs) are 19-22 nucleotide long single stranded non protein coding RNAs that act as guide molecules in post transcriptional gene silencing by base pairing with target mRNA leading to its cleavage or translational

repression.(CULLEN 2003; Bartel 2004; VICTOR AMBROS 2008). With >200 members per species in higher eukaryotes, miRNAs form one of the largest gene families accounting for approx 3% of the genome and targeting 30% of coding regions of the genome (Lewis, Shih et al. 2003; Bartel 2004). Since their discovery back in 1993 by Victor Ambros and colleagues, who identified the first miRNA targeting lin-4 gene in C.elegans, miRNAs have come a long way in being recognized as a major player in post transcriptional gene regulation. Even though we are far from unraveling the complex role of miRNAs in gene regulation, it is evident that they have important role in almost all biological process known, including developmental timing, growth control, differentiation and apoptosis. Accordingly, altered miRNA expression can have serious implication on the regular metabolism of the cell and be a potential cause for diseases like cancer.

Gene Structure and miRNA gene transcription

Location in the genome

Early annotation to find out the genomic position of miRNAs indicated that most them are located in the intergenic regions , although a sizeable minority are found in the intronic region of known genes in sense or anti sense orientation.(Lau, Lim et al. 2001; Kim 2005) This implied that most of the miRNAs are transcribed as autonomous replication unit with their own promoter. Approximately 50% of the miRNAs are found in close proximity to known genes, (Lagos-Quintana, Rauhut et al. 2001; Lim, Lau et al. 2005) which allows one to speculate that they are transcribed from single polycistronic transcription unit. So the conclusion drawn was that they can be transcribed as discrete as independent transcription unit or in a cluster by polycistronic transcription unit.

MiRNA biogenesis

miRNAs are transcribed as long primary transcripts by RNA polymerase II; subsequently trimmed into short hairpin intermediates called primary miRNAs and finally processed into mature miRNAs.(Lee, Kim et al. 2004). The catalytic activity

of the enzymes responsible for first and second miRNA processing is compartmentalized into the nucleus and cytoplasm and is tightly regulated. (Figure 1.6)

Nuclear processing by Drosha

RNase III endonuclease Drosha is a 160 kDa protein that is highly conserved in higher eukaryotes. (Filippov, Solovyev et al. 2000; Wu, Xu et al. 2000; Kristine R Fortin 2002) It contains two random RNAIII domains (RIIID) and a double stranded RNA binding domain (dsRBD) that are critical for its catalytic activity. Transcription of miRNA genes by RNA pol II yields primary transcripts (pri-miRNA) that are usually several kilobases long and contain a local hairpin structure. The pri-miRNA is first cleaved by RNase III endonuclease Drosha at sites near the base of stem loop, that releases ~60-70 nucleotide precursor miRNA. (Lee, Ahn et al. 2003) Drosha cleaves the pri-miRNA duplex with a staggered cut typical of RNase III endonucleases, leaving 2 nucleotide overhang at the 3' end.(Lee, Ahn et al. 2003) The specificity with which Drosha recognizes and cleaves pri-miRNA has been under investigation. It was proposed on the basis of evidences from mutational studies that the tertiary structure of the pri miRNA is the factor that decides the specificity of Drosha. (CULLEN 2003; Lee, Ahn et al. 2003; Zeng, Yi et al. 2005)

Nuclear export by exportin-5

Following nuclear processing by Drosha, pre-miRNA is translocated to the cytoplasm through the nuclear pore complex embedded in the nuclear membrane.(Nakielny and Dreyfuss 1999) Export of the pre-miRNA is mediated by one of the nuclear transport receptors, exportin-5. (Rui Yi 2003; Lund, Güttinger et al. 2004) . Owing to the compartmentalization of the two processing events, the nuclear transport of pre-miRNA is a crucial step in miRNA biogenesis. (Kim 2004; Murchison and Hannon 2004)

Cytoplasmic processing by Dicer

On reaching the cytoplasm, pre-miRNA is cleaved by cytoplasmic RNase III endonuclease Dicer, into ~ 22 nucleotide miRNA duplex. (Bernstein, Caudy et al. 2001; Grishok, Pasquinelli et al. 2001; Hutvágner, McLachlan et al. 2001; Ketting 2001; Knight and Bass 2001). Dicer is a highly conserved protein, found in all eukaryotes including Saccharomyces pombe, plants and animals. Apart from the two RIIID domains and dsRBD domain, Dicer contains long N terminal segment containing 'dead box RNA helicase' domain and PAZ domain.(Zhang, Kolb et al. 2004) PAZ domain belongs to a group of highly conserved proteins called Argonaute preteins. Structural and biochemical studies of AGOI and AGOII in D.melanogaster revealed that PAZ domain binds to the 3' protruding end of the small RNAs.(Song, Liu et al. 2003; Yan, Yan et al. 2003; Lingel, Simon et al. 2004) . Usually one strand of this short lived duplex disappears, whereas the other strand remains as mature miRNA. Mature miRNAs are incorporated into effector complexes that are known as microRNP (miRNA containing ribonucleoprotein complex), migronaute' or microRISC (miRNA containing RNA inducing silencing complex). The strand that gets loaded in the microRISC complex is thought to be the one that has unstable base pairing at the 5' end. (Khvorova, Reynolds et al. 2003; Schwarz, Hutvágner et al. 2003).
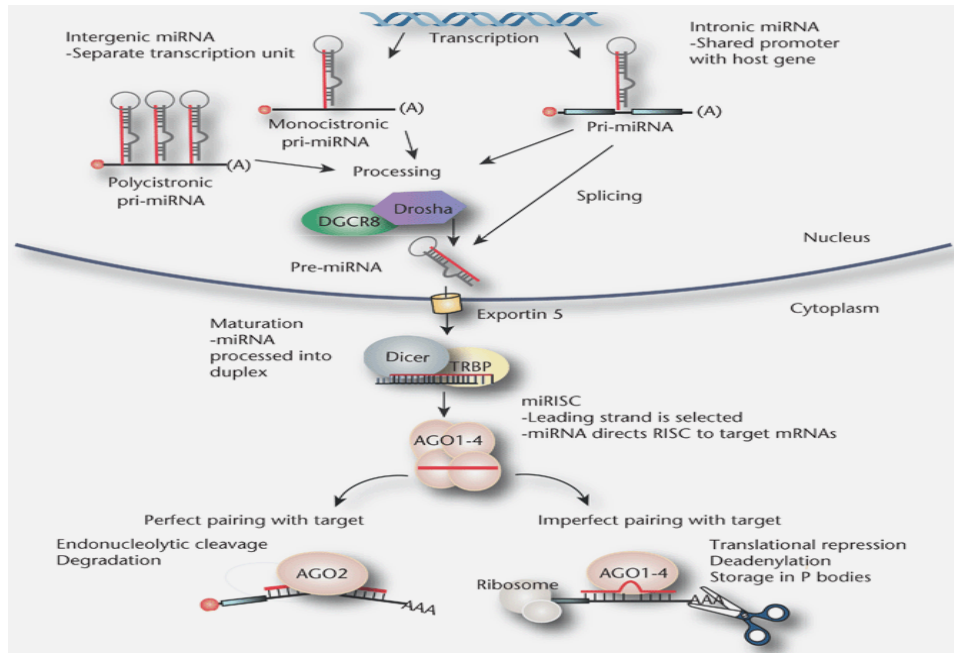
Figure 1.6 Biogenesis of miRNA. Intergenic miRNAs are transcribed from independent promoters as monocistronic whereas most of the miRNAs are intragenic and found in clusters with other genes. They are transcribed as polycistronic units. miRNAs are transcribed by RNA polymeraseII as several kilobases long pri-miRNA. Pri- miRNA is processed in the nucleus by RNAIII endonuclease (Drosha) which cleaves it into 60-70nt long pre-miRNA with a 2 nucleotide long overhang at the 3'end. This pre-miRNA is transported to the cytoplasm by nuclear transporter exportin-5. In the cytoplasm, pre-miRNA is cleaved by another RNAIII endonuclease (Dicer), which generates the mature miRNA. One of the strands of the resulting duplex is loaded onto miRNA inducing silencing complex called miRISC. Mature ss miRNA guides RISC to the target mRNA. After that there are several mechanisms to downregulate mRNA/protein expression depending on the degree of sequence complimentarity. (adapted and modified from Christian Marin-Muller Oct 2010)

Mechanism of miRNA mediated gene regulation

miRNA can direct RISC to downregulate gene expression by either of the two post-transcriptional mechanisms:

1.) Translational repression

2.) mRNA degradation/sequestration

Mechanism of translational repression

Once incorporated in the RISC complex, miRNA directs translational repression or target cleavage depending on degree of sequence complimentarity with the target.(Hutvágner, McLachlan et al. 2001). In animals, there are several evidences suggesting that miRNAs recognize their target mRNA by limited base pairing between the 2-8 nucleotide seed region at the 5'end of the miRNA and the 3' UTR of target mRNA.(Lewis, Shih et al. 2003; Farh, Grimson et al. 2005; Stark, Brennecke et al. 2005) The interaction between microRNPs and mRNA can have several consequences which can be direct or indirect. Direct effects include inhibition of translational initiation by preventing ribosome association with the internal ribosome entry site (IRES) on the target mRNA or inhibition post translation. Post translational repression includes premature ribosome drop off, stalled or slowed down elongation, and/or co-translational protein degradation; the repressed mRNA seems to be present in polyribosomes.(Nilsen 2007) (Figure 1.7)

Mechanism of mRNA degradation/sequestration

Perfect sequence complimentarity between the seed region of miRNA and the 3'UTR of target sequence leads to the destabilization and subsequent degradation of the transcript. miRNAs achieve this by deadenylation and decapping of the target sequence, which results in degradation and increased turnover of the transcript. The site of miRNA directed target sequestration are thought to be the processing bodies (P bodies) also known as the GW bodies. The P bodies, which were originally described in budding yeasts, are known as the cytoplasmic foci rich in decapping , deadenylation enzymes and exonucleases required for mRNA degradation.

(THEOPHANY EYSTATHIOY 2003 October; Liu, Rivas et al. 2005; Eulalio, Behm-Ansmant et al. 2007) miRNA bound to the Argonaute protein in the RISC complex recognizes its target by complimentarity base pairing. The Argonaute protein interacts with the GW182 protein; miRNA-mRNA and argonaute protein complex is delivered to P bodies. In the P bodies, the mRNA is decapped and deadenylated, subsequently degraded or held in stasis i.e spatially isolated from the translational machinery since P bodies do not have ribosomes. (THEOPHANY EYSTATHIOY 2003 October; Liu, Valencia-Sanchez et al. 2005) In stasis, the P bodies are thought to act as repository for untranslated mRNAs and this has been experimentally proved in budding yeast as well as mammalian cells.(Brengues, Teixeira et al. 2005; Bhattacharyya, Habermacher et al. 2006)
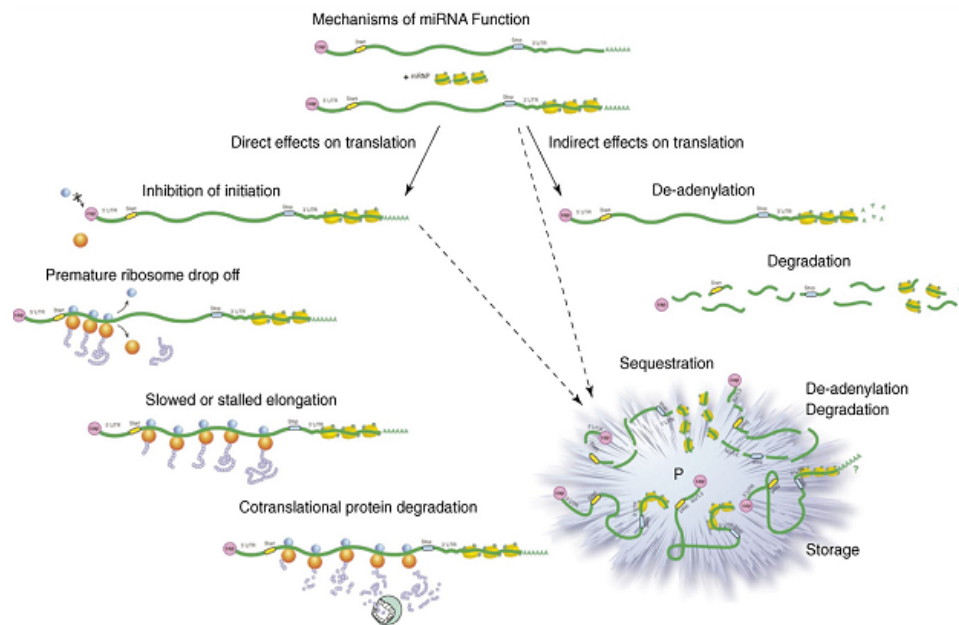
Figure 1.7 Mechanisms of miRNA mediated repression of gene expression.
Mature single stranded miRNA loaded in a RISC complex targets and represses the translation of mRNA in more than one way, either directly or indirectly. In direct interaction, miRNA inhibits the initiation of translation by blocking the entry of ribosomes into the internal ribosomal entry site(IRES). Post translational initiation, miRNA can cause premature ribosome drop off , slowed or stalled elongation or co-translational protein degradation. In indirect effects, miRNA targets the mRNA to P-

bodies where they might undergo deadenylation and decapping by the enzymes present in the P-bodies and hence degraded or they might simply be sequestered from the translational machinery of the cell. In the later case, which is also called 'stasis' the P-bodies act as repository for all those mRNA that indirectly targeted by miRNA. (adapted and modified from Timothy Nilsen, 2007)

MiRNA signatures in cancer

Cancer is a complex genetic disease that involves aberrant expression pattern of coding as well as non-coding genes. Earlier it was thought that only the protein coding genes and their altered expression are involved in the pathogenesis of cancer but with the discovery of miRNAs, the understanding of cancer mechanisms and genomic complexity of cancer cell has completely changed. miRNAs have been proposed to contribute to oncogenesis either as tumor suppressors (eg- miR-15a and miR-16-1) or oncogenes (eg- miR-155 or members og the miR-17-92 cluster). The genomic abnormalties influencing miRNA expression in cancer are the same as for the protein coding genes eg- chromosomal rearrangement, genomic amplification, deletion and polymorphisms in the target site of the mRNA.(Abelson, Kwan et al. 2005; He, Jazdzewski et al. 2005)

Oncogenesis and tumor suppression - dual role of miRNAs

Majority of the miRNAs are located in regions of genomic instability that undergo amplification or deletion which is why they have a huge role to play in the regulation of all major cancer pathways. miRNAs lying in regions that undergo genomic amplification act as oncogenes eg-miRNA 17-92 cluster; whereas the chromosomal regions that undergo deletion in cancer, act as tumor suppressors eg- miRNAs 15s-miR-16-1 cluster. O'Donnels in his work on cell model system showed that, human B-cell line P493-6, which overexpresses c-MYC, shows tumor suppressor activity through miRNA 17-92 cluster, which targets E2F1 and hence inhibits c-MYC regulated cell proliferation.(O'Donnell, Wentzel et al. 2005) The same cluster of miRNA when undergo amplification in the genome, act as oncogene by cooperating with c-MYC and promoting cell proliferation and blocking apoptosis. Hence it can

by concluded that miRNAs can participate in distinct cancer pathways with either the role of oncogene or tumor suppressor depending on the cell type and expression pattern. Apart from their dual role, miRNAs can also act by targeting more than one mRNAs at the same time and have varied response .For eg-miRNA 15a and miRNA-16-1 target the anti-apoptotic Bcl-2 in leukemia cells , however in 293 fetal kidney cells, where they are expressed, they don't show any such response.(Calin, Liu et al. 2004). The loss of miRNA 15a and miRNA 16-1, leads to the over-expression of Bcl-2 and consequently B-cell malignancies. In a different tissue, the over-expression of miRNA 15a and miRNA 16-1 causes the loss of activity of a tumor suppressor that is critical to check cell proliferation and promote apoptosis in malignant cells. There are different mechanisms that can lead to aberrant miRNA expression in a cancerous cell-

1.) Location of miRNA in cancer associated genomic regions

More than 50% of known miRNAs are found in genomic regions that are prone to alteration in cancer cells.(Calin, Sevignani et al. 2004). Such regions include minimal regions of LOH (loss of heterozygosity), which are thought to harbor tumor suppressors genes, minimal regions of amplification, which mostly harbor oncogenes, common breakpoint region in or near possible oncogenes or tumor suppressor genes and fragile sites.

2.) Epigenetic regulation of miRNA expression

DNA hypomethylation, CpG island hypermethylation and histone modification losses represent epigenetic markers of malignant transformation and have been known to be one of the mechanisms involved in miRNA abnormal expression in cancer.((Fraga and Esteller 2005)

3.) Abnormalities in miRNA processing genes and proteins

Alterations in the proteins machinery that is involved in miRNA biogenesis, has dramatic effects on the miRNA expression. Failure of Drosha processing step or

Dicer mutation, could explain the downregulation of miRNA observed in primary tumors.(J. Michael Thomson1 2006)

MiRNAs in colorectal cancer

Dysregulation of miRNA targeting tumor suppressors and oncogenes, have been reported in carcinogenesis of colorectal cancer. Two approaches are applied today to investigate the connection between miRNAs and CRC: functional and profiling studies. Many proteins involved in key signaling pathways of CRC, such as members of the Wnt/β-catenin and phosphatidylinositol-3-kinase (PI-3-K) pathways, KRAS, p53, extracellular matrix regulators as well as epithelial-mesenchymal transition (EMT) transcription factors (Fearon and Vogelstein 1990) are altered by miRNA in CRC (Figure 1.8). Study of these miRNAs crucial for better understanding CRC pathogenesis with an aim to eventually identify novel therapeutic targets. Expression profiling of miRNAs have shown to have same potential for identification of biomarkers as profiling of their mRNA or protein counterparts. Together these studies help to determine the clinical prognosis of the disease along with identification of therapeutic targets.

Few pathways that are prominent in CRC and provide evidence of involvement of miRNAs, are detailed below-

MiRNA altering regulation of Wnt/β-catenin pathway in CRC

The Wnt/β-catenin pathway plays a central role in an early colorectal tumor development. Inactivation of the adenomatous polyposis coli (APC) gene is a major initiating event in colorectal carcinogenesis occurring in more than 60% of colorectal adenomas and carcinoma. (Fearon and Vogelstein 1990). According to a recent study by Nagel et al.(Nagel, le Sage et al. 2008), miRNAs represent a novel mechanism for APC regulation in CRC. miR-135a and miR-135b decrease translation of the APC transcript in vitro. miR-135a and miR-135b were also found to be up-regulated in vivo in colorectal adenomas and carcinomas and correlated with low APC levels.

(Nagel, le Sage et al. 2008). These observations suggest that alteration in the miR-135 family can be one of the early events in CRC's molecular pathogenesis.

MiRNA regulating EGFR signaling (KRAS and phosphatidylinositol-3-kinase pathways)

The epidermal growth factor receptor (EGFR) pathway contribute to promotion and progression of several solid tumors including colonic tumor. Stimulation of the EGFR and KRAS signaling lead to the activation of numerous signal transduction molecules initiating a cascade of downstream effectors that mediate tumor growth, survival, angiogenesis and metastasis. (Ciardiello and Tortora 2008) KRAS oncogene has been reported to be a direct target of the let-7 miRNA family.(Johnson, Grosshans et al. 2005). Another miRNA associated with KRAS regulation in CRC is miR-143.(Chen, Guo et al. 2009). Inhibition of KRAS expression by miR-143 blocked constitutive phosphorylation of MAPK.(Chen, Guo et al. 2009). Another central signaling pathway downstream from EGFR and important in CRC development is the phosphatidylinositol-3-kinase (PI-3K) pathway. The p85β regulatory subunit involved in stabilizing and propagating the PI-3K signal was mechanistically proven to be a direct target of miR-126. Furthermore, this p85β reduction mediated by miR-126 was accompanied by a substantial reduction in phosphorylated AKT levels in the cancer cells, suggesting an impairment in PI-3K signaling. Another important regulatory component of the PI-3K pathway, the tumor suppressor gene PTEN, is strongly repressed by miR-21.(Meng F 2007 Aug) miR-21 is the miRNA most frequently up-regulated in CRC.(Slaby O 2007; Schetter, Leung et al. 2008; Krichevsky and Gabriely 2009) It appears that suppression of PTEN controlled by miR-21 is associated with augmentation of PI-3K signaling and progression of CRC.

MiRNAs regulating p53 pathway

Tumor suppressor gene p53 is mutated in about 50-75% of all CRCs and many other human tumors. (Hussain, Amstad et al. 2000) It has been speculated that p53-mediated control of miRNA expression could allow it to indirectly repress target

gene expression at the post-transcriptional level. Recently, several groups have unraveled important aspects of connection between p53 and the miRNA network.(Hermeking 2007). The miRNA 34a-c family was found to be direct transcriptional targets of p53 and their targets regulate cell-cycle progression , cellular proliferation, apoptosis, DNA repair and angiogenesis. (Chang, Wentzel et al. 2007). Among the downregulated targets of the miR-34 family were well-characterized p53 targets like CDK4/6, cyclin E2, E2F5, BIRC3 and Bcl-2.Epigenetic silencing was found to be the mechanism of downregulation of miRNA 34a-c in several CRC tumors. (Lodygin, Tarasov et al. 2008) Hence, miRNA 34 a-c was identified as a tumor suppressor that is downregulated by epigenetic silencing in CRC, whereas in a normal cell it is associated with the p53 network.

MiRNAs in extracellular matrix breakdown and epithelial-mesenchymal transition

Epithelial mesenchymal transition (EMT) is the conversion of an epithelial cell into a mesenchymal cell. ECM remodeling is one of the processes in tumor growth, survival, invasiveness, and metastasizing. The key enzymes involved in ECM breakdown are the urokinase plasminogen activator (uPA) cascade and the matrix metalloproteinases (MMPs). (Takayama, Miyanishi et al. 2006) Morphologically, EMT is characterized by a decrease of E-cadherin, loss of cell adhesion, and increased cell motility leading to promotion of metastatic behavior of cancer cells (including CRC). (Asangani, Rasheed et al. 2007). The functional links to EMT comes from members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429).

MiRNAs regulating other signaling pathways in CRC

miR-145 has been proposed as a tumor suppressor and is known to target the 3' UTR of insulin receptor substrate-1 (IRS-1) which dramatically inhibits the growth of colon cancer cells. (La Rocca, Badin et al. 2009) More recently, IGF-IR was proven to be another direct target of miR-145. (Gregersen, Jacobsen et al. 2010) Cyclooxyygenase-2 (COX-2) strongly contributes to the growth and invasiveness in CRC. miRNA 101 was found target COX-2 directly and cause translational

repression, hence playing the role of a tumor suppressor miRNA. (Strillacci, Griffoni et al. 2009)
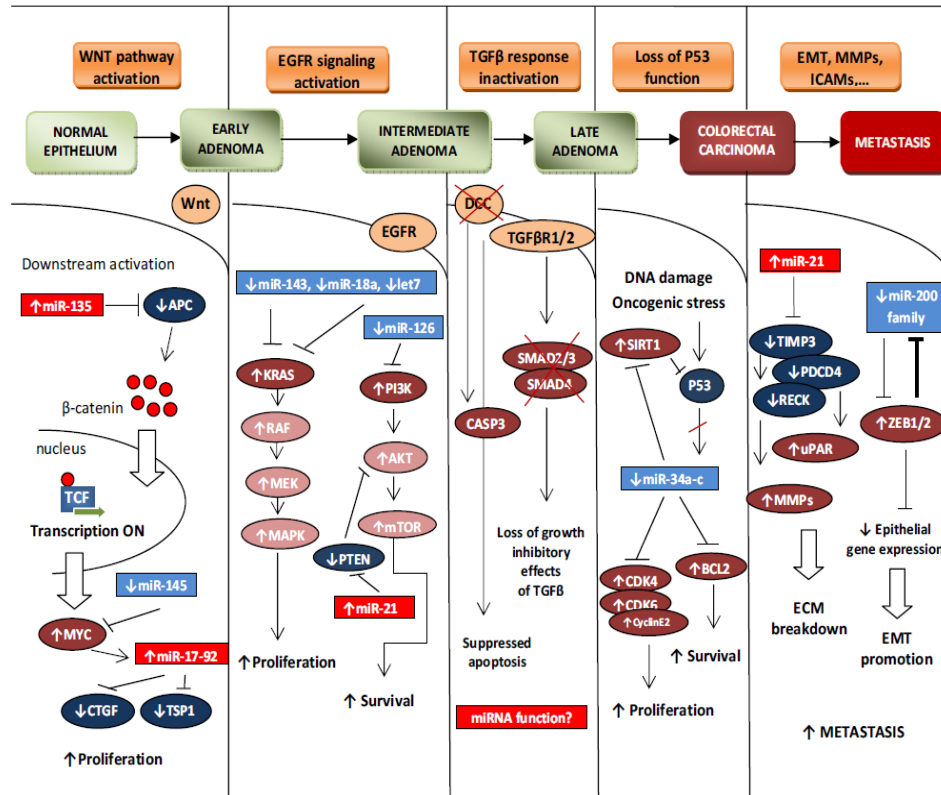
Figure 1.8 Aberrant miRNA expression affecting key pathways in colon carcinogenesis. Several miRNAs are involved in various stages of colon carcinogenesis. Inactivation of the APC gene of the Wnt/β catenin signaling pathway by miRNA 135 which is up-regulated in most CRC cases, leads to the stabilization and constitutive activation of β-catenin. This leads to transcription of genes promoting cell proliferation. Similarly, down-regulation of miRNA 145, which directly targets c-MYC increases cell proliferation. miRNA 143 and miRNA 126, which are down-regulated in CRC target KRAS and p85β subunit of P-I-3 kinase AKT pathway which leads to increased cell proliferation and survival. miRNA 21,up-regulated in majority of CRC cases directly targets tumor suppressor PTEN. miRNA 34 a-c are p53 regulated miRNAs that target several of downstream p53 targets eg- CDK4/6, cyclin E2, E2F5, BIRC3 and Bcl-2. miRNA21 also targets RECK amd TIMP3 which are inhibitors of invasion and malignancy . Members of

miRNA 200 are negatively regulated by transcriptional activator ZEB3 which induces metastasis in CRC cells.

MiRNAs as mediators of inflammation in colon cancer

Specific miRNAs can be regulated by inflammatory stimuli and alternately certain miRNAs can act as mediators of inflammatory stimuli. Expression profiling reveal that lipopolysaccharide (LPS) induced inflammation causes altered expression of several miRNAs.(Taganov, Boldin et al. 2006; Sterghios A Moschos 2007). Studies showed that treatment with either of the pro-inflammatory cytokines, IL1b or TNFα stimulated the expression of miR-146a. (Schetter, Heegaard et al. 2010)The promoter region for miR-146a was found to contain NFκB binding sites, indicating that NFκB was probably responsible for driving the expression of miR-146a. (Taganov, Boldin et al. 2006). Lung inflammation due to allergic reactions showed increased miR-21 expression.(Lu, Munitz et al. 2009). Rheumatoid arthritis which is a chronic inflammatory autoimmune condition affecting joints and tissues showed elevated levels of miR-146a.(Nakasa, Miyaki et al. 2008) Similarly, active inflammation in ulcerative colitis leads to increased expression of several miRNAs, including miR-21.(Wu, Zikusoka et al. 2008). It is now thought that miRNA act as mediators in inflammation associated carcinogenesis such as colon cancer. It was seen that IL6, a pro-inflammatory cytokine, can induce the expression of miRNA-21 in a STAT3 dependent manner. (Lu, Munitz et al. 2009) miRNA-155 stimulated in proinflammatory conditions, can target suppressor of cytokines 1 leading to the induction of NOS2.(Wang, Zhao et al. 2009).

Distal and proximal colon gives rise to different colonic tumors

In the last two decades, histological assessment of tumors, molecular evidences from population based and clinical studies in addition to tumor behavior studies in preclinical models have presented a case supporting the idea of existence of distal and proximal colonic tumor as separate diseases. (Iacopetta 2002; Li and Lai 2009).

Differences in physiology, embryology, molecular features like gene expression pattern etc exist between the normal right and left colon that are responsible for the

differences seen in the tumors appearing along the length of the colon. For example, the proximal colon originates from the embryonic midgut and is perfused by the superior mesenteric artery, whereas the distal colon derives from the hindgut and is served by the inferior mesenteric artery. The capillary network surrounding the proximal colon is multilayered, whereas that of the distal colon is single layered, possibly relating to the greater water absorption and electrolyte transport capacity of the former. (Araki, Furuya et al. 1996; Skinner and O'Brien 1996). The different absorptive nature of right and left colon might be duw to the differences in the average crypt length which is greater in case of distal than proximal.(Arai T 1989) (Table 1.1)

Table 1.1- Differences in right sided (proximal) and left sided (distal) colon.

Features	Proximal/ right sided colon	Distal/left sided colon
Anatomical location	Right of splenic flexure consisting of caecum, ascending colon and transverse colon	Left of splenic flexure consisting of descending colon and sigmoid colon
Embryological origin	Midgut	Hindgut
Blood supply	Branches from superior mesenteric artery Capillary network is multi-layered	Branches from inferior mesenteric artery Capillary network is single layered
Function	Short chain fatty acid and ethanol fermentation	Protein fermentation, neutral muco-polysaccharide breakdown
Average crypt length	Lesser compared to distal colon	Greater compared to proximal colon
Location of stem cells and Direction of migration of colonocyte	Stem cells are located in mid crypt, colonocyte migration is bidirectional i.e, up towards the luminal surface and down towards the crypt base	Stem cells are located at crypt base and colonocyte migration is upwards towards luminal surface.
Type of cells in the crypt	Lower one third has predominantly mucous cells and upper one third has columnar cells.	Small number of mucous cells at the base, highest proportion of goblet cells.
Morphology of tumors	Sessile serrated adenomas, hyperplastic polyps with low grade dysplasia	Traditional serrated adenomas, mostly pedunculated or villiform in appearance with high grade dysplasia
Molecular	Microsatellite instability	Chromosomal instability

pathway for carcinogenesis	pathway, in which the DNA repair enzymes are silenced by promoter methylation, causing mutations in microsatellite regions of genes involved in cell proliferation, apoptosis.etc. BRAF mutation and high level of CpG island methylation leading to inactivation of MLH1 and MGMT, result in high and low level MSI respectively	pathway ,in which there is allelic loss of chromosomes harbouring the APC, P53, K-Ras genes. CpG island methylation in KRAS, leading to microsatellite stable (MSS) or low grade microsatellite instable (MSI-L) carcinogenesis.
Gene expression pattern	Genes overexpressed include-phospho- sulfotransferases, genes associated with ions, electrolyte, heterocyclic compounds, polycyclic hydrocarbons and steroids absorption e.g-cytochrome P450 family genes, glutathione S-transferase Z1, 3-β-hydroxysteroid dehydrogenase1 , hydroxysteroid(17β) dehydrogenase.	Genes overexpressed include-phospholipase A2, genes associated with cell cycle control (cyclin D1,D2,G1, cyclin dependent kinase 2, PCNA), DNA replication, DNA damage repair, DNA adduct metabolism, TNFR2, NF-κB, IGR-IRα, IRα, and IRβ
Tumor behavior in response to to exogenous agents	Azoxymethane –less tumor High fat diet – no significant effect	Azoxymethane – more tumor High fat diet – promotes growth of tumor
	Fish oil diet- no significant effect	Fish oil diet- retards growth of tumor
	High calcium diet- promotes growth of ACF/tumor Sulindac-induces new inflammatory lesions with malignant potential	High calcium diet-reduces growth of ACF Sulindac-regresses tumor growth

Approximately 60% of CRC in high-incidence populations arise in the left colon (distal), whereas in low-incidence regions there is a predominance of right-sided (proximal) cases. (Haenszel W 1973). The incidence of proximal tumors in Western countries has steadily increased in the past decade while that of distal tumors has

shown a corresponding decrease.(Rhodes, Holmes et al. 1977).(Gervaz, Bucher et al. 2004; Fenoglio, Castagna et al. 2010). The cases have been more common at an advanced age and in female gender of the population. (Okamoto, Stiratori et al. 2002). Taken together, the geographic location, age and gender influences on CRC subsite distribution, in concert with the observations on proximal shift and migrant studies, suggests the existence of two broadly different mechanisms of CRC.

Biological heterogeneity in colonic tumors depending on their spatial origin

Morphologically and histologically colonic tumors exhibit different features. The adenomatous polyps differ in the degree of epithelial dyplasia, whereas hyperplastic polyps have serrated appearance without dysplasia. The mixed polyps are the ones that display both dysplasia as well as serrated appearance. Tumors could appear as sessile, pedunculated, polypoid, villous as well as flat lesions. These features allude to the possibility that the origin and biology of tumors differ. (Warnecke, Engel et al. 2009) Serrated neoplasia's (SNs) with the hyperplastic pattern are located predominantly in the proximal colon and have superficial appearance, whereas SNs with the adenomatous pattern were observed in the distal colon and have a protruding appearance. (Noffsinger 2008). It has been noticed that the distribution of polyps with high grade dysplasia is more predominant in the distal region than proximal.(Goldstone, Itzkowitz et al. 2011)

Behavioral heterogeneity in colonic tumor

 The concept that distal and proximal tumors could respond differently to cancer preventive agents and growth modulators, has been explored in preclinical models. Some of these require cancer induction by a carcinogen, whereas some models are genetically manipulated to address the role of specific pathway in carcinogenesis.(Vignjevic, Fre et al. 2007; Raju 2008). It has been previously established that pre-neoplastic lesions (ACF) appearing in response to chemical carcinogens like AOM are different in distal colon compared to proximal.(Bird RP 1989) It has been reported that distally located ACF grow more rapidly in response

to multiple injections of azoxymethane compared to their proximal counterparts.(Raju and Bird 2003). The interpretation here could be that proximal colonic regions resist growth modulating environment and respond slowly or that the test agent may not be as readily available to proximal colonic tissue. For e.g; high fat tumor promoting diet promotes the growth of distally located tumors and it is less effective in proximal tumors.(Kim, Chung et al. 1998; Hambly, Saunders et al. 2002). A high fish oil diet in a high risk preclinical model of colon carcinogenesis is more effective in retarding the growth of distal developing tumors. (Hong, Chapkin et al. 2001) Similarly a high calcium diet retards the growth of ACF in distal colon and tumors whereas has an opposite effect on the ACF or tumors developing in the proximal region. (Li, Kramer et al. 1998; Pierre, Taché et al. 2003) Recently, it was observed that a non-inflammatory drug Sulindac is able to regress distal colonic tumor in azoxymethane injected mice, whereas in the proximal region it induces new inflammatory lesions which develop into adenocarcinomas in upto 18-25% of p53 or mismatch repair deficient mice.(Mladenova, Daniel et al. 2011).In Zucker obese model, distally located tumors exhibited a phenotype compatible to enhanced growth than the proximal tumors. TNFα signaling pathway, insulin and expression of IGF receptors were higher in the distal colonic tumors.(Jain and Bird 2010) The most notable finding was that the ERK1 and 2 were differently expressed. Elevated expression of TNFR2, NF-κB, IGR-IRα, IRα, and IRβ was seen in the distal tumor mass compared to proximal tumor. Distal tumor showed high levels of NF-κB, IKKβ and lower level of IκB-α, which suggests that the pathway leading to activation of NFκB and subsequent survival genes is more active in distal colonic tumor compared to proximal. The presence of elevated levels of insulin, IGF-1R and TNF-α in the distal colon could also act synergistically to provide superior survival conditions to developing preneoplastic lesions in distal colon compared to proximal colon. (Jain and Bird 2010)

Molecular heterogeneity evident in profiling studies in clinical system

The distinct clinico-pathological characteristics as well as differences seen in tumor behavior along the colonic axis, is also reflected in the pattern of gene expression.

Some of these differences are thought to be acquired embryonic and post natal development, whereas some are speculated to be the result of interaction with the environmental risk factors, aging, diet etc. Microarray profiling of human colonic tumor showed >1000 genes that were differentially expressed in distal versus proximal tumor, seventy percent of which were highly expressed in distal colon compared to proximal colon.(Joyce and Pintzas 2007). The genes found to be differentially expressed in distal and proximal tumor are known to be involved in many cellular processes which include cell cycle, proliferation, cell death, response to external stimuli, stress response, and DNA replication and damage repair. These are also implicated in key signaling pathways known to be involved in colon carcinogenesis eg-EGF. The genes found to be differentially expressed in distal and proximal tumor are known to be involved in many cellular processes which include cell cycle, proliferation, cell death, response to external stimuli, stress response, and DNA replication and damage repair. These are also implicated in key signaling pathways known to be involved in colon carcinogenesis eg- EGF, TGF-β, Wnt, Ras, insulin and integrin signaling.(Glebov, Rodriguez et al. 2003).

miRNA profiling in distal and proximal colonic tumor: evidences from clinical studies

In recent years miRNAs have been been used as diagnostic tool to differentiate between right (proximal) and left sided (distal) colonic tumors. A comparitive study between normal colonic tissue and colon tumor from distal and proximal regions, showed several unique miRNAs that were differentially expression for microsatellite instability positive (MSI+) colon tumors found mostly in the proximal .There were several miRNAs that were differentially expressed for KRAS2-mutated colon tumors, and 139 miRNAs were differentially expressed for TP53-mutated colon tumors mostly seen in the distal region of the colon. (Slattery, Wolff et al. 2011). miRNA profiling of colon cancer samples characterized by microsatellite stability(MSS) and samples characterized by microsatellite instability showed differential expression of several miRNAs. miRNA 17-92 family showed

upregulation in MSS colonic tumor which is predominantly found in distal colonic region.

miRNA has been used as diagnostic tool and biomarker for prognosis and predicting clinical outcomes in colon cancer. With evidences from clinical and preclinical models supporting the concept of proximal and distal colonic tumor as distinct diseases, there is need to get deeper insight into the regulation of gene expression in these two regions of the colon. miRNA have shown distinct expression pattern in these regions further strengthening the molecular evidences in this regard. However, they are very few evidences from animal model study that look into miRNA expression pattern in distal and proximal colon in control or altered physiological state.

CHAPTER 3

DESIGN AND METHODOLOGY

Animals

Female Zucker obese fa/fa (Zk-Ob) and lean (Zk-Ln) rats (n=10/group), weighing approximately 300 and 80 Kg respectively ,were obtained from Charles River Laboratories (Montreal, QB, Canada) at 12 weeks of age. They were housed in standard plastic cages with woodchip bedding and stainless steel wire mesh lid in the Biology Animal facility, University of Windsor, under controlled environmental conditions (22° C temperature, 50% humidity, 12 hour light/dark photo-period). Animal care and all investigative procedures adhered to guidelines of the Office of Research Ethics and Animal care committee of University of Windsor (AUPP# 10-19) and the Canadian Council of Animal Care. Body weights of the animals was monitored twice a week and finally at termination.

Following one week of acclimatization, animals were terminated by CO_2 asphyxiation. After recording weight of the body, liver and kidney, blood was obtained in heparinized or EDTA vacutainer tubes (BD Vacutainer Systems, NJ from a cardiac puncture using a multiple sample Luer adapter). Organs collected were stored in liquid nitrogen and kept at -80°C for future studies.

Blood analysis

Blood samples were centrifuged for 10 min at 2000 rpm to separate plasma, which was then aliquoted. Samples were either stored for further analysis at -80ºC, or sent to Animal Health Laboratories (University of Guelph, Guelph, ON) for biochemical analysis of inflammatory cytokines (Interleukins), adhesion molecules like inter cellular adhesion molecules (ICAM), chemokines, Monocyte Chemoattractant Protein (MCP) (appendix A.1)

Colon preparation

Colon from each animal was removed, flushed with cold phosphate buffer saline and placed on a cold plate set at 4°C. A longitudinal cut was made from the rectal to the ceacal end. Mucosa from the colon was scraped separately from distal and proximal region of the colon and snap from in liquid nitrogen as well as RNA later (Sigma) for biochemical and molecular analysis.

Total RNA isolation

50-100 mg of frozen tissue was used to isolate total RNA using miRNeasy Mini Kit (Qiagen). The miRNeasy Mini Kit combines phenol /guanidine based lysis of samples and silica-membrane based purification of total RNA. Tissue samples were homogenized in QIAzol Lysis Reagent which is a monophasic solution of phenol and guanidine thiocyanate similar to TRIazol, which facilitates lysis of tissues, inhibits RNases, and removes most of the cellular DNA and proteins from the lysate by organic extraction. After addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA was partitioned to the upper, aqueous phase, while DNA was partitioned to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol (50%) was added to provide appropriate binding conditions for all RNA molecules from 18 nucleotides (nucleotide) upwards. The sample was then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water.

MiRNA isolation

The flow through from the above step (using RNeasy Mini spin column), was mixed with 0.65 volumes (450µl) of 100% ethanol and vortexed. This sample was loaded into RNeasy MinElute spin column which was placed in 2ml collection tube. The samples were centrifuged for 1 minute at approximately 8000 x g (10,000 rpm) at room temperature (15-25°C). Flow through was discarded. Column was washed twice with 700µl of RWT buffer (wash buffer containing guanidine thiocyanate).

Column was finally washed with 80% ethanol. miRNA was eluted in 30µl of RNase free water.

Spectrophotometric quantification of miRNA-enriched fractions

MiRNA enriched fractions prepared is assumed to contain many types of small RNA, including miRNA, snoRNA, and piRNA, as well as tRNA and a significant proportion of the 5S and 5.8S rRNA. Larger species, including tRNA, dominate the OD measurement of these fractions and also gel staining or assays which use RNA-binding dyes. For this reason, it is not possible to quantify miRNA using OD measurements. Hence to quantify miRNA, qRT PCR was performed using miScript System (Qiagen).

MiRNA to cDNA (Reverse-transcription step)

10-20 ng of miRNA enriched fraction of total RNA was converted to cDNA using RT^2 mi RNA first strand kit (Qiagen) and primer specific probes. miScript Reverse Transcriptase Mix is an optimized blend of enzymes comprising of a poly (A) polymerase and Reverse Transcriptase. The buffer system enables maximum activity of both enzymes besides containing Mg2+, dNTPs ,oligo-dT primers, and random primers. Unlike mRNAs, miRNAs are not polyadenylated in nature.During the reverse-transcription step, miRNAs are polyadenylated by poly (A) polymerase. Reverse transcriptase converts RNA (including precursor miRNA, mature miRNA, other small noncoding RNA, and mRNA) to cDNA using oligo-dT and random primers. Polyadenylation and reverse transcription are performed in parallel in the same tube. The oligo-dT primers have a universal tag sequence on the 5' end. This universal tag allows amplification in the real-time PCR step.

For each 10µl reaction, the components (Appendix B.1) were added to a regular pcr tube, mixed and stored in ice. The reaction was incubated at 37°C for 60 minutes and then for 5 minutes at 95°C to inactivate Reverse Transcriptase.

Real Time Quantification of miRNA

MiRNA abundance was quantified using conventional two step quantitative reverse transcriptase real time PCR (q PCR) using miScript housekeeping primer assay for small nuclear RNA (RNU6 and 4.5s_V1). Template cDNA was characterized by qRT PCR using RT² SYBR Green/ROX qPCR Master Mix on the 7500 FAST Real-Time PCR System (Applied Biosystems). miRNAs were amplified using the miScript Universal Primer (Qiagen) , which primes from the universal tag sequence, together with the miScript Primer Assay for housekeeping small nuclear RNA.

Reaction setup for real-time PCR based quantification of miRNA (Appendix B.2)

For 20µl reaction volume, the components are added to a PCR tube, mixed thoroughly and kept in ice.

Cycling conditions for real-time PCR for miRNA quantification – (Appendix B.3)

Manual C_T threshold value was adjusted to 0.02, again as per instrument specifications. Fold change in gene expression was calculated using the $\Delta\Delta\ C_t$ method and analyzed with Sequence Detection System software (SDS version 2.0.5 Applied Biosystems).

MiRNA Rat Cancer PCR Array

The differential expression of miRNA in colonic mucosa tissue was profiled using RT2 miRNA PCR Array system (SAbioscience / Qiagen). The universal primer in every assay of the RT2 mi RNA PCR Arrays is specific for the unique sequence incorporated into the cDNA by the universal RT primer in the RT2 mi RNA First Strand Kit.

Step 1- cDNA First Strand synthesis (Appendix B.4)

The contents were mixed, followed by brief centrifugation. The samples were then incubated at 37°C for 2 hours; and then heated at 95°C for 5 minutes to degrade the RNA and deactivate the Reverse Transcriptase. The samples were then chilled on ice and 90µl of RNase free water was added to each 10 µl reaction volume.

Step 2- Real Time PCR

The 96 well RT^2 miRNA cancer PCR array (SAbiosciences /Qiagen) was used for screening cancer miRNA coding genes from distal and proximal mucosa samples obtained from Zk-Ob and Zk -Ln rats.

The components for were added to a 15 ml tube and mixed thoroughly with a pipette as per the manufacturer's protocol. (Appendix B.5)

25µl of the cocktail was added to each well of the 96 well Rat Cancer RT2 miRNA PCR Arrays (stored at -20°C). The array profiles the expression of 88 miRNA sequences (annotated by the Sanger miRBase Release 14.) which are implicated in cancer and related pathways directly or indirectly. Cycling conditions for real time PCR for the array experiment (Appendix B.6).

The 10-minute step at 95°C was required to activate the HotStart DNA polymerase. SYBR Green fluorescence was detected and recorded from each well at the annealing step for each cycle. The threshold cycle C_T was calculated using the instrument software, i.e Sequence Detection System software (SDS ver 2.0.5) Applied Biosytems. To define the Baseline, the Linear View of the amplification plots were used and the instrument was set to use the readings from cycle number three (3) to cycle number fifteen (15).Other amplification kinetics were measured on Applied Biosystems 7500 FAST sequence detector and analysed with Sequence Detection System software (SDS ver 2.0.5) Applied Biosytems. Data was analysed using $\Delta\Delta C_t$ method.

MiRNA Sequence Specific Assays contained within each RT2 mi RNA PCR Array include one universal primer and one gene-specific primer for each mi RNA

sequence. Details of array layout (Appendix B.7) Controls used in the array are a panel of housekeeping Assay consisting of small nuclear RNA housekeeping assay panel which include Rnu6, U87, 4.5S_V1 and Y1. Duplicate Reverse Transcription Control (RTC) in the rray tests the efficiency of first strand reaction with a primer set miRNA called External RNA Control (ERC) detecting the cDNA template synthesized. Positive PCR Control (PPC) tests the efficiency of the polymerase chain reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it.

Statistical Analysis

DataAssist^{TM} v3.0 Software (Applied Biosystems) was used as analysis tool for sample comparison using relative comparative C_T ($\Delta\Delta C_t$) method (Livak and Scmittgen, 2008) . It provides relative quantification analysis of gene expression through combination of statistical analysis by performing t-test for biological group. For graphical representation, relative quantification (RQ plot) was plotted that displays RQ (fold change) versus target assay. The cluster analysis and heat map was created using the DataAssist^{TM} software v3.0 that graphically displays results of unsupervised hierarchical clustering. Distance between samples and assays were calculated for hierarchical clustering based on the ΔC_T values using Pearson's Correlation application of the DataAssist software.

Target Prediction by bioinformatics analysis

Several algorithms were used to predict potential targets of miRNA, such as miRanda (http://miRNA.sanger.ac.uk/targets/v4/), PicTar (http://pictar.bio.nyu.edu/), and TargetScan 3.1 (http://www.targetscan.org/). Potential target sequences were pooled for both rat and human mRNA and only those targets were considered that were conserved between the two species. Finally, the predicted targets were aligned against published Affymetrix gene expression profile from rat distal and proximal colonic tissue.(Su, Bush et al. 2007).

Chapter 4

ANALYSIS OF RESULTS

Body weight, liver weight, kidney weight

The average body weight of Zk-Ob (fa/fa) rats was 315gm. The average liver and kidney weight was 19.286 gm and 4.536 gm respectively. The average body weight of Zk-Ln(Fa/fa) rats was 86gm.The average liver and kidney weight was 10.77 gm and 3.87 gm respectively.

Blood parameters

A multiplex assay using antibody bead array technology (Appendix A.1) was used to assess the levels of specific cytokines and markers associated with obesity and inflammation eg- ICAM-1, E-selectin, IL-1α, IL-6 and MCP-1. The markers that showing significantly higher levels in obese plasma compared to lean are as follows:

 ICAM - obese 11569.8 pg/mol ±1582.64 versus lean 7810.48 pg/mol ±623.67

 MCP - obese 389.06 pg/mol ±161.16 versus lean 167.55 pg/mol ± 19.95

 E-Selectin - obese 94405.26 pg/mol ± 8415.09 versus lean 87697.38 pg/mol ± 5588.32

 IL-1α - obese 1996.3275 pg/mol ± 987.09 versus lean 1170.4025 pg/mol ± 284.98

 IL-6 - obese 1875.13 pg/mol ± 887.62 versus lean 711.8825 pg/mol ± 90.37

Results indicate a significant rise in levels of inflammatory cytokines (IL-1α, IL-6), chemokines (MCP) and cellular adhesion molecules (ICAM and E-selectin) in the blood plasma of Zk-Ob rats compared to their lean (control) counterparts.

Differential expression of miRNA in Zk-Ob and Zk-Ln liver

This study was carried out to meet specific aim1 i.e establishing method of isolation and quantification of miRNA in rat tissue. Liver tissue was used to optimize the miRNA isolation since it is a large organ that provides sufficient start material to establish a methodology that was subsequently used to meet specific aim 2 and 3. Liver steotosis or abnormal accumulation of fats in the hepatic cell is a common symptom seen in obesity. It is a part of metabolic syndrome associated with obesity and is speculated to play an important role in inflammation associated metabolic disorders. Hence, liver along with colon can provide an insight into the role of obesity in regulation of miRNA expression.

The miRNA expression pattern of Zk-Ob and Zk-Ln liver tissue, showed that 20 miRNAs were significant up-regulated in Zk-Ob liver compared to Zk-Ln. These include miRNA 125a-5p, miRNA 132, mi RNA 137, miRNA 146 b, miRNA 15b, mi RNA 181a, mi RNA 181b, miRNA 183, miRNA 191, mi RNA 199a-3p, miRNA 199a-5p, miRNA 200, miRNA 21, miRNA 218, mi RNA 223, mi RNA 23a, miRNA 27a, miRNA 34a, miRNA 429 and miRNA 96. (Figure 4.1) There were few that showed more than 2 fold up-regulation but were not found to be statistically significant. Similarly, few miRNA were found to be almost two fold down-regulated in Zk-Ln, though not significant. Out of the 20 miRNAs that showed significant differential regulation in Zk-Ob liver compared to lean, 3 prominent ones, namely miRNA 21, miRNA 199-3p and miRNA -5p were picked for independent validation studies. In their individual validation studies, all three of them showed significant up-regulation in Zk-Ob liver compared to Zk-Ln liver in accordance with the array studies (Figure 4.2).

The method of miRNA isolation and quantification was successfully established (specific aim1). Several miRNAs were found up-regulated in obese liver compared to lean, establishing the fact that obesity influences miRNA expression irrespective of genotype.(Table 4.1) Through validation studies, it was further confirmed that miRNA 21, miRNA 199-3p and miRNA 5p are up-regulated under obese conditions.
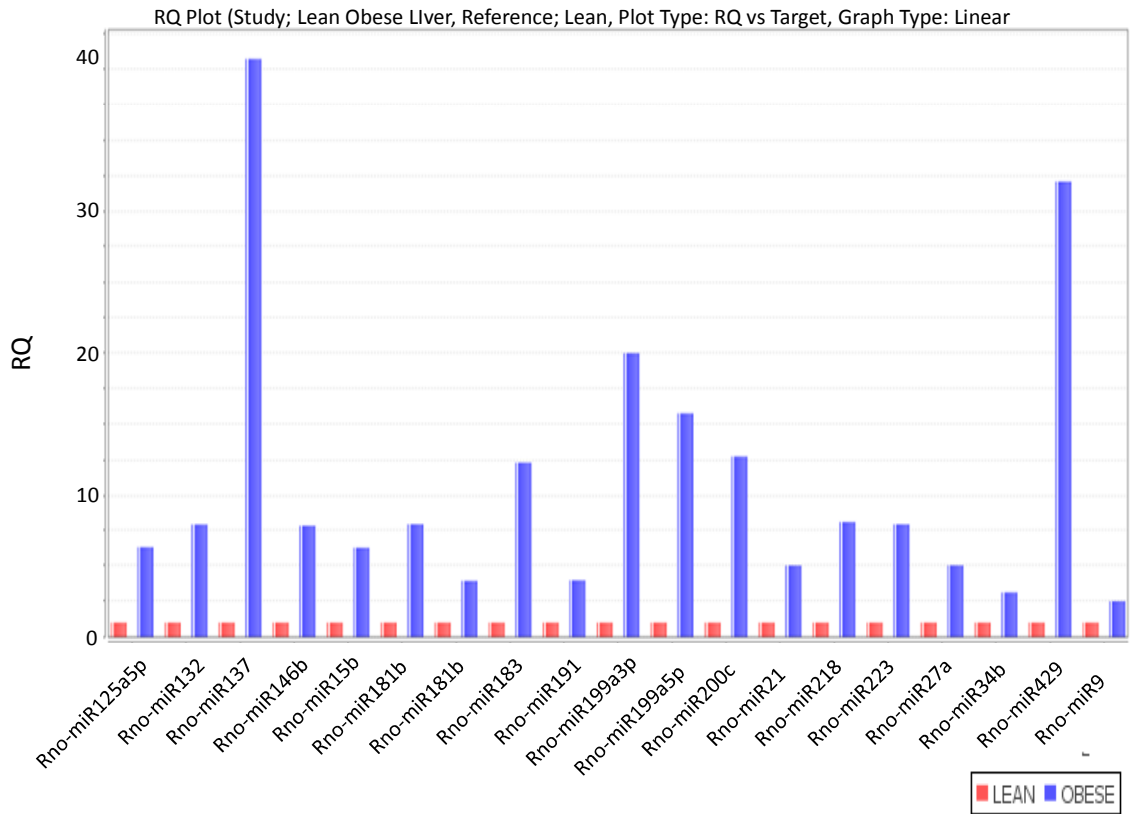
Figure 4.1 Differential expression of miRNAs in liver tissue of Zk-Ob versus Zk-Ln. miRNA abundance in the samples was normalized using miRNA 4.5s_V1 as endogenous control. The abundance of each of the candidate miRNAs is represented as linear RQ or fold change of obese compared to lean (reference sample). P-values are calculated using student t-test application of DataAssist software v3.
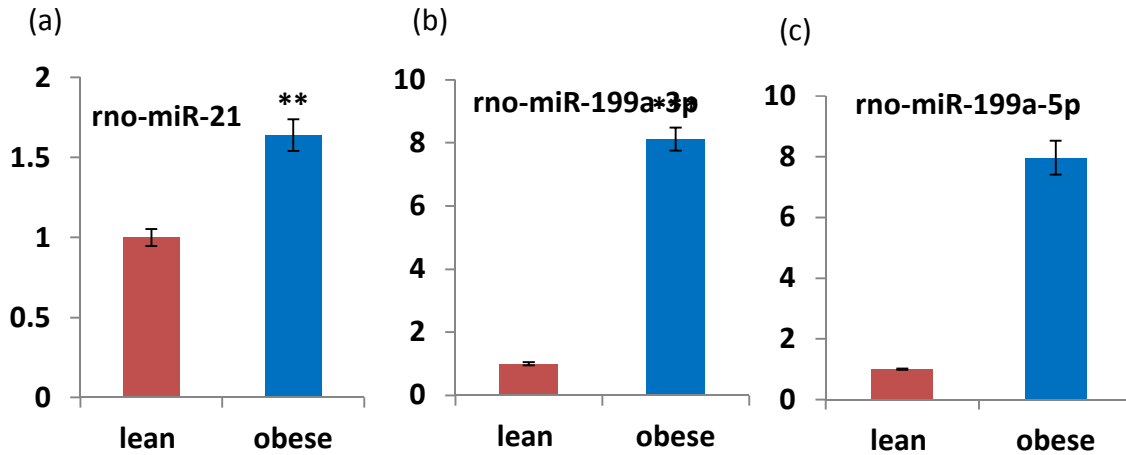
Figure 4.2 Validation of selected miRNAs in independent sample set. miRNA was isolated from idependent set of liver tissue from Zk-Ob and Zk-Ln rats (n=3). Individual miRNA primer assays for miRNA 21, miRNA, 199-3p and miRNA 199-5p were used to measure of miRNA abundance in obese versus lean liver tissue using q RT PCR. Here the individual miRNA abundance is plotted against fold change (RQ). Data was normalised using 4.5s_v1 as endogenous control and significance was calculated by student t-test($**p<0.05$, $**p<0.01$, $***p<0.001$) Panel (a)-miRNA 21 shows approx.1.5 fold abundance in Zk-Ob liver compared to Zk-Ln; panel (b.) miRNA 199-3p shows 4 fold abundance in Zk-Ob liver compared to Zk-Ln; panel(c.) miRNA 199-5p shows 4 fold abundance in Zk-Ob liver compared to Zk-Ln.

Table 4.1-Significantly up-regulated miRNAs in Zk-Ob liver compared to Zk-Ln liver and their function/target

miRNA	Fold change	Direction	Adjusted P-value	Cellular function/target	Reference
miRNA 125a-5p	6.32	up-regulation	0.02	directly targets oncogene ERBB2 in gastric carcinoma cells, negatively regulates cell proliferation and metastasis, EGFR regulated tumor suppressor, regulates the inflammatory response, lipid uptake in monocytes/macrophages	(Chen, Huang et al. 2009; Nishida, Mimori et al. 2011; Guofu Wang 2009 October)
miRNA 132	7.91	up-regulation	0.003	targets tumor suppressor protein rein retinoblastoma (pRb) in panc nreatic cancer cells, thus increasing cecell proliferation, potentiates Cholinergic Anti-Inflammatory Signaling by Targeting Acetylcholinesterase	(Shaked, Meerson et al. 2009; Park, Henry et al. 2011)
miRNA 137	40.77	up-regulation	0.015	acts as a tumor suppressor in colon and is often silenced by promoter methylation, targets carboxyl terminal binding protein1 that acts as co-repressor for many tumor suppressor genes, targets Cdc2 expression, induces G1 cell	(Liu, Lang et al. 2011; Zhang, Hao et al. 2011)

				cycle arrest and inhibits invasion in CRC cells.	
miRNA 146b	7.82	up-regulation	0.022	supresses NF-κB activity in breast cancer cells,expressed in response to rising inflammatory cytokine levels as part of negative feed back loop that restrains excessive senescence associated secretory phenotype.	(Bhaumik, Scott et al. 2008; Dipa Bhaumik1 2009)
miRNA 15b	6.82	up-regulation	0.024	E2F (promotes cell proliferation) regulated which in turn regulates E2F activity, associated with increased cell proliferation and decreased apoptosis.	(Satzger, Mattern et al. 2010; Ofir, Hacohen et al. 2011)
miRNA 181a	7.92	up-regulation	0.037	induced by Wnt/β catenin signaling, including Wnt10 over-expression.	(Bioscience 2011)
miRNA 181b	3.9398	up-regulation	0.013	expression promotes G1 to S transition, targets CBX7 and cell death associated genes eg-PCD4, BCl11etc	(Gelsomina Mansueto 2010)
miRNA 183	12.32	up-regulation	0 0.009	functions as oncogene by targeting transcription factor EGR1,PTEN and promoting cell migration	(Sarver, Li et al. 2010)

miRNA 191	3.97	up-regulation	0.014	oncogene that promotes cell proliferation and inhibits apoptosis in hepatocarcinoma, targets MDM4 which down-regulates p53.	(Elyakim, Sitbon et al. 2010; Wynendaele, Böhnke et al. 2010)
miRNA 199-3P	19.97	up-regulation	0.006	regulates cell proliferation and survival by targeting Caveolin-2,targets mTOR and c-Met in human hepato-carcinoma cells	(Fornari, Milazzo et al. 2010; Shatseva, Lee et al. 2011)
miRNA 199-5p	15.77	up-regulation	0.008	targets hypoxia inducible factor-α in hepatocellular carcinoma, discoidin domain receptor-1 (DDR1) tyrosine kinase, involved in cell invasion-related signaling pathway is targeted by miRNA 199-5p,targets HES which negatively regulates cell proliferation	(Livia Garzia 2009; Qingli Shen1 2010; Wang, Song et al. 2011)
miRNA 200c	12.74	up-regulation	0.009	suppresses E-Cadherin and other genes related with EMT transition, by targeting ZEB1 and ZEB2	(Howe, Cochrane et al. 2011)
miRNA 21	4.98	up-regulation	0.036	acts as oncogen by targeting tumor suppressor gene tropomyosin (TPM1), programmed cell death 1(PCD4) and maspin, both implicated in metastasis and invasion,directly targets PTEN tumor suppressor, EGFR	(Zhu, Wu et al. 0000; Hurt and Farrar 2007; Meng, Henson et al. 2007) **9**:227 (Seike, Goto et al. 2009)

regulated anti apoptotic factor

miRNA 218	8.09	up-regulation	0.036	targets EGFR that regulates NF-κB activity and is associated with apoptotic response, tumor suppressor activity in lung squamous cell carcinoma, targets mTOR	(Davidson, Larsen et al. 2010; Gao, Zhang et al. 2010; Uesugi, Kozaki et al. 2011)
miRNA 223	7.92	up-regulation	0.03	targets starthmin1(onco-protein) in hepatocarcinoma cells, promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3, negatively correlated with oncogene c-myc, targets tumor suppressor FBW7, that targets oncoproteins like cyclin E, c-myc for degradation, induces expression of GLUT3	(Wong, Lung et al. 2008; Lu, Buchan et al. 2010; Xu, Sengupta et al. 2010; Li, Zhang et al. 2011; Zhao WY 2011 Feb)
miRNA 23a	3.95	up-regulation	0.013	acts as tumor supprerssor by promoting and antiapoptotic factor in hepatocellular carcinoma cells, downregulates interleukin -6 receptor	(Zhu, Liu et al. 2010)
miRNA 27a	5.00	up-regulation	0.035	oncogenic, targets ZBTB10 and Myt1 which cataylses phosphorylation of cdc2 to inhibit progression of cells from	(Sudhakar Chintharlapalli 2009, october 15)

G2 to M phase

miRNA 34a	3.14	up-regulation	0.042	p53 dependent tumor suppressor, targets CDK4/6, E2F5, BcL2, SIRT1 (negative regulator of apoptosis under stress conditions)	(Munekazu Yamakuchi*† June 25, 2008)
miRNA 429	32.08	up-regulation	0.006	modulates expression of c-myc, playes important role in pathogenesis of gastric carcinoma	(Sun, Wang et al.)
miRNA 96	24.84	up-regulation	0.006	targets KRAS, acts as tumor suppressor in pancreatic cancer	(Yu, Lu et al. 2010)

Differential expression of miRNA in distal and proximal colon of Zucker lean rat (control)

This study was conducted to determine the miRNA expression profile in right and left sided colon using Zk-Ln rats as control. It was found that 9 out of the 88 miRNAs on the array showed statistically significant fold increase (P< 0.05) in proximal colonic mucosa compared to distal. There were few miRNAs that showed down-regulation for eg- miRNA 221 and miRNA 222 showed 0.6223 and 0.2513 down-regulation respectively. However, they did not show any statistically significance. miRNAs that showed significant up-regulation(P <0.05) in proximal colon mucosa compared to distal include miRNA-143, miRNA-152, mi-RNA 206, miRNA-218, miRNA- 223, miRNA 29b, miRNA-30c,miRNA-363, miRNA 335. (Figure 4.3) There were atleast 10 more miRNAs that showed more than 2 fold up-regulation but they were all found to be statistically insignificant.

Looking at the lean (control) group alone, it can be concluded that miRNA are differentially expressed in distal and proximal regions of the colon (specific aim2).(Table 4.2) The possibility of few or all of these miRNAs serving as biomarkers for specific colonic regions would depend on the consistency of their trend of expression under other physiological conditions.

Figure 4.3 Differential expression of miRNAs in Zk-Ln proximal colonic mucosa versus Zk-Ln distal colonic mucosa. miRNA abundance in the samples was normalized using miRNA 4.5s_V1 as endogenous control. The abundance of each of the candidate miRNAs is represented as linear RQ or fold change of Zk-Ln proximal colonic mucosa compared to Zk-Ln distal colonic mucosa (reference sample). P-values are calculated using student t-test application of DataAssist software v3.0.

Table 4.2- Significantly up-regulated miRNAs in Zk-Ln proximal colon compared to Zk-Ln distal colon and their function/targets

mi RNA	Fold change	Direction	Adjusted -P value	Cellular function/target	Reference
miRNA 143	25.74	up-regulation	0.046	targets KRAS oncogene, operational in colon cancer, targets DNA methyl transferases3 in colon cancer	(Ng, Tsang et al. 2009; Gao, Zhang et al. 2011)
miRNA 152	8.04	up-regulation	0.046	induces aberrant DNA methylation in Hepatitus B virus related Hepatocarcinoma by targeting DNA methyl transferaseI	(Jinfeng Huang July 2010)
mi RNA 206	32.32	up-regulation	0.032	targets notch3 ,activates apoptosis,inhibits tumor cell migration	(Song, Zhang et al. 2009)
miRNA 218	130.73	up-regulation	0.046	targets EGFR that regulates NF-κB activity and is associated with apoptotic response, tumor suppressor activity in lung squamous cell carcinoma, targets mTOR	(Davidson, Larsen et al. 2010; Gao, Zhang et al. 2010; Uesugi, Kozaki et al. 2011)
miRNA 223	41.17	up-regulation	0.047	targets starthmin1(oncoprotein) in hepatocarcinoma cells, promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3, negatively correlated with oncogene c-myc,	(Wong, Lung et al. 2008; Xu, Sengupta et al. 2010; Li, Zhang et al. 2011; Zhao WY 2011 Feb)

				targets tumor suppressor FBW7, that targets oncoproteins like cyclin E, c-myc for degradation, induces GLUT4 expression	
miRNA 29b	10.12	up-regulation	0.047	induces global DNA hypomethylation by directly targeting DNMT1	(Garzon, Liu et al. 2009)
miRNA 30c	16.28	up-regulation	0.032	associated with HER and RAC1 signaling pathways in breast cancer	(F Germán Rodríguez-González 2011)
miRNA 363	16.57	up-regulation	0.032	regulated by insulin growth factor receptor IGF-R, down-regulated in colon cancer.	(Adhip PN Majumdar1 April 2010)
miRNA 335	258.02	up-regulation	0.047	miRNA-335 acts as a metastasis suppressor in gastric cancer by targeting Bcl-w	(Xu, Zhao et al. 2011)

Differential expression of miRNA in distal and proximal colon of Zk-Ob rats under conditions of chronic inflammation

The aim of this study was to identify the key miRNAs that are differentially expressed in proximal versus distal colon under obese conditions. It was found that 15 out of the 88 miRNAs on the array, showed significant fold decrease (P<0.05) in proximal colon compared to distal colon. (Figure 4.4) Interestingly, most of the miRNAs were found to be down-regulated in proximal colon compared to distal. Among the miRNAs that were found to be significantly down-regulated, are miRNA 125a-5p, miRNA 132, miRNA 133b, miRNA 181d, miRNA 183, miRNA199a-5p , miRNA 21, miRNA 223, miRNA301a, miRNA320, miRNA 34a, miRNA 378, miRNA 708, miRNA 7a and miRNA 92a. miRNA 223, that was found significantly up-regulated in lean proximal colon was found significantly down-regulated in proximal colon under conditions of obesity. miRNA 21, which is associated with inflammation was down-regulated in proximal colon of Zk-Ob rat as well. Most of the other miRNAs that were down-regulated in proximal colon compared to distal were not statistically significant.

In this study, several observations were made regarding the role of obesity in regulation of miRNA expression. Firstly, obesity seems to have a profound on several miRNAs in the colon which shows that inflammation is a key factor involved. (Table 4.3) Secondly, miRNA 223 that was significantly up-regulated in proximal colon compared to distal in the control lean group, was significantly down-regulated under obese conditions. Interestingly, this miRNA is also down-regulated in hepatocellular carcinoma, where it acts as tumor suppressor by directly targeting Stathmin1 oncogene. (Wong, Lung et al. 2008) Similar trend was observed in case of few others miRNAs also, however they were not significant.
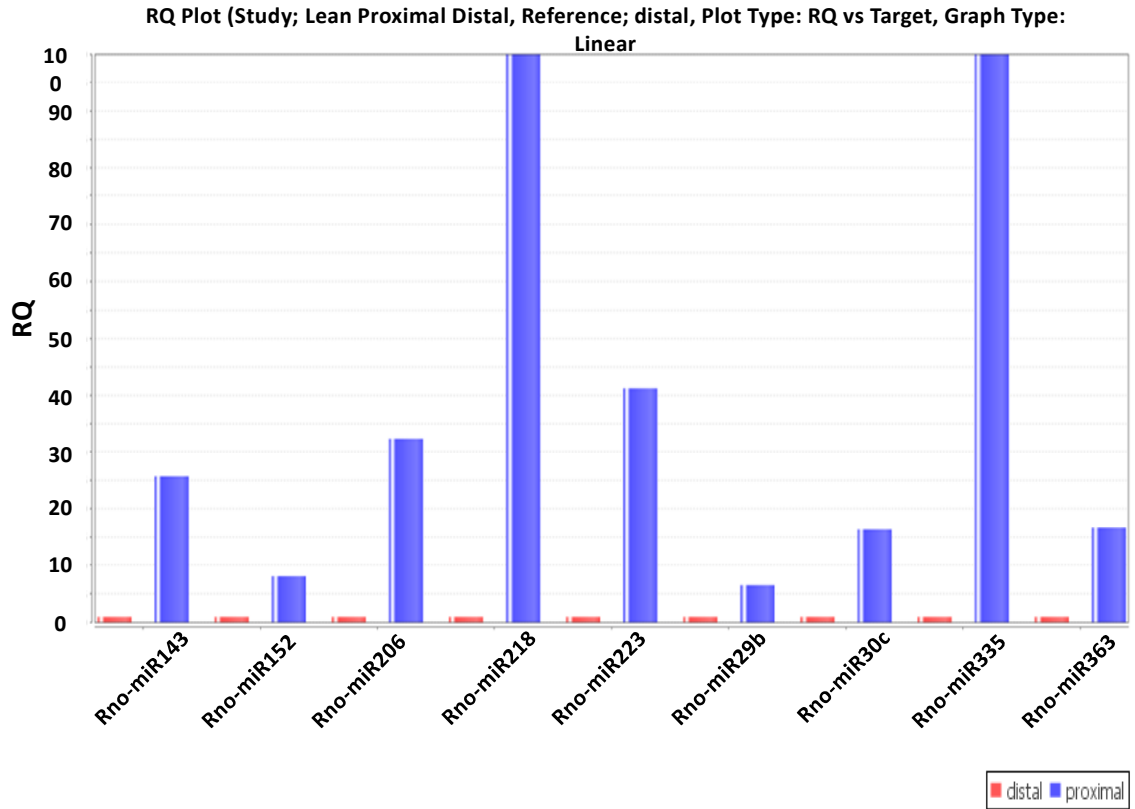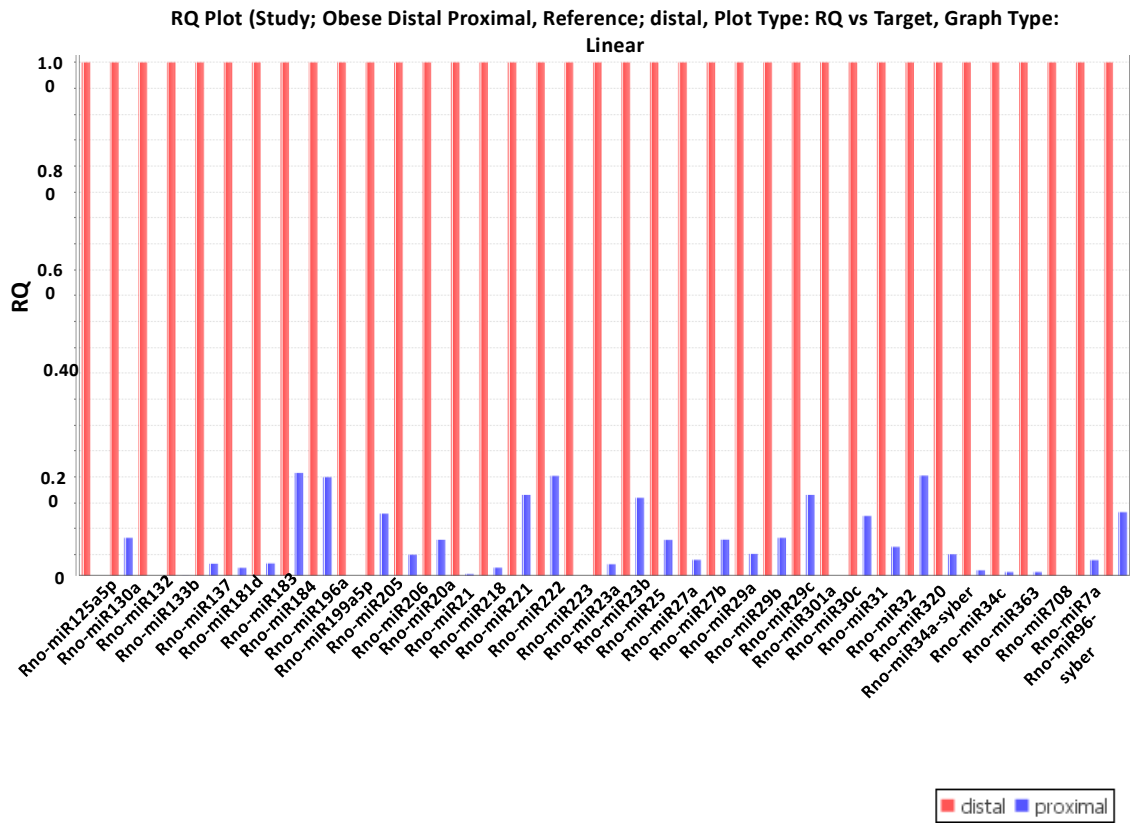
Figure 4.4 Differential expression of miRNAs in Zk-Ob proximal colonic mucosa versus Zk-Ob distal colonic mucosa. miRNA abundance in the samples was normalized using miRNA 4.5s_V1 as endogenous control. The abundance of each of the candidate miRNAs is represented as linear RQ or fold change of Zk-Ob proximal colonic mucosa compared to Zk-Ob distal colonic mucosa (reference sample). P-values are calculated using student t-test application of DataAssist software v3.0

Table 4.3- Significantly down-regulated miRNAs in Zk-Ob proximal colonic mucosa compared to Zk-Ob distal colonic mucosa

miRNA	Fold regulation	Direction	P value	Cellular function/target	Reference
miRNA-125a-5p	0.004	down-regulation	0.008	directly targets oncogene ERBB2 in gastric carcinoma cells, negatively regulates cell proliferation and metastasis, EGFR regulated tumor suppressor, regulates the inflammatory response, lipid uptake in monocytes/macrophages	(Chen, Huang et al. 2009; Nishida, Mimori et al. 2011; Guofu Wang 2009 October)
miRNA 132	0.009	down-regulation	0.023	targets tumor suppressor protein retinoblastoma (pRb) in pancreatic cancer cells, thus increasing cell proliferation, Potentiates Cholinergic Anti-Inflammatory Signaling by targeting acetylcholinesterase	(Shaked, Meerson et al. 2009; Park, Henry et al. 2011)
miRNA 133b	0.010	down-regulation	0.048	targets pro survival signals MCL-1 and BCL2L2 in lung cancer	(Melissa Crawford August 2009)
miRNA 181d	0.0247	down-regulation	0.049	targets tissue inhibitor of matrix metalloproteinase (TIM3) in hepatocarcinogensis, induced by β catenin signalling	(Wang, Hsu et al. 2010; Junfang Ji 2011 January 18)
miRNA 183	0.032	down-	0.013	functions as oncogene by targeting transcription factor EGR1 and promoting cell	i et al. 2010)

		9	regulation		migration	
miRNA 199a-5p	0.0032	down-regulation	0.008	targets hypoxia inducible factor-α in hepatocellular carcinoma, discoidin domain receptor-1 (DDR1) tyrosine kinase, involved in cell invasion-related signaling pathway is targeted by miRNA 199-5p	(Qingli Shen1 2010)
mi RNA 21	0.012	down-regulation	0.049	acts as oncogene by targeting tumor suppressor gene tropomyosin(TPM1), programmed cell death 1(PCD4) and maspin, both implicated in metastasis and invasion,directly targets PTEN tumor suppressor, EGFR regulated anti apoptotic factor	(Zhu, Wu et al. 0000; Hurt and Farrar 2007; Meng F 2007 Aug; Seike, Goto et al. 2009)
mi RNA 223	0.008	down-regulation	0.024	targets starthmin1(oncoprotein) in hepatocarcinoma cells, promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3, negatively correlated with oncogene c-myc, targets tumor suppressor FBW7, that targets oncoproteins like cyclin E, c-myc for degradation, induces GLUT4 expression	(Wong, Lung et al. 2008; Xu, Sengupta et al. 2010; Li, Zhang et al. 2011; Zhao WY 2011 Feb)
miRNA 301	0.005	down-regulation	0.012	oncogenic role in breast cancer,controls cell proliferation, clonogenicity, migration, invasion, tamoxifen resistance, tumor growth, and micro vessel density	(Shi, Gerster et al. 2011)
miRNA 320	0.051	down-regulation	0.038	oncogenic role, cell proliferation, detected in highly proliferative coloin crypts in CRC	(Schepeler, Reinert et al. 2008)
miRNA 34a	0.019	down-regulation	0.008	p53 dependent tumor suppressor, targets CDK4/6, E2F5, BcL2, SIRT1 (negative	(Munekazu Yamakuchi*† June

				regulator of apoptosis under stress conditions)	25, 2008)
miRNA 378	0.031	down-regulation	0.031	oncogenic, promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression	(Lee, Deng et al. 2007)
miRNA 708	0.004	down-regulation	0.009	tumor suppressor role, induces apoptosis and suppresses tumorigenicity in renal cancer cells	(Saini, Yamamura et al. 2011)
miRNA7a	0.039	down-regulation	0.035	inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2	(Dong, Meng et al. 2010)
miRNA 92a	0.032	down-regulation	0.037	biomarker for detection of leukemia, controls angiogenesis	(Tanaka, Oikawa et al. 2009),(Angelika Bonauer 2009)

Differential expression of miRNA in Zk-Ob compared to Zk-Ln rat colon

As part of specific aim 3, the purpose of this study was to find out if obesity alters the miRNA expression levels when compared to its lean control. Secondly to identify miRNAs those are affected by obesity independent of location on the colon.

In the first part of this study, the differential expression of miRNA in the proximal colon of Zk-Ob compared to proximal colon of Zk-Ln was studied. It was found that compared to Zk-Ln proximal colon, the Zk-Ob proximal colon showed down-regulation of most the miRNAs, though majorities were not statistically significant. The miRNAs that were significantly down-regulated in Zk-Ob proximal colon compared to Zk-Ln proximal colon include miRNA132, miRNA 125a-5p, miRNA 134, miRNA 138, miRNA 15b, miRNA 16, miRNA 191, miRNA 195, miRNA 199a-5p, miRNA 223, miRNA 27a, miRNA 29a, miRNA 301a, miRNA 30c, miRNA 378, miRNA 429, miRNA 708, miRNA 7a and miRNA 92a. (Figure 4.5) Whereas, miRNA 181-b (fold change =18.0851,p value=0.281) , miRNA 200c (fold change=3.5934, p value=0.8087) , miRNA 210 (fold change=2.233, p value=0.7937) and miRNA 222 (fold change=4.4617, Pvalue = 0.2822) were found to be up-regulated in Zk-Ob proximal colon compared to lean proximal but none were statistically significant. Interestingly, miRNA 223 which acts as tumor suppressor in hepatocellular carcinoma was again found to be down-regulated in Zk-Ob proximal colon compared to Zk-Ln proximal. (Table 4.4)

In the other part of the study, the differential expression of miRNA in distal colon of Zk-Ob versus distal colon of Zk -Ln rats was studied. Here, most of the miRNAs are up-regulated in Zk-Ob distal colon compared to Zk-Ln distal. The miRNAs that show significant up-regulation include- miRNA 142-5P, miRNA 146b, miRNA 150, miRNA 218, miRNA 223, miRNA 29b and miRNA 9. (Figure 4.6) All the other miRNAs that show up-regulation in Zk-Ln distal colon in comparison to Zk-Ln distal do not show statistical significance. Very few miRNA like miRNA let -7a, miRNA 103, miRNA 29a , show down-regulation but they are statistically insignificant. miRNA 223, shows significant up-regulation in distal region of Zk Ob rat compared to it lean counterpart.(Table 4.5)

Looking at the two study groups, it is clearly evident that obesity does alter miRNA expression levels when compared to control lean group. What is interesting to note here is that obesity also seems to have a region specific effect on the colon which reconfirms the fact that distal and proximal colon are distinct regions of the colon. miRNAs that are up-regulated in proximal region of obese colon compared to lean are down-regulated in distal region. For eg- miRNA 223 is up-regulated in Zk-Ob proximal colon compared to its lean counterpart. However it is significantly down-regulated in distal colon of Zk Ob rats compared to distal colon of Zk-Ln. There are few other miRNAs eg- miRNA134, miRNA 199-5p and miRNA 708 that showed a similar trend but were not statistically significant. (not included in result) This reiterates the fact that proximal and distal colon indeed have distinct miRNA expression profile and falls in place with the existing theory of colon having two biologically distinct parts called proximal(right) and distal(left) colon. Secondly, obesity plays a major role in regulating miRNA expression in region specific manner.
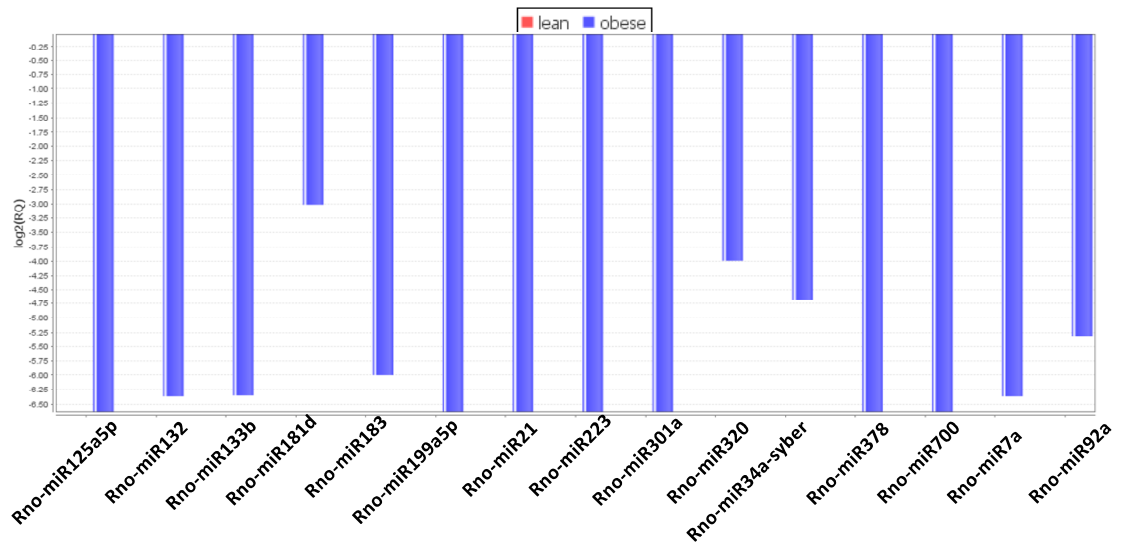
Figure 4.5 Differential expression of miRNAs in Zk-Ob proximal colonic mucosa versus Zk-Ln proximal colonic mucosa. miRNA abundance in the samples was normalized using miRNA 4.5s_V1 as endogenous control. The abundance of each of the candidate miRNAs is represented as linear RQ or fold change of Zk-Ob proximal colonic mucosa compared to Zk-Ln proximal colonic mucosa (reference sample). P-values are calculated using student t-test application of DataAssist software v3.0

Table 4.4-Significantly down-regulated miRNAs in Zk-Ob proximal colon compared to Zk-Ln proximal colon and their function/targets

mi RNA	Fold change	Direction	Pvalue	Cellular function/target	Reference
miRNA 132	0.043	down-regulation	0.0497	targets tumor suppressor protein rein retinoblastoma(pRb) in pancreatic cancer cells, thus increasing cell proliferation, potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase	(Shaked, Meerson et al. 2009; Park, Henry et al. 2011)
miRNA 125a-5p	0.008	down-regulation	0.024	directly targets oncogene ERBB2 in gastric carcinoma cells, negatively regulates cell proliferation and metastasis, EGFR regulated tumor suppressor, regulates inflammatory response and lipid uptake in macrophages/ monocytes	(Chen, Huang et al. 2009; Nishida, Mimori et al. 2011; Guofu Wang 2009 October)
miRNA 138	0.027	down-regulation	0.041	tumor suppressor, associated with over-expression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines.	(Mitomo, Maesawa et al. 2008)
miRNA 15b	0.043	down-regulation	0.042	oncogenic, correlated with tumor cell proliferation and apoptosis in malignant melanoma	(Satzger, Mattern et al. 2010)
miRNA 16	0.0137	down-	0.0433	tumor suppressor, inhibits the growth	(Takeshita,

		regulation		of metastatic prostate tumors via down-regulation of multiple cell-cycle genes	Patrawala et al. 2009)
miRNA 191	0.005	down-regulation	0.031	oncogene that promotes cell proliferation and inhibits apoptosis in hepatocarcinoma, targets MDM4 which down-regulates p53	(Elyakim, Sitbon et al. 2010; Wynendaele, Böhnke et al. 2010)
miRNA 195	0.003	down-regulation	0.017	tumor suppressor promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells	(Liu, Chen et al. 2010)
miRNA 199-5p	0.017	down-regulation	0.010	targets hypoxia inducible factor-α in hepatocellular carcinoma, discoidin domain receptor-1 (DDR1) tyrosine kinase, involved in cell invasion-related signaling pathway is targeted by miRNA 199-5p, targets HES which negatively regulates cell proliferation	(Livia Garzia 2009; Qingli Shen1 2010; Wang, Song et al. 2011)
miRNA 223	0.022	down-regulation	0.022	targets starthmin1(oncoprotein) in hepatocarcinoma cells,promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3,negatively correlated with oncogene c-myc, targets tumor suppressor FBW7, that targets oncoproteins like cyclin E, c-myc for degradation, induces GLUT4 expression	(Wong, Lung et al. 2008; Lu, Buchan et al. 2010; Xu, Sengupta et al. 2010; Li, Zhang et al. 2011; Zhao WY 2011 Feb)

miRNA 27a	0.043	down-regulation	0.022	regulates basal transcription by targeting the p44 subunit of general transcription factor IIH that display activities involved in transcription and DNA repair processes.	(Portal 2011)
miRNA 29a	0.139	down-regulation	0.035	tumor suppressor, regulates the expression of numerous proteins and reduces the invasiveness and proliferation of human carcinoma cell lines	(Muniyappa, Dowling et al. 2009)
miRNA 301a	0.027	down-regulation	0.011	oncogenic role in breast cancer,controls cell proliferation, clonogenicity, migration, invasion, tamoxifen resistance, tumor growth, and microvessel density	(Shi, Gerster et al. 2011)
miRNA 30c	0.044	down-regulation	0.010	independent predictor of clinical benefit of endocrine therapy in advanced estrogen receptor positive breast cancer	(Antony Rodriguez 2004)
miRNA 378	0.017	down-regulation	0.025	oncogenic, promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression	(Lee, Deng et al. 2007)
miRNA 429	0.055	down-regulation	0.012	modulates expression of c-myc, playes important role in pathogenesis of gastric carcinoma	(Sun, Wang et al.)

miRNA 708	0.011	down-regulation	0.002	tumor suppressor role, induces apoptosis and suppresses tumorigenicity in renal cancer cells	(Saini, Yamamura et al. 2011)
miRNA 7a	0.047	down-regulation	0.03	inhibits Proliferation of Human Prostate Cancer Cells in vitro and in vivo by targeting E2F2 and CCND2	(Dong, Meng et al. 2010)
miRNA 92a	0.088	down-regulation	0.04	MYCN-regulated miRNA-92 inhibits secretion of the tumor suppressor DICKKOPF-3 (DKK3) in neuroblastomas	(Haug, Henriksen et al. 2011)
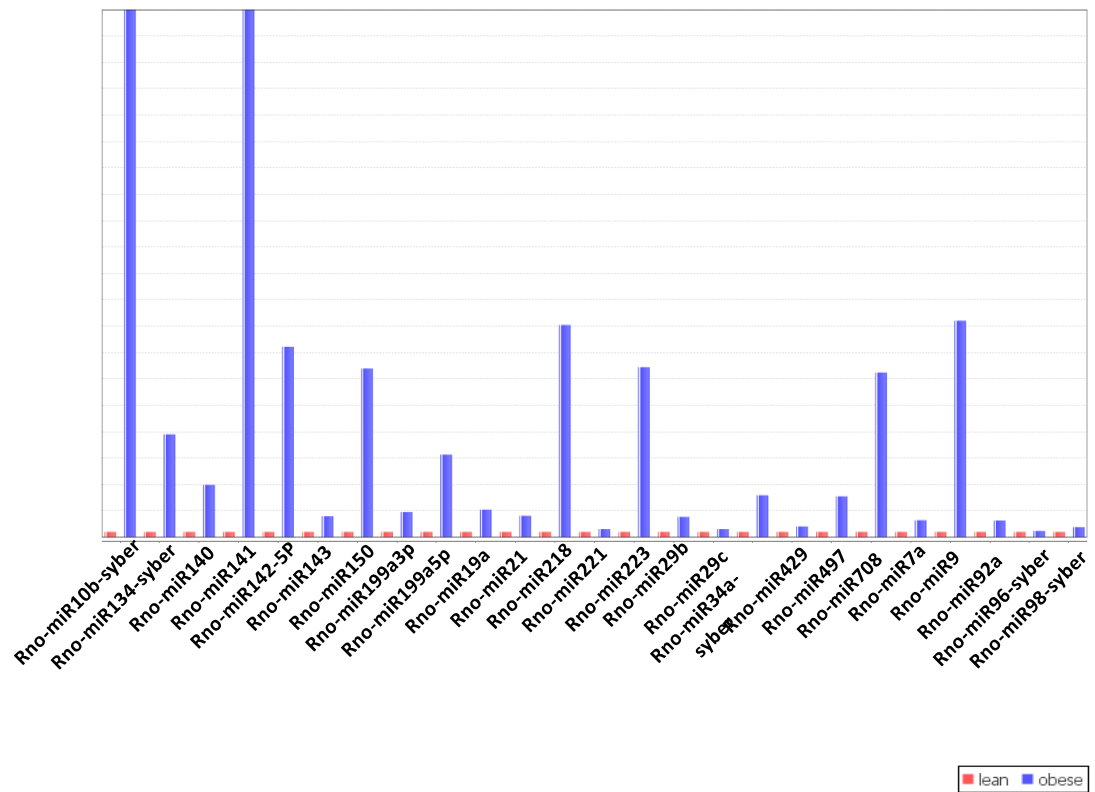
Figure 4.6 Differential expressions of miRNAs in Zk-Ob distal colonic mucosa versus Zk-Ln distal colonic mucosa. miRNA abundance in the samples was normalized using miRNA 4.5s_V1 as endogenous control.The abundance of each of the candidate miRNAs is represented as linear RQ or fold change of Zk-Ob distal colonic mucosa compared to Zk-Ln distal colonic mucosa (reference sample). P-values are calculated using student t-test application of DataAssist software v3.0

Table 4.5- Significantly up-regulated miRNAs in Zk-Ob distal colon compared to Zk-Ln distal colon

mi RNA	Fold change	Direction	Adjusted P value	Cellular function/target	Reference
miRNA 142-5p	23.12	up-regulation	0.005	regulates target genes in several oncogenic signal pathways, such as TP53, MAPK, Wnt and vascular endothelial growth factor.	(Zhang, Yan et al. 2011)
miRNA 146b	9.84	up-regulation	0.021	supresses NF-κB activity in breats cancer cells,expressed in response to rising inflammatory cytokine levels as part of negative feed back loop that restrains excessive senescence associated secretory phenotype.	(Bhaumik, Scott et al. 2008; Dipa Bhaumik1 2009)
miRNA 150	21.64	up-regulation	0.018	tumor suppressor in malignant lymphoma, increases the incidence of apoptosis and reduced cell proliferation, directly downregulated expression of DKC1 and AKT2, reduced levels of phosphorylated AKT$^{ser473/4}$ and increased levels of tumor suppressors such as Bim and p53.	(Watanabe, Tagawa et al. 2011)
miRNA 218	25.78	up-regulation	0.013	Targets EGFR that regulates NF-κB activity and is associated with apoptotic response, tumor suppressor activity in lung squamous cell carcinoma, targets mTOR	(Davidson, Larsen et al. 2010; Gao, Zhang et al. 2010; Uesugi, Kozaki et al. 2011)

miRNA 223	20.65	up-regulation 0.029	Targets starthmin1 (oncoprotein) in hepato carcinoma cells, promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3, negatively correlated with oncogene c-myc, targets tumor suppressor FBW7, that targets oncoproteins like cyclin E, c-MYC for degradation, induces GLUT4 expression	(Wong, Lung et al. 2008; Lu, Buchan et al. 2010; Xu, Sengupta et al. 2010; Li, Zhang et al. 2011; Zhao WY 2011 Feb)
miRNA 29b	2.45	up-regulation 0.029	Induces global DNA hypomethylation by directly targeting DNMT1	(Garzon, Liu et al. 2009)
miRNA 9	26.29	up-regulation 0.043	Regulates Neurogenesis in Mouse Telen cephalon by Targeting Multiple Transcription Factors E-cadherin (encoded by CDH1) is a direct target of miR-9.	(Mikihito Shibata1 2 March 2011),(Almeida, Reis et al. 2010)

Discussion

The blood plasma results indicate elevated levels of inflammatory cytokines like IL-1α, IL-6, chemokines like MCP and cellular adhesion molecules like ICAM and E-selectin, in Zk-Ob compared to Zk-Ln. Obesity is a patho-physiological condition that shows heightened inflammatory activity. Early feature of inflammation is the release of chemokines like MCP. These factors increase the expression of interstitial and vascular cellular adhesion molecules like ICAM and E-selectin that attracts monocytes and immune cells. Chemokines like MCP also induce the proliferation and pro-inflammatory gene activation producing cytokines like IL-1α, IL-6, IL-18 etc. Other factors that stimulate gene expression of pro-inflammatory cytokines in obese state are RONS, oxidized lipids, free fatty acids.

Optimising method of miRNA isolation, detection and quantification using Zucker rat tissue was the first aim which was achieved using Zk-Ob and Zk- Ln liver tissue. The study showed that several miRNAs were differentially expressed in Zk-Ob liver compared to Zk-Ln (control) liver. Out of these, 20 miRNAs were significantly up-regulated, prominent among those were miRNA 137, miRNA 183, miRNA 199-3p, miRNA 199-5p, miRNA 200c, miRNA 218, miRNA 223 and miRNA 429. miRNA 137 is known to be highly expressed in chronically inflammed model of colon cancer. (Necela, Carr et al. 2011) Target prediction and pathway analysis suggests that this miRNAs regulates signaling pathways related to MAPK, PI3K, WNT, and TGF-β, all of which are known to be involved in transformation. In hepato-carcinoma cells (HCC), miRNA 183, inhibits apoptosis by targeting programmed cell death 4 protein (PDCD4) .(Jipeng Li 2010) miRNA 199-3p target mTOR and c-Met in HCC.(Fornari, Milazzo et al. 2010) miRNA 199-5p targets discoidin domain receptor1 in HCC.(Qingli Shen1 2010) Similarly miRNA 200c, miRNA 218, miRNA 223 and miRNA 429 have roles either as oncogenes or tumor suppressor or pathways related to inflammation, apoptosis, cell growth, cell cycle etc.(Table 4.6) It seems that obesity has a profound effect on the expression levels of these miRNAs. The

underlying factors of obesity that might be regulating the expression of these miRNAs remains to be seen.

The primary objective of second and third aim was to use miRNA as a molecular tool to understand the regional differences in the colon under control and obese conditions. The findings of the present study demonstrated that both the anatomical regions of the colonic tissue as well as the physiological state of the animal influenced the miRNA expression pattern. However, obesity seems to be the dominating factor that influences the expression of miRNA in the two regions of the colon. Indeed, a selected group of miRNAs showed significant differential expression in proximal versus distal colon in both control (lean) as well as obese group. However, we were unable to identify common miRNAs that showed similar expression pattern in both the groups. Hence, we can conclude that though proximal and distal colonic regions show differential expression of miRNAs in the individual groups which coincides with the concept of these two regions of colon being biologically separate organs, we cannot say the same when comparing one genotype with another. On the other hand, obesity was found to have a profound effect on the expression pattern of miRNAs in proximal and distal colon. The miRNA that stood out in the study, as being regulated differentially in proximal and distal colon by obesity factor, is miRNA 223. This miRNA was significantly up-regulated in the proximal colon compared to distal in the control lean group. However, in obese group, the same miRNA was down-regulated significantly in the proximal colon compared to distal. Obesity seems to be influencing the expression of miRNA 223 in such a way that it is highly expressed in the distal colonic region and has a very low expression in proximal colon. There were several other miRNAs that seemed to be influenced by obesity such that expression is drastically altered in the two regions of the colon. However, they were not significant and hence not included. It would be interesting to find out the factors associated with obesity that could be playing a role here. Pro-inflammatory cytokines like IL-6, IL-1α, chemokines like MCP and adhesion molecules like ICAM, E-selectin which were found elevated in obese group when compared to lean (control) might be involved in regulation of miRNA expression in site specific manner.

There were 16 miRNAs shortlisted from all the analysis that was found to be significantly up or down-regulated. (Table 4.6) Target prediction of these miRNAs, using a combination of bioinformatics tool like miRanda, PicTar and Target Scan, indicated their role in inflammation, cell proliferation, cell growth, apoptosis, DNA damage and repair etc . These findings suggest that the molecular make up of the tissue is profoundly affected by genotype and tissue biology.

miRNA 218 was found to be influenced by obesity so that its expression is up-regulated significantly in the distal colon under obese conditions. It has been experimentally proven that miRNA 218 has tumor suppressor activity in squamous cell carcinoma, where it may directly or indirectly target MYC and SRC oncogenes. (Davidson, Larsen et al. 2010) Interestingly, one of the several targets of miRNA 218, as found by miRanda bioinformatics tool was CDC 27 (cell division cycle protein 27 homologue) . This protein is a part of the anaphase promoting complex subunit3, a cell cycle-regulated E3 ubiquitin ligase that controls progression through mitosis and the G1 phase of the cell cycle. The APC/C complex acts by mediating ubiquitination and subsequent degradation of target proteins. miRNA 218 binds to the 3'UTR of this cell cycle protein, hence has an oncogenic role.

miRNA 223 was down-regulated in the proximal region of the colon under obese conditions. In liver the expression was 46 fold high in the obese group compared to control lean liver. miRNA 223 is known to have tumorigenic functions in hepato-carcinoma. Overexpression of miRNA 223 in mouse embryonic fibroblasts cell lines lead to reduced expression of tumor suppressor FBW7, which targets oncogenes like cMYC and cyclinE for ubiquitination and degradation.(Xu, Sengupta et al. 2010) It has been found to be negatively correlated with cMYC oncogene in hepato-carcinoma (Zhao WY 2011 Feb). Interestingly, p53 was also found to be one of the targets of miRNA 223, though not experimentally validated. (Source: Uniprot/ SWISSPROT ; Acc : P10361). Other predicted target genes identified by more than one algorithm were myotrophin (Mtpn), Ras related protein rab-10 (Rab10), nuclear factor IA (NfIa), myocyte enhancer factor 2c (Mef2c), and insulin-like growth factor 1receptor (Igf1r). Glut4 could be a direct targetof miR-223 across species, possible

through the interaction of multiple target sites. Over-expression of miR-223 in vitro inhibited insulin-stimulated Akt and GSK3b phosphorylation in cardiomyocytes. miRNA 223 is also known to induce the expression of GLUT4 (Glucose transporter4) and increases the basal glucose uptake in cardiomycetes.(Lu, Buchan et al. 2010)

miRNA 199-5p showed down-regulation in the proximal colon compared to distal colon of both lean and obese group. However, liver showed significant up-regulation (16 fold) in obese rat compared to control lean rat. This might be because of independent mechanisms of miRNA regulation in liver and colon. In hepato-carcinoma (HCC) tumors, the under expression of miRNA 199-5p, which happens via hypoxia inducible factor (Hif1α) , is inversely correlated with survival and directly with malignancy of HCC.

 miRNA 125-5p showed similar trend. i.e down-regulation in proximal region of the colon under obese condition and up-regulation in obese liver. miRNA 125-5p is known to modulate inflammatory response and lipid uptake in macrophages and monocytes. (Chen, Huang et al. 2009). miRNA 125-5p directly targets oxysterol binding protein related protein 9(ORBP-9) , which is a cholesterol transfer protein that regulates golgi-structure and function.(Ridgway 2009 March 1) ORBP9 maintains the integrity of the early secretory pathway by mediating transport of sterols between the ER and trans-Golgi/TGN. Down-regulation of ORBP-9 by miRNA 125-5p, causes Golgi fragmentation, inhibition of vesicular somatitus virus glycoprotein transport from the ER and accumulation of cholesterol in endosomes /lysosomes.(Ridgway 2009 March 1)

miRNA 132 also shows significant down-regulation in proximal colon compared to distal colon under obese condition. It is highly expressed in liver. In the brain, miRNA 132 directly targets Acetylcholinesterase (AChE) attenuating inflammation by reducing AChE amounts.(Shaked, Meerson et al. 2009). In pancreatic adenocarcinoma, miRNA 132 acts as oncogene by targeting tumor suppressor Retinoblastoma protein (pRb).

miRNA 183 showed significant differential regulation only in the obese group. In Zk-Ob rats, it was down-regulated 23 fold times in proximal colon compared to distal colon. In the liver tissue, the expression was 7 fold more in the obese group compared to the lean group. miRNA 183 is overexpressed in several cancer types eg-colon cancer, synovial sarcoma . It targets the tumor suppressor EGR1 and PTEN.(Sarver, Li et al. 2010).

miRNA 191 shows significant down-regulation in proximal colon compared to distal colon in the obese group. It is up-regulated in the obese liver (185 fold) compared to lean liver .miRNA 191 targets MDM4 which down-regulates p53.(Wynendaele, Böhnke et al. 2010) In hepato-carcinoma, miRNA 191 has an oncogenic role where they increase cell proliferation and inhibits apoptosis. (Elyakim, Sitbon et al. 2010) miRNA 191 does show a region specific difference in expression and is induced under obese conditions which might the factor regulation its differential expression.

miRNA 301a showed very high expression in distal colon compared to proximal colon under obese conditions. miRNA 378 enhances cell survival, tumor growth and angiogenesis by targeting two tumor suppressors Sufu and Fus-1 (Lee, Deng et al. 2007) in a normal cell. It has been reported that over-expression of miRNA 378 during adipogenesis increases triacylgylcerol accumulation due to increased de novo lipogenesis. Interestingly, miR-378/378∗ is located within the peroxisome proliferator-activated receptor gamma(PPAR-γ) coactivator-1 beta gene and is transcribed during adipocyte differentiation .

 Other miRNAs like miRNA 708, 92a, 15b and 21 expressions were also significantly altered in the two different regions of the colon under obese conditions. Some of them were exclusively expressed in colon and not liver. miRNA 708 induces apoptosis . In renal cancer it targets the regulators of E-cadherin such as ZEB2 and BMI1, suppresses epithelial to mesenchymal transition(EMT) and hence acts as a tumor suppressor.(Saini, Yamamura et al. 2011). In liver miRNA 92a did not show any significant differential regulation in obese versus lean. Among the miRNAs that were expressed at lower levels in proximal colon under obese conditions, miRNA 21 stands out as an important miRNA . This miRNA has been

extensively studied as a major player in inflammation and inflammation related disorders including cancer. Hence its high expression in liver was well expected. However its downregulation in proximal colon compared to distal shows how distal and proximal colon stand as distinct organs with independent mechanisms of gene regulation. Pro-inflammatory cytokines like IL-6, activate STAT-3 transcription factor, which directly binds to the promoter of miRNA21 and activates it. miRNA 21 targets tumor suppressors like PTEN, leading to increased NF-κB activity, which promotes cell survival and cell proliferation. (Iliopoulos, Hirsch et al. 2009) PTEN is a tumor suppressor gene encoding a phosphatase that regulates cell cycle, Akt and p53 activity. Tropomyosin 1 (TPM1) and programmed cell death 4 (PDCD4) are other tumor suppressors targeted by miRNA21. miRNA 21 expression has been correlated as a part of inflammatory response in many cancers including colon cancer. IL-6 is thought to drive the expression of miRNA 21 in a STAT dependent manner. (Lu, Munitz et al. 2009) There is also a predicted binding site for miR-21 in the 3′ untranslated region(3'UTR) of IL-12a as indicated by Targetscan and miRanda. Aberrant over-expression of miRNA 21 has been attributed to activated EGFR signaling. (Seike, Goto et al. 2009) miRNA 21 has strong connection with inflammation and hence its up-regulation in obese conditions both in liver as well as colon, is expected. However, the role of obesity associated inflammation in regulating the differential expression of miRNA 21 in colon is not clear at this point. In addition to the miRNAs discussed in the preceeding section a number of miRNAs such as 29, 29b, 30c and 34a were also found altered in different colonic regions under obese conditions.

Table 4.6- Summary of miRNAs showing significant differential regulation in Zk-Ob versus Zk-Ln and proximal versus distal colonic mucosa

micro RNA	Physiological state	Colon/liver	Reference sample	Fold change/direction	Biological function
miRNA 218	Lean	proximal colon	distal colon	130 X up-regulation	tumor suppressor activity in squamous cell carcinoma, targets EGFR, targets mTOR
	obese	distal colon	lean distal	25 X up-regulation	
	obese	liver	lean liver	8X up-regulation	
miRNA 223	Lean	proximal colon	distal colon	41 X up-regulation	acts as tumor suppressor by targeting stathmin1 oncoprotein, targets tumor suppressor EPB4IL3, negatively correlated with oncogene c-myc, targets tumor suppressor FBW7, which targets oncogenes like cMYC and cyclin E for ubiquitination and degradation, induces GLUT4 (glucose transporter4) expression
	obese	proximal colon	distal colon	42 X down-regulation	
	obese	proximal colon	lean proximal	46 X down-regulation	
	obese	distal colon	lean distal	34 X up-regulation	
	obese	liver	lean liver	8 X up-regulation	

miRNA 199-5p	obese	proximal colon	distal colon	312 X fold down-regulation	tumor suppressor in hepato-cellular carcinoma, oncogenic in medullo-blastoma tumor cells
	obese	proximal colon	lean proximal	57 X down-regulation	
	obese	liver	lean liver	16 X up-regulation	
miRNA 125a-5p	obese	proximal colon	distal colon	208 X down-regulation	targets oncogene ERBB2 in gastric carcinoma cells, regulates inflammatory response and lipid uptake in monocytes/macrophages.
	obese	proximal colon	lean proximal	116 X downregulation	
	Obese	liver	lean liver	6 X upregulation	
miRNA 132	Obese	proximal colon	distal colon	102 X down-regulation	Oncogene, targets pRb tumor suppressor in pancreatic cancer, Potentiates Cholinergic Anti-inflammatory Signaling by targeting acetylcholinesterase
	Obese	proximal colon	lean proximal	23 X down-regulation	
	obese	liver	lean liver	7 X up-regulation	
miRNA 183	obese	proximal colon	distal colon	30 X down-regulation	oncogene, promotes cell migration
	obese	liver	lean liver	12 X up-regulation	
mi RNA 191	obese	proximal colon	lean proximal	185 X down-regulation	oncogene, promotes cell proliferation and inhibits apoptosis, tumor suppressor targets MDM4
	obese	liver	lean liver	4 X up-regulation	

mi RNA 301a	obese	proximal colon	distal colon	196 X down-regulation	oncogenic role in breast cancer
	obese	proximal colon	lean proximal colon	37 X downregulation	
mi RNA 378	obese	proximal colon	distal colon	25 X down-regulation	oncogenic , promotes cell survival and tumor growth
	obese	proximal colon	lean proximal	40 X down-regulation	
mi RNA 708	obese	proximal colon	distal colon	104 X down-regulation	tumor suppressor, induces apoptosis
	obese	proximal	lean proximal	526 X down-regulated	
miRNA 92a	obese	proximal colon	distal colon	27 X down-regulation	tumor suppressor, controls angiogenesis in leukemia
	obese	proximal colon	lean proximal	25 X down-regulated	
miRNA 15b	obese	proximal colon	lean proximal	24 X down-regulated	oncogenic role in malignant melanoma
	obese	Liver	lean liver	7 X up-regulation	
miRNA 21	obese	proximal colon	distal colon	20 X down-regulation	oncogenic role, targets PTEN
	obese	liver	lean liver	5 X up-regulation	
miRNA 29b	obese	distal colon	lean distal	2 X up-regulation	both oncogenic and tumor suppressor rolerole, targets DNMT
	lean	proximal	distal colon	10 X up-regulation	

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colon

miRNA 30c	lean	proximal colon	distal colon	16 X up-regulation	tumor suppressor
	obese	proximal colon	lean proximal	99 X down-regulation	
miRNA 34a	obese	proximal colon	distal colon	123 X down-regulation	p53 dependent tumor suppressor, targets CDK4/6, E2F5, BcL2, SIRT1 (negative regulator of apoptosis under stress conditions)
	obese	liver	lean liver	3 X up-regulation	

CHAPTER 5

CONCLUSION AND RECOMMENDATION

When one looks at an overall trend it is apparent that miRNAs are expressed in a tissue specific manner. Differential miRNA expression in distal and proximal colon of both lean and obese group strengthens the molecular evidences supporting the idea of existence of proximal and distal colon as separate organs. However, obesity seems to be significantly altering the pattern of miRNA expression in these two regions when compared with control lean group. Several miRNAs that showed up-regulation in distal colon compared to proximal colon in control lean group, show completely reverse trend in the obese group. The results obtained demonstrated that obesity seem to profoundly affect the expression levels of several miRNAs. Moreover, the findings also demonstrated that distinct regional differences exist in the colon with respect to expression levels of miRNA. The miRNAs that showed significant changes are oncogenic as well as tumor suppressors in cancerous cells and also have role in apoptosis, cell growth and differentiation. The findings of the present research supports the hypothesis that selected miRNAs could serve to distinguish molecular differences among tissue types and that colonic regions expressing different miRNAs expression pattern and levels further confirm that these sites are biologically different entities. These differences may in part be responsible for emergence and selection of preneoplastic lesions with different mutations and growth rates. However, physiological state such as obesity in this case, cannot be overlooked especially when it seems to be altering the miRNA expression pattern in the different regions of the colon, in such a significant manner.

Summary and Future research

The findings of the present research have provided a strong foundation to further explore the role of miRNAs in colon carcinogenesis. Whether some of these miRNAs are secreted in the blood to detect the presence of an early disease state remains to be seen. Role of selected miRNAs particularly the miRNAs involved in pathways like inflammation, oxidative stress, cholesterol biosynthesis, DNA damage, repair and apoptosis will be of interest. In this study, we have focused on the expression of miRNA in distal versus proximal colon in obese model. In our study we could not identify common miRNAs which could serve as biomarkers for distal and proximal colon, in both lean and obese group. However, we conclude that proximal and distal colon show distinct pattern of miRNA expression which depends on the host's physiology. Our results indicate that miRNA expression is indeed influenced by obesity and also show site specific variation in colon. In future, the connection between obesity and colonic region specific miRNA expression needs to be validated. Also, it would be interesting to see if the pattern of miRNA expression is the same in the different stages of colon cancer such as ACF, adenoma and adenocarcinoma. The study of underlying mechanisms connecting obesity with miRNA expression in proximal versus distal colonic tumor will give a deeper insight into the complexity of colon carcinogenesis.

APPENDIX A

A.1 Blood Plasma analysis of Zk-Ob and Zk-ln rats

Commercially available assay using Luminex liquid antibody bead array technology was used to measure a panel of cytokines, inflammatory biomarkers and adhesion molecule endpoints in plasma, e.g intercellular adhesion molecules(ICAM), E-selectin, monocyte chemoattractant protein (MCP), IL-1α, IL-6 according to manufacturer's instructions. (Lincoplex, Millipore Bioplex and BioRad). Values   are mean ± SE, n=4 samples per group.

A.2 Results
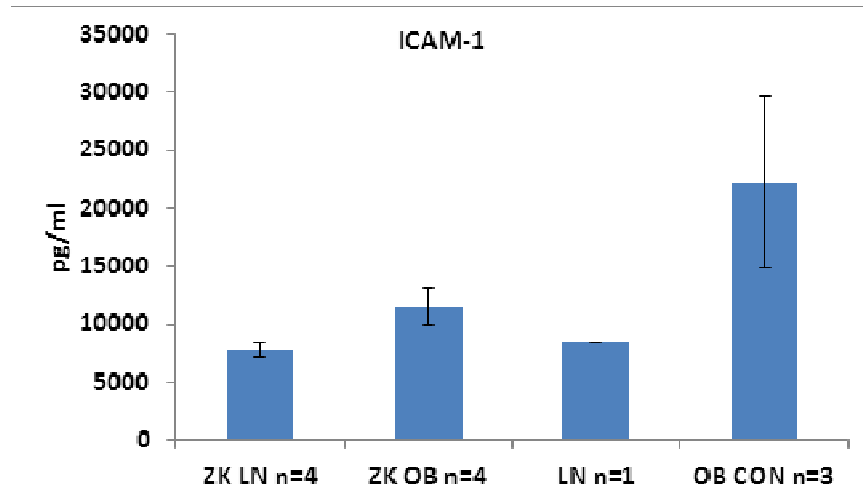
Figure 1- Plasma levels of ICAM-1 in Zk-Ob and Zk-Ln (control)
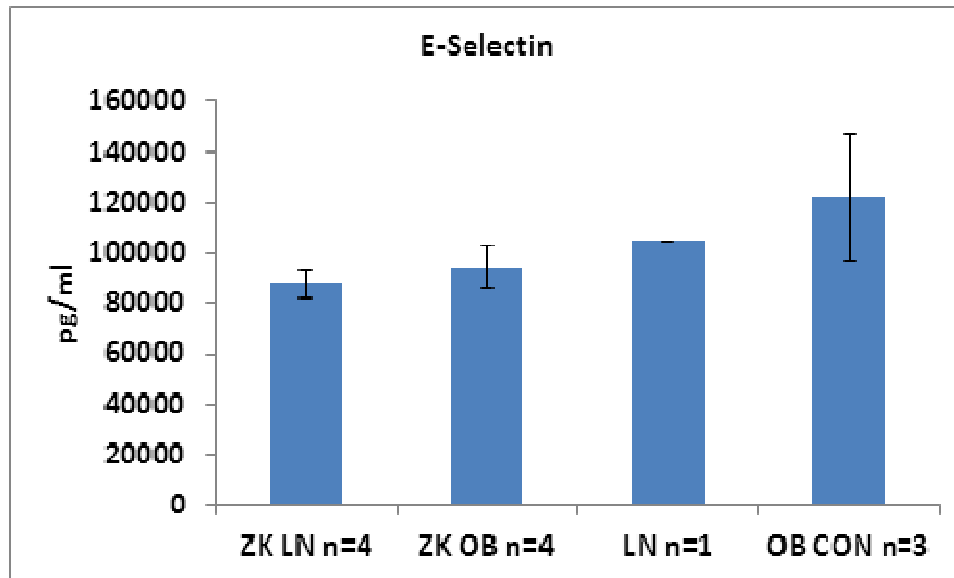
Figure 2- Plasma levels of E-selectin in Zk-Ob and Zk-Ln (control)
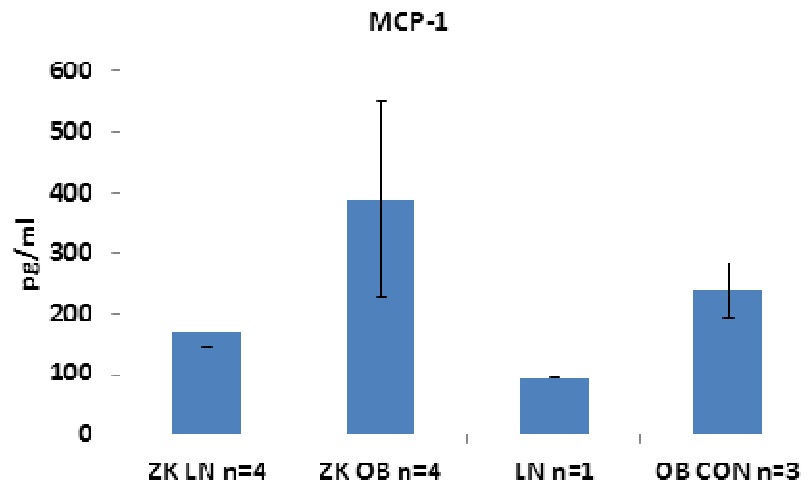
Figure 3- Plasma levels of MCP-1 in Zk-Ob and Zk-Ln(control)
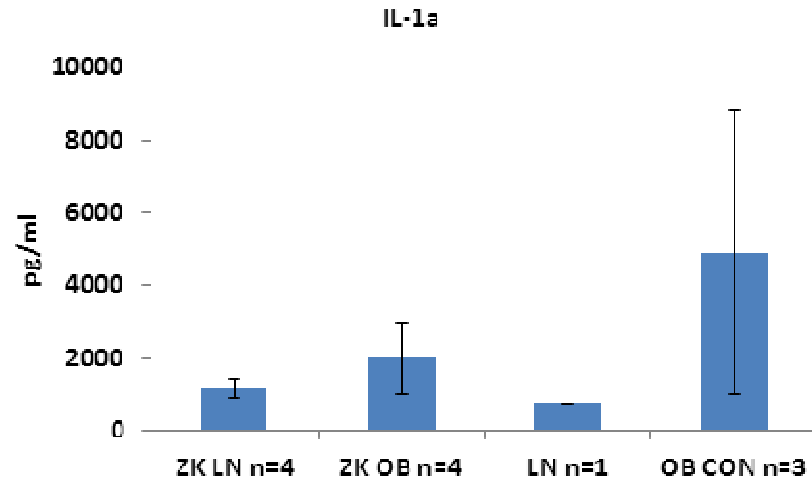
Figure 4- Plasma levels of IL-1α in Zk-Ob and Zk-Ln(control)
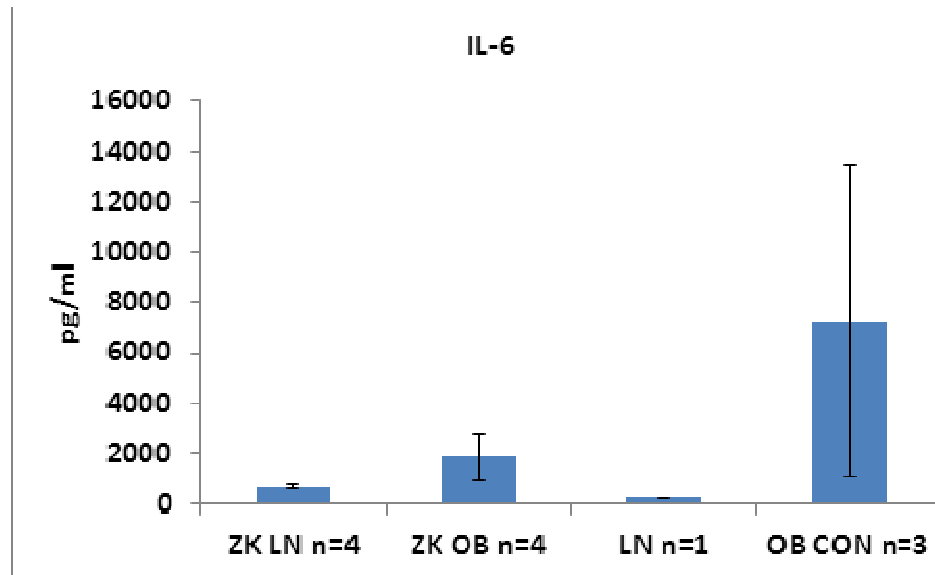
Figure 5 – Plasma levels of IL-6 in Zk-Ob and Zk-Ln (control)

APPENDIX B

B.1 Reaction Components for cDNA synthesis for miRNA quantification

Template miRNA	10-20 ng
miScript RT buffer(5X)*	2µl
miScriptReverse Transcriptase Mix	1µl
RNase free water	variable
Total reaction volume	10µl

B.2 Real time PCR for miRNA quantification

2x Quanti Tect SYBR Green PCR Master Mix	10µl
10x mi Script Universal Primer	2µl
10x mi Script Primer Assay	2µl
Template cDNA	3 ng/reaction
RNase free water	variable
Total volume	20 µl

B.3 Cycling conditions for real-time PCR for miRNA quantification

Step	Time	Temperature	Specifications
PCR initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	15 sec	94°C	
Annealing	30 sec	55°C	
Extension	34 sec	70°C	Fluorescence data collection.
Cycle number	40 cycles		

B.4 Components for cDNA synthesis for miRNA array

For each reaction, the following components were added in a sterile PCR tube
Small RNA – 50-400 ng of mi RNA enriched fraction
5x miRNA RT Buffer 2 - 2.0 µl
RT Enzyme Mix - 1.0 µl
Nucleotide Mix - 1.0 µl
RT primer + ERC mix – 1.0 µl
RNase-free H2O to a final volume of 10.0 µl

B.5 Components for real time PCR step in array experiment

2X RT2 SYBR Green qPCR Mastermix –1275 µL
Diluted first strand reaction-100 µL
RNase free H_2O- 1175 Ml
Total volume- 2550 µL

B.6 Cycling conditions for real time PCR for array experiment

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 seconds 40 seconds 32 seconds	95°C 60°C 72°C

B.7 RT2 miRNA array layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	m01	m02	m03	m04	m05	m06	m07	m08	m09	m10	m11	m12
B	m13	m14	m15	m16	m17	m18	m19	m20	m21	m22	m23	m24
C	m25	m26	m27	m28	m29	m30	m31	m32	m33	m34	m35	m36
D	m37	m38	m39	m40	m41	m42	m43	m44	m45	m46	m47	m48
E	m49	m50	m51	m52	m53	m54	m55	m56	m57	m58	m59	m60
F	m61	m62	m63	m64	m65	m66	m67	m68	m69	m70	m71	m72
G	m73	m74	m75	m76	m77	m78	m79	m80	m81	m82	m83	m84
H	m85	m86	m87	m88	HK1	HK2	HK3	HK4	RTC	RTC	PPC	PPC

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