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Isoform-Specific Regulation of miRNA by HNF4α

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by

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ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Genetics,Genomics & Bioinformatics
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Hepatocyte nuclear factor 4α (HNF4α/NR2A1) is a highly conserved member of the nuclear receptor superfamily of ligand-dependent transcription factors which plays a crucial role in the early development of the liver, intestine/colon, pancreas and kidney as well as maintaining metabolic and organismal homeostasis. Based on usage of alternative promoters (P1 and P2) and alternate splicing, it is estimated that nine different splice variants of HNF4α exist. The P1- and P2-driven variants differ by 16 amino acids in their N-terminal amino terminal (AB) domain that encodes an activation function (AF-1). It has been shown previously that expression of P1-driven HNF4α protein is decreased in different human cancers such as hepatocellular, gastric, renal and colorectal carcinomas, while P2-driven HNF4α is either unchanged or up regulated. HNF4α is known to play an important role during early embryonic colon development where it regulates genes related to epithelial function. In addition, HNF4α has been shown to function in protecting the epithelium during experimental colitis in young adult mice. The functional

consequences of the up regulation of the P2-driven HNF4α in human cancer are not known.

In Chapter 2 a potential link between levels of specific splice variants of HNF4α and miRNA 21, an oncogenic miRNA is investigated. miR-21 has been implicated in the development, growth, progression and metastasis of different types of cancers including colorectal, hepatocellular and pancreatic cancers. A series of *in vitro* assays using human colorectal cancer cells are performed, miRNA-21 levels is detected using stem loop RT-PCR. The results show that ectopic expression of P2-driven HNF4α preferentially regulates miRNA 21 in human embryonic kidney and colon cancer cell lines that do not express endogenous HNF4α. Knockdown of P2-driven HNF4α in a colon cancer cell line that expresses endogenous HNF4α, results in preferential decrease of miRNA 21 levels in a different human colorectal cancer cells (Caco-2). In order to elucidate the potential functional significance of miRNA-21 regulation by HNF4α, both P1- and P2-driven HNF4α were knocked down using siRNA. This results in downregulation of miRNA 21 as well as reduction in cell number. Ectopic expression of a miRNA 21 mimic in the HNF4α knockdown cells partially restored the cell count. The molecular mechanism by which HNF4α regulates miRNA 21 expression is explored in experiments that show that HNF4α binds the promoter region of miRNA 21 and that P2-driven HNF4α preferentially activates the human miRNA 21 promoter in a transient transfection system. Taken together, these observations lead us to propose that P2-driven HNF4α preferentially activates miRNA 21, an oncogenic miRNA, thus suggesting a functional significance of P2-driven HNF4α upregulation in colon cancer.

In Chapter 3, the potential functional role of isoform-specific HNF4α and miRNA 21, in a mouse model of colitis is investigated in which colitis is induced by the addition of DSS in the drinking water. Mortality, rectal bleeding, colon length, and spleen/body weight ratios were all differentially affected in both male and female transgenic mice (expresses only P2- driven HNF4α) compared to WT indicating an enhanced susceptibility of the transgenic mice to DSS. Immunoblotting and stem loop RT PCR performed on colonic tissue isolated from the DSS-treated mice showed that the expression of both HNF4α and miRNA 21 correlates with better recovery from DSS-induced colitis. These findings suggest that HNF4α-mediated regulation of miRNA 21 may play an important role in colitis and could be used as a marker for improved prognosis of colitis.

In Chapter 4, a potential link between specific splice variants of HNF4α and miRNA 194-2 are explored. miR-194-2 has been implicated in intestinal epithelial differentiation, cell invasion and inhibition of Epithelial-Mesenchymal-Transition (EMT). Ectopic expression of P2-driven human HNF4α resulted in a preferential upregulation of miRNA 194-2 in human embryonic kidney and colon cancer cell lines. miRNA 194-2 levels were also found to be elevated in the distal colon of transgenic mice that expresses only P2-driven HNF4α. These results suggest that miRNA-194-2, like miRNA-21, may be preferentially regulated by P2-driven HNF4α. Further investigation is required to address the potential functional significance of this regulation.

Finally, in Chapter 5 the implications of the results of Chapters 2, 3, and 4 are discussed in detail. The importance of the role of HNF4α in general regulating these

two microRNAs (miR-21 and miR 194-2) is considered as well as the relevance of the differential expression by the P1- and P2-driven isoforms. Finally, the role of these microRNAs and HNF4α splice variants are discussed in the broader context of the function of the colon in both the normal and diseased states.

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Chapter 1
Introduction

Hepatocyte nuclear factor 4α (HNF4α)

HNF4α was originally identified as a tissue-specific transcription factor that binds to the promoter of the liver-specific genes, transthyretin *(TTR)* and apolipoprotein CIII (*Apo CIII*) (Costa et al, 1989; Sladek et al, 1990). Based on sequence homology, HNF4α was designated as a member of the ligand dependent transcription factors which the sex steroid receptors (estrogen, androgen, progesterone) as well as metabolic receptors (tyroid hormone, retinoic acid and vitamin D) and stress receptors (glucocorticoid) as well as several other recetors that do not have identifiable ligands. HNF4α is a unique member of the nuclear receptor family as it binds DNA exclusively as a homodimer and is predominantly localized in the nucleus (Jiang et al, 1995). In addition, HNF4α is considered to be a constitutively active transcription factor as it has considerable transactivation activity without the addition of an exogenous ligand.

HNF4α contains six modular domains: (1) an amino-terminal A/B domain which contains a ligand-independent activation function 1 (AF1) (Green et al, 1998); (2) a central DNA binding domain (DBD domain) which contains two zinc finger motifs and binds specific DNA response elements represented by direct repeats of AGGTCA (half-site) separated by a one nucleotide spacer (DR1) (Jiang and Sladek, 1997); (3) a hinge region (domain D) (4) ligand binding domain (LBD domain) which contains the activation function 2 (AF2) region (helix 12) and a hydrophobic pocket that has been reported to bind a mixture of fatty acids (Dhe-Paganon et al, 2002; Wisely et al, 2002) fatty acyl-coenzyme A thioesters (Hertz et al, 1998) and linoleic acid (Yuan et al,

2009); a and 5) an unique C-terminal (F domain) which contains a repressor region (Hadzopoulou-Cladaras et al, 1997; Sladek et al, 1999).

Splice variants of HNF4α

HNF4α is derived from two different promoters, a proximal (P1) and a distal (P2) promoter, which are ~45.5 kb apart (Nakhei et al, 1998; Torres-Padilla et al, 2001) (Figure 1.1). As many as nine different splice variants have been proposed for HNF4α based on the alternate promoter usage and splicing (Sladek and Siedel, 2001). Isoforms HNF4α1, HNF4α2 and HNF4α3 are derived from the P1-promoter, whereas HNF4α7, HNF4α8 and HNF4α9 are derived from the P2-promoter. The P1- and the P2-driven isoforms differ in the A/B domain; the P2-driven HNF4α lacks the AF1 function. Alternative splicing creates an insertion of 10 amino acids in the middle of the F domain in HNF4α2 and HNF4α8, but not HNF4α1 and HNF4α7, which modulates interaction with co-activators (Sladek et al, 1999). The F domain of HNF4α3 and HNF4α9 is entirely different from the other isoforms.

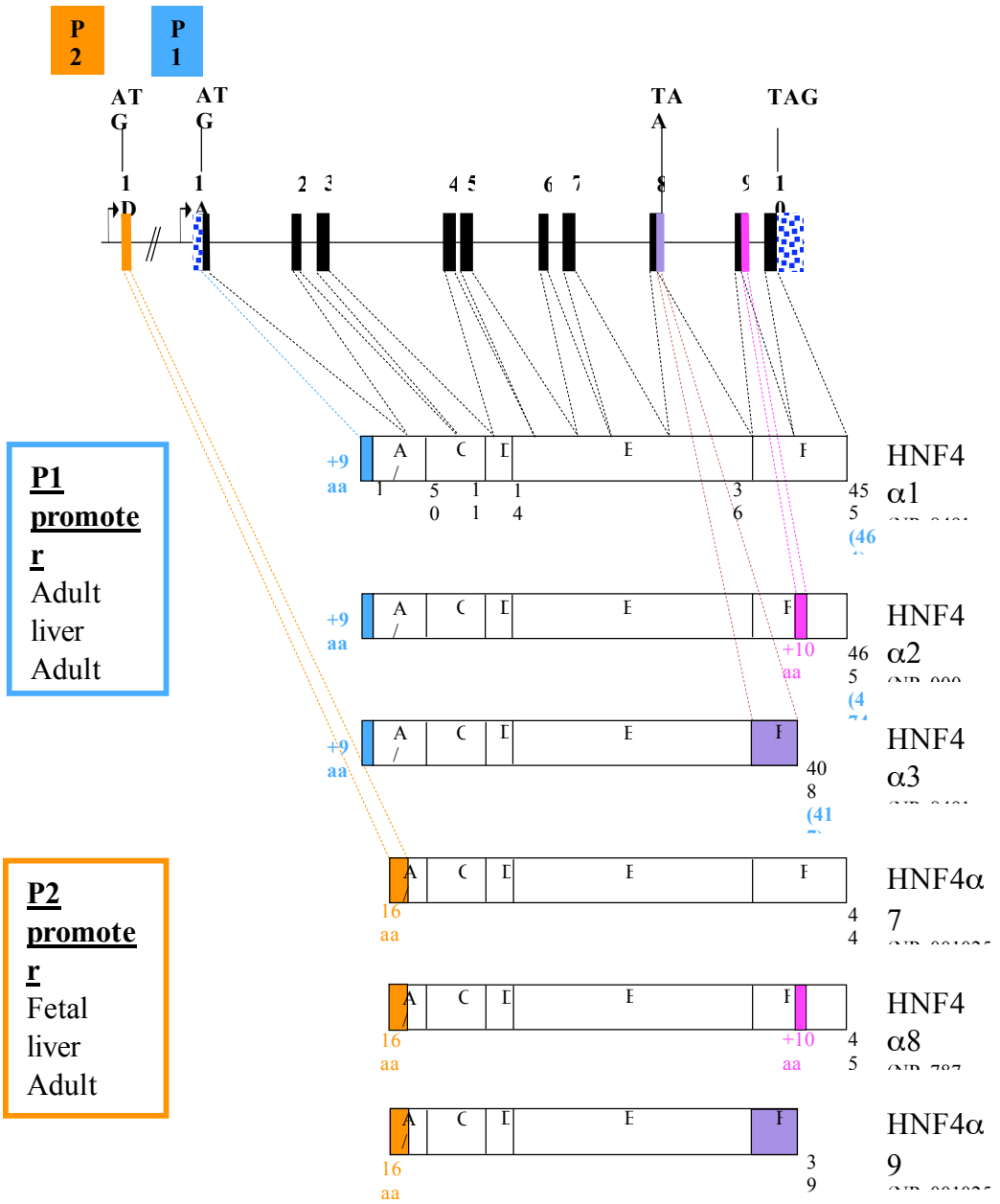
The spatial-temporal expression of HNF4α isoforms suggests a isoform-specific role during development and pathological conditions. The difference in the A/B and F domain in HNF4α splice variants contributes to their differential transcriptional activity (Huang et al, 2008). The tissue distribution and function of four isoforms, HNF4α1, HNF4α2, HNF4α7 and HNF4α8 are well characterized, whereas, the isoforms HNF4α3 and HNF4α9 are less well characterized. P1-driven isoforms (HNF4α1 and HNF4α2) are expressed in the adult liver, proximal tubular cells of the kidney, intestine,

colon and fetal pancreas (Harries et al, 2008; Jiang et al, 2003; Kritis et al, 1996; Tanaka et al, 2006). There are also reports of expression of P1-driven isoforms in thymus and ovary (Kanazawa et al, 2008). On the other hand, P2-driven isoforms (HNF4α7 and HNF4α8) are expressed in fetal liver, adult pancreas, stomach, intestine, colon, ovary, and mammary tissue (Harries et al, 2008; Ishikawa et al, 2008; Jiang et al, 2003; Kanazawa et al, 2008; Torres-Padilla et al, 2001). The expression of HNF4α3 has been detected in the liver cancer cell lines HepG2 and Hep3B (Huang et al, 2008; Kritis et al, 1996), whereas, HNF4α9 is expressed in mouse islets, rat insulinoma cells (Ins-1), human hepatocellular carcinoma cells (Hep3B) and gastric carcinoma cell line (Kato III) (Huang et al, 2008; Tanaka et al, 2006). P1- and P2-driven isoforms of HNF4α differ in their N-terminal A/B domain. In the case of the P1-driven isoforms (HNF4α1 & HNF4α7), the A/B domain contains a activation function 1 (AF-1) corresponding to amino acids 1 to 24, whereas the A/B domain of the P2-driven isoforms (HNF4α2 & HNF4α8) lack AF-1 (Hadzopoulou-Cladaras et al., 1997). It has been found that mutations in the common coding region of the P1- and P2-driven HNF4α are associated with maturity onset diabetes of the young 1 (MODY1) (Ellard and Colclough, 2006). There are few studies that also point to the functional differences between P1- and P2-driven HNF4α in regulating cell proliferation and apoptosis (Erdmann et al., 2007; Gupta et al., 2007; Niehof and Borlak, 2008). Taken together, these studies suggest that P1-driven HNF4α is anti-proliferative, while P2-driven HNF4α tends to be pro-proliferative. This is consistent with reports indicating that expression of P1-driven HNF4α isoforms are decreased in different human cancers such as hepatocellular, gastric, renal and colorectal carcinomas,

while the P2-driven isoforms are either unchanged or upregulated (Oshima et al., 2007; Tanaka et al., 2006). Nonetheless, the mechanism responsible for the differential dysregulation of P1- and P2-driven HNF4α isoforms is not known. In this study, we investigated a differential regulation of miRNA 21 and miRNA 194-2 by isoform-specific HNF4α (P1- and P2- driven HNF4α.

Figure 1.1 Human HNF4α gene structure
The human *HNF4A* gene spans ~74 kb on chromosome 20 and contains two promoters (P1 and P2) that drive the expression of at least six splice variants (isoforms). The numbers in black are those used for the MODY1 mutations and are based on the original amino acid sequence (Sladek et al, 1990). A conserved alternate translation start site in the P1 promoter 9 residues upstream of the original ATG is shown in blue. Letters refer to the functional domains of the nuclear receptors. Indicated are the major tissues in which the P1 and P2 promoters are expressed in approximate order of expression (Harries et al. 2008); fetal kidney, gut and stomach, as well as the visceral endoderm also express HNF4α although promoter usage has not been established (Duncan et al, 1994). Solid bars, coding regions; stippled bars, untranslated regions. Exons 1C and 1B were originally proposed to create an insertion in the A/B domain giving rise to isoforms HNF4α4/5/6 (Drewes et al, 1996; Furuta et al, 1997). However, recent reports using qPCR methods have not detected these transcripts (Harries et al. 2008, Huang et al. 2008), and it now appears that if those exons are expressed, they would result in a minor transcript encoding a truncated protein that would contain neither the canonical zinc finger nor the ligand binding domains of the nuclear receptors. The mouse *HNF4α* gene on chromosome 2 has a similar structure and expression pattern, with minor variations; the mouse HNF4α2 protein is ~96% percent identical to human.
Figure made by Dr. Frances M Sladek (http://cisreg.ca/tfe)

Fig 1.1


P 2
P 1
ATG
ATG
TAA
TAG
1D
1A
2 3
4 5
6 7
8
9
10
P1 promoter
Adult liver
Adult
P2 promoter
Fetal liver
Adult
+9 aa
+10 aa
16 aa
A
C
D
E
F
HNF4 α1
HNF4 α2
HNF4 α3
HNF4α 7
HNF4 α8
HNF4α 9

Function of HNF4α during normal development

Liver

The liver is a reddish brown organ with five to six lobes of unequal size and shape. A human liver normally weighs 1.44–1.66 kg (3.2–3.7 lb), and is a soft, pinkish-brown, triangular organ. It is both the largest internal organ (the skin being the largest organ overall) and the largest gland in the human body. It is located in the right upper quadrant of the abdominal cavity, resting just below the diaphragm. It is connected to two large blood vessels, one called the hepatic artery and one called the portal vein. The gall bladder sits under the liver, along with parts of the pancreas and intestines. The liver and these organs work together to digest, absorb, and process food. The liver's main job is to filter the blood coming from the digestive tract, before passing it to the rest of the body. The liver also detoxifies chemicals and metabolizes drugs and the liver also secretes bile acids that eventually goes back into the intestines. The liver also makes proteins important for blood clotting and other functions. There are two major types of cells populate the liver lobes: parenchymal and non-parenchymal cells. Parenchymal cells commonly referred to, as hepatocytes constitute 80% of the liver volume. Non-parenchymal cells constitute 30% of the total number of liver cells but only 6.5% of the liver volume. Sinusoidal endothelial cells, Kupffer cells and macrophage-like and hepatic stellate cells are some of the non-parenchymal cells in the liver. Hepatocytes make up 70-85% of the liver's cytoplasmic mass. These cells are involved in protein storage, transformation of carbohydrates, synthesis of cholesterol, bile salts and phospholipids.

Hepatocytes also have the ability to metabolize, detoxify, and inactivate exogenous compounds such as drugs, (drug metabolism), and insecticides, and endogenous compounds such as steroids.

HNF4α is the central regulator of liver development, differentiation and function as suggested by both *in vitro* and *in vivo* studies (Costa et al, 2003). HNF4α mRNA is expressed in the visceral endoderm of the yolk sac; in the embryonic tissue its expression is restricted to the liver and gut primordial, kidney and pancreas. HNF4 mRNA was first detected in embryonic tissues at day 8.5, in the liver diverticulum and the hindgut. At later times HNF4 transcripts were observed in the mesonephric tubules, pancreas, stomach, and intestine and, still later, in the metanephric tubules of the developing kidney. This expression pattern suggests that HNF4 has a role in the earliest stages of murine postimplantation development as well as in organogenesis (Duncan et al, 1994). Chen et al found that homozygous deletion of the *Hnf4a* gene in mouse resulted in embryonic lethality at day 9.5 and that the expression of HNF4α in the visceral endoderm is essential for embryonic ectoderm survival and normal gastrulation (Chen et al, 1994). Hayhurst et al found that deletion of the *Hnf4a* gene in liver lead to significant decrease in high density lipoprotein (HDL) cholesterol and triglycerides but a large increase in bile acids in the serum of mice lacking hepatic HNF4α suggesting its important role in maintaining lipid homeostatis (Hayhurst et al, 2001). Further examination of the mice lacking hepatic HNF4α revealed that HNF4α regulates ammonia detoxification by inducing the expression of ornithine transcarbamylase (*OTC*), an enzyme that is critical for ureagenesis (Inoue et al, 2002; Sladek and Siedel, 2001). In

addition, HNF4α modulates gluconeogenesis by inducing the expression of phospho*enol*pyruvate carboxykinase (PEPCK, *Pck1*) and glucose-6-phosphatase (*G6Pase*) (Hall et al, 1995; Rhee et al, 2003). Another group also established a role for HNF4α in morphological and functional differentiation of hepatocytes using the HNF4α liver-specific knock out mice. HNF4α regulates liver differentiation and epithelial transformation by inducing the expression of the genes involved in cell adhesion and junction, such as E-cadherin (*Cdh1*), carcinoembryonic antigen-related cell adhesion molecule 1 (*Ceacam1*), connexin 32 (*Gjb1*) and 26 (*Gjb2*) (Parviz et al, 2003; Garrison et al, 2006).

Pancreas

HNF4α alpha transcription is driven almost exclusively by the P2 promoter in pancreatic islets although recently couple of reports showed that P1-driven HNF4α expression in fetal or rodent pancreas. The role of HNF4α in pancreas was established mainly in three independent studies in which HNF4α was over expressed as a dominant negative protein or knocked down. In the first study, over expression of a dominant negative form of HNF4α in insulinoma cells showed that several genes involved in glucose metabolism as well as *HNF1A* were differentially expressed, suggesting that HNF4α regulates β cell glucose metabolism through the regulation of *HNF1A* and several glycolytic and mitochondrial genes (Erdmann S et al, 2007). In the second study, conditional knockout of mouse HNF4α in pancreatic β cells resulted in impaired glucose tolerance. This study also suggested that the effect of HNF4α on insulin secretion is mediated through the ATP-dependent potassium channel pathway, mainly by increasing the expression of

kir6.2 and *Sur1* mRNA, but is independent of HNF1α (Gupta et al, 2005). In a third study, Miura et al. also used β-cell specific HNF4α knock out mice to show that HNF4α is required for the glucose mediated stimulation of insulin secretion (Miura et al, 2006). The authors also show that HNF4α knockout leads a to defect in K_{ATP} channel activity but in contrast to the second study they found that the effects on channel are not due to changes in the expression of mouse *kir6.2* and *SUR1* mRNA.

Type 2 diabetes is characterized by the failure of the pancreatic β cells to expand in response to increased metabolic demand. Kaestner and collegues showed that knock out of HNF4α (P2-driven) in pancreatic β cells abolished the ability of β cells to undergo expansion in response to increased metabolic demand during pregnancy (Gupta et al, 2007). This result suggests that HNF-4α is essential for the physiological expansion of adult β-cell mass in response to increased metabolic demand. The importance of HNF4α in β-cell proliferation is attributed to the increase in the expression of the target gene suppressor of tumorigenicity 5 (*St5*), which regulates the ERK signaling pathway.

Genome wide analysis indicates that HNF4α is bound to the promoter of 11% genes in the pancreatic islets, suggesting an important role for HNF4α in islet function (Odom et al, 2004). Recently it was also found that through adenoviral-mediated overexpression of an isoform of HNF4 (HNF4α8) initiates cell cycle entry, but is not sufficient to promote β-cell expansion in human islets (Rieck et al, 2012). HNF4α alpha transcription is driven almost exclusively by the P2 promoter in pancreatic islets, although couple of reports showe that P1-driven HNF4α is expressed in fetal pancreas (Gupta et al, 2007; Harries et al, 2008), though the importance of temporal regulation of

P1- and P2-driven isoforms in regulating pancreatic function needs to be investigated.

Intestine and Colon

The colon is the last part of the digestive system in most vertebrates. It extracts water and salt from solid wastes before they are eliminated from the body and is the site in which flora-aided (largely bacterial) fermentation of unabsorbed material occurs. Unlike the small intestine, the colon does not play a major role in absorption of foods and nutrients. However, the colon does absorb water, sodium and some fat soluble vitamins.

During mouse development, HNF4α is expressed in the gut primordia at embryonic day 8.5 (e8.5) suggesting a role for HNF4α in the formation of the gut (Duncan et al, 1994). To investigate the role of HNF4α later on in the colon development, mouse embryos lacking HNF4α in the colon were generated using Cre-loxP technology. HNF4α-null colons (e18) were characterized by the loss of crypt formation, thinning of muscle layers, decrease in the number of epithelial cells and defects in goblet cell maturation. Analysis of gene expression profiles showed that a set of several genes involved in transport, metabolism and cell adhesion were decreased in the HNF4α-null colon compared to the control mice (Garrison et al, 2006).

To investigate the role of HNF4α in the adult gastrointestinal tract, an intestinal epithelial cell-specific HNF4α-null mouse line was produced using Cre transgenic mice in which the Cre recombinase gene is expressed in the large and small intestine under the control of the mouse villin promoter (Ahn et al, 2008). Electron microscopy and biochemical analysis revealed a decrease in the number of mucosal

granules and secretion of mucous from goblet cells in the HNF4α-null colon mice compared to the control. In addition, the intestine-specific HNF4α-null mouse showed a significantly increased risk to inflammatory bowel disease (IBD) induced by dextran sulfate sodium (DSS) compared to the control mice. This could be attributed to one or more of the following: (1) increased expression of *IL-1β, TNFα, IFNγ, and CCR2* mRNA levels (chemokines responsible for the inflammatory response) in the HNF4α-null mouse in response to DSS treatment; (2) altered expression of aquaporins, mucins and mephrins; and for (3) increased permeability following inflammation.

While these studies clearly show a central role for HNF4α in the development and function of the intestine and colon, none of them differentiated the various isoforms of HNF4α. Infact Intestine is the only adult tissue that expresses both P1-driven HNF4α and P2-driven HNF4α are expressed in adult intestine (Tanaka et al, 2006) All the knockdown studies used strategies that eliminated the expression of both P1- and P2 driven HNF4α by deletion therefore essentially nothing is known about the functional differences between the various HNF4α isoforms.

Function of HNF4α during disease pathogenesis

Type 2 Diabetes

Diabetes is a metabolic disorder that affects more than 347 million people and accounts for 5% of global death (http://www.who.int/diabetes/en/; World Health Organization (WHO)). The WHO projects that the number of people with diabetes will more than doubled by 2030, indicating that there is an urgent need to address the global heath issue

of diabetes by taking steps to prevent and treat this disease. Diabetes can be classified into two major types: Type 1 and Type 2 diabetes. The Type 1 diabetes (T1D) is caused by insulin deficiency due to autoimmune destruction of pancreatic β cells or mutations in the insulin or insulin receptor genes. In contrast, type 2 diabetes (T2D) is characterized by the inability of muscle, fat and liver to respond to insulin (insulin resistance) and the inability of the β cells to respond normally to glucose by appropriately increasing insulin secretion (Doria et al, 2008; Kahn, 1994). Type 2 diabetes accounts for more than 90% of diabetic cases both in the United States and worldwide (Skyler and Oddo, 2002) and it typically affects adults and is heavily influenced by diet and environmental factors as well as genetics.

Maturity onset diabetes of the young (MODY) is characterized by autosomal dominant inheritance and impaired insulin secretion. It accounts for 2%-5% of type 2 diabetes. MODY can result from mutations in at least six different genes namely HNF4α (*HNF4A*, MODY1), glucokinase (*GCK*, MODY2), HNF1α (*HNF1A,* MODY3), insulin promoter factor-1 (*PDX1*, MODY4), HNF1β (*HNF1B*, MODY5) and neurogenic differentiation factor 1 (*NEUROD1*, MODY6) (Froguel and Velho, 1999; Shih et al, 2002, Yamagata et al, 1996).

MODY1 is associated with the loss of function mutations in *HNF4A*, both in the coding and regulatory regions (promoter) of the gene (Ellard and Colclough, 2006; Owen and Hattersley, 2001). Some of these mutations occur in the P2-promoter region of HNF4α (Hansen et al, 2002; Thomas et al, 2001). Genotyping for single nucleotide polymorphism (SNP) indicates that additional genetic variation in the P2 promoter of

HNF4α might predict susceptibility to late-onset type 2 diabetes among different ethnic groups (Bonnycastle et al, 2006; Love-Gregory et al, 2004; Raeder et al, 2006; Silander et al, 2004; Weedon et al, 2004). Since MODY1 is characterized by a defect in nutrient-stimulated insulin secretion, the function of P2-driven HNF4α in pancreatic β-cell is considered to be critical in regulating insulin sensitivity (Gupta et al, 2007; Gupta et al, 2005; Miura et al, 2006). However, a recent study showed that point mutation in *HNF4A* (T130I) that was identified in a Japanese population with late-onset diabetes, decreased the transactivation of potential HNF4α in cultured primary hepatocytes but not in the MIN6 insulinoma cell line (Zhu et al, 2003). This suggests that the loss of HNF4α function in the liver, as well as the pancreas, might contribute to the progression of the disease. Knockout of HNF4α in the adult leads to a fatty liver, a condition that can lead to metabolic disease and potentially diabetes (Hayhurst et al, 2001).

HNF4α and Cancer

HNF4α and hepatocellular carcinoma

Differentiation and maintenance of the hepatic phenotype is carried out by transcriptional network composed of HNF4α, HNF1α, HNF1β, FoxA3, HNF6, C/EBPα (Schrem et al, 2002). It has been proposed that dedifferentiation of mature hepatocytes is one of the mechanisms by which cancer develops (Sell, 1993). The other proposed mechanism is via expansion of resident stem cell population. There have been a number of studies that have looked at the expression of HNF4α during hepatocellular carcinoma (HCC). In rat models of chemically induced (diethyl nitrosoamine) HCC there was only a marginal

effect on HNF4α gene expression and DNA binding activity (Kalkuhl et al, 1996; Stumpf et al, 1995). In clinical human HCC samples a decrease in C/EBPα and an increase in HNF1, HNF3β, HNF4α, and HNF4γ gene expression have been reported (Xu et al, 2001). In contrast in a mouse model of HCC, highly differentiated, slow-growing, HCC (sgHCC) progresses into a highly invasive, fast-growing, dedifferentiated variant (fgHCC), the expression of the entire group of liver transcription factors is downregulated (Lazarevich et al, 2004).

During normal development, P2-driven HNF4α is expressed in fetal liver, whereas only the P1-isoform is expressed in adult liver (Briancon et al, 2006; Torres-Padilla et al, 2001). In contrast, immunohistochemical staining with monoclonal antibody specific to either P1-driven or P2-driven HNF4α show that P1-driven HNF4α protein expression is lost, and P2-driven HNF4α is newly expressed in human HCC tissues (Tanaka et al, 2006). The mRNA level of P1- and P2-driven HNF4α was not examined in these tissues, hence, it is not clear at this point if the decrease in P1-driven HNF4α is on the mRNA or protein level. More recently, a decrease in P1-driven gene expression was reported in 7 out of 13 clinical HCC cases (54%) (Yin et al, 2008). Exogenous expression of HNF4α (P1-isoform) in hepatocellular cancer cell lines HepG2 and Hep3B retards tumor growth by decreasing cell proliferation, increasing apoptosis and senescence (Lazarevich et al, 2004; Yin et al, 2008). It has also been reported that increasing the expression of HNF4α in liver cancer lines (HepG2 and Hep3B) can significantly decrease the population of cancer stem cell progenitor cells, suggesting that re-expression of HNF4α can be used as a gene therapy for HCC (Yin et al, 2008).

While most of the studies mentioned above were performed inliver cancer cell lines, the most definitive role for HNF4α in liver cancer comes from few recent works *in vivo*. In the first study HNF4α expression was decreased in cirrhotic liver tissue and further decreased in hepatocarcinoma relative to healthy tissue. Notably, there was an inverse correlation between HNF4α expression and epithelial-mesenchymal transition (EMT). Enforced expression of P1-driven HNF4α attenuated hepatocyte EMT during hepatocarcinogenesis, alleviated hepatic fibrosis, and blocked HCC occurrence. In parallel, stem cell marker gene expression was inhibited along with cancer stem/progenitor cell generation. Further, forced expression of HNF4α inhibited activation of β-catenin, which is closely associated with EMT and HCC. Taken together, these results suggest that the inhibitory effect of HNF4α on HCC development might be attributed to suppression of hepatocyte EMT and cancer stem cell generation through an inhibition of β-catenin signaling pathways (Yin et al, 2008; Ning et al, 2010). In the second study, Walesky et al has shown that hepatocyte specific deletion of HNF4α, in adult mice resulted in increased hepatocyte proliferation with a significant increase in liver-body weight ratio. Global gene expression changes using Illumina HiSeq-based RNA sequencing revealed that a significant number of up-regulated genes following deletion of HNF4α were associated with cancer pathogenesis, cell cycle control, and cell proliferation. To determine whether deletion of HNF4α affects cancer pathogenesis the authors treated mice, with the known hepatic carcinogen diethylnitrosamine (DEN), and knockdown for HNF4α after the appreance of liver tumors. Deletion of HNF4α significantly increased the number and size of DEN-induced hepatic tumors (Walesky et

al, 2013). In the third study Haziapostolou et al showed that transient inhibition of HNF4α initiates hepatocellular transformation through a microRNA-inflammatory feedback loop circuit consisting of miR-124, IL6R, STAT3, miR-24, and miR-629. They also show that once this circuit is activated, it maintains suppression of HNF4α and sustains oncogenesis (Haziapostolou et al, 2011). None of the above studies distinguished the role of P1-driven and P2- driven HNF4α in HCC.

HNF4α and intestinal and colon cancer

In contrast to the liver, both P1- and P2-driven HNF4α are expressed in the normal adult gut (Tanaka et al, 2006). Three recent studies have looked closely at the expression of HNF4α isoforms in colorectal carcinoma (CRC). The first study revealed that P2-driven HNF4α was found in all normal and cancerous tissues examined, while P1-driven HNF4α was expressed in only 5 out of 18 primary colorectal carcinomas. Furthermore, in P1-HNF4α positive cases, the nuclear HNF4α signal was weaker than in the normal tissue (Tanaka et al, 2006). The second study examined the HNF4α levels in different stages of CRC. Colorectal cancer can be classified into four stages as devised by Dr Cuthbert Dukes: Dukes A (invasion into but not through the bowel wall); Dukes B (invasion through the bowel wall but not involving lymph nodes); Dukes C (involvement of lymph nodes); and Dukes D (widespread metastases) (Kyriakos, 1985). Oshima et al showed that the frequency of P1-positive expression of HNF4α was higher in Duke's A and B than in Duke's C and D tumors. Furthermore, the frequency of P1-positive expression in CRC patients with metachronous liver metastasis was significantly lower than those

without liver metastasis, suggesting that the loss of P1-HNF4α is a poor prognostic factor in CRC and is associated with liver metastasis (Oshima et al, 2007). Finally a third study from our lab examined a larger set of tumor samples (450 total) and revealed that approximately 80% of tumor samples showed P1-HNF4α staining that was not exclusively nuclear ie was either completely lost or present partially in the cytoplasm, in contrast to normal tissues where HNF4α staining was observed exclusively in the nucleus (Chellappa et.al, 2012). Therfore the emerging view is that P1-driven HNF4α acts as a tumor suppressor in colon as well as in liver cancer. In contrast, P2-driven HNF4α appears to be permissive to cancer growth however more work is required to define the role of both P1- and P2- driven HNF4α in both normal and diseased colon tissue.

HNF4α and renal cell carcinoma

Only P1-derived HNF4α is expressed in the kidney where it is localized to the proximal tubular epithelial cells in kidney, (Chabardes-Garonne et al, 2003; Jiang et al, 2003; Mietus-Snyder et al, 1992; Tanaka et al, 2006). The role of HNF4α in kidney is still not well known. However, several groups have reported that HNF4α gene expression is decreased in renal cell carcinoma (RCC) (Anastasiadis et al, 1999; Lenburg et al, 2003; Sel et al, 1996), while another group found no change in protein expression of HNF4α in RCC (Tanaka et al, 2006).

HNF4α Isoform -specific mice

Briancon et al generated HNF4α knock in mice that express only one type of HNF4α

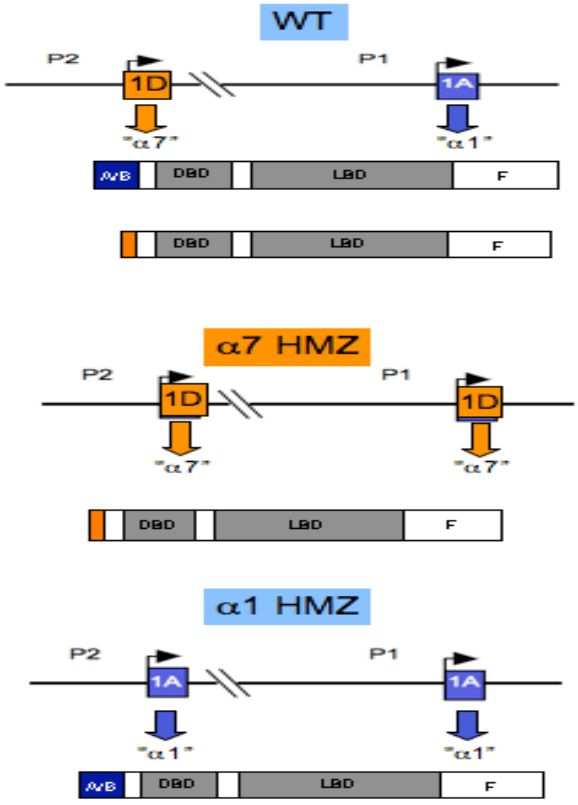
isoform (HNF4α1 or HNF4α7 and their corresponding splice-derived variants) under control of the two promoters, P1 and P2 (Fig 1.2). The isoform-specific mice were generated by an exon swap strategy in which exon 1D driven by the P2- promoter was replaced by exon 1A driven by the P1 promoter. These mice are referred to as α1 HMZ mice as they express only the HNF4α isoforms containing exon 1A (HNF4α1, HNF4α2, HNF4α3) regardless of the promoter that is activated, similarly exon 1A driven by the P1- promoter was replaced by exon 1D driven by the P2 promoter. These mice are referred to as α7 HMZ mice as they express only the HNF4α isoforms containing exon 1D (HNF4α7, HNF4α8, HNF4α9) regardless of the promoter activated. This minimal intervention strategy permits conservation of the ratio among the internal splice variants as well as the native promoters.

The α1-HMZ and α7-HMZ mice did not present any evident phenotype, contrasting with the embryonic lethality of the whole body knockout (Chen et al, 1994; Duncan et al, 1997). This suggests functional redundancy of the isoforms in the visceral endoderm and other tissues. In addition, in contrast to the severe architectural defects reported in the fetal-liver-specific HNF4αKO (Parviz et al, 2003), α7-HMZ fetal livers appear indistinguishable from WT. In the adult, the α7-HMZ mice exhibit a non lethal dyslipidemia associated with slight hepatic steatosis, reminiscent, in more subtle form, of the phenotype of the liver-specific *Hnf4*a null mice. This indicates not only that HNF4α7 is sufficiently redundant functionally with HNF4α1—the main isoform in the WT liver—to enable long-term survival of the α7-HMZ mice, but also that there are specificities inherent to each isoform due to the presence or absence of the AF-1 domain. These exon

swap mice provided the first evidence *in vivo* of the HNF4α AF-1 function and has also allowed for identification of AF-1-dependent target genes (Briancon et al, 2006). Both P1- and P2-driven HNF4α are expressed in distal colon of the WT mice while only P2-driven HNF4α is expressed in α7 HMZ mice (Fig 1.2).

In order to investigate a potential differential role of P1- and P2-derived isoforms of HNF4α in the distal colon, we employed isoform-specific mice generated by weiss group (α7 HMZ and WT) (Briancon et al, 2006). Both P1- and P2-driven HNF4α are expressed in the distal colon of WT mice while only P2-driven HNF4α is expressed in the α7 HMZ mice. Although recent studies indicate that P1- and P2-driven HNF4α are differentially regulated during HCC and CRC, (Niehof and Borlak, 2008; Tanaka et al, 2006; Chellapa et al, 2012) the isoform-specific role of P1- and P2-driven HNF4α during cancer and colitis has never been investigated. In this study we investigated a potential functional role for HNF4α and miRNA 21 during cancer and colitis.

Fig 1.2: HNF4α Isoform-specific mice: Isoform- specific mice were generated by Nadege Briancon at Pasteur institute in 2006 (Briancon et al, 2006) through reciprocal swapping of the first exons driven by each of the two promoters (P1 or P2) in the *HNF4A* gene. This figure shows that Both P1- and P2-driven HNF4α are expressed in distal colon of the WT mice while only P2-driven HNF4α is expressed in α7 HMZ mice and only only P1-driven HNF4α is expressed in α1 HMZ mice.



MicroRNA

Lin-4 was identified in a mutant screen in 1981 as a mediator of *Caenorhabditis elegans* development (Chalfie et al, 1981). Unexpectedly, twelve years later, cloning of lin-4 indicated that it was not a protein-coding gene, rather it encoded a short small, 21-nucleotide RNA molecule that has no open reading frame (Lee et al, 1993). Furthermore, binding of lin-4 to the 3'UTR of a protein-coding mRNA, lin-14, repressed its translation (Lee et al, 1993). In 2000 another small non protein coding RNA was identified in *C. elegans* (let-7) (Reinhart et al, 2000), followed by confirmation of the existence of small RNA were identified in several other species, including humans (Pasquinelli et al, 2000). In 2001 the term "microRNA" was introduced as nomenclature for these small, regulatory RNA (Lagos-Quintana et al, 2001). Since then, thousands of miRNAs have been identified in a multitude of organisms (microRNA.sanger.ac.uk/) and miRNAs have led to an explosion in our knowledge of the role that post-transcriptional gene regulation plays in organ function. It is predicted that 1-5% of genes encode miRNAs, and that they regulate the expression of as many as 30% of mRNAs (Berezikov et al, 2005; Lewis et al, 2005). However, the functional importance of individual miRNAs and the identity of their specific mRNA targets are just now beginning to be elucidated in rodents and human cell lines. It remains to be determined if the function of the miRNAs are conserved across species in the same manner as protein-encoding genes.

MicroRNA Biogenesis

MicroRNA genes reside between protein-coding genes (intergenic) and within genes (intragenic). In a few cases miRNA genes within exons or comprise the entire intron in which case they are called mirtrons (Berezikov et al, 2007; Saini et al, 2007). As a result miRNAs can be transcribed either independently (intergenic and intronic) from protein coding genes or in succession with mRNAs if they reside within an intron or exon (Corcoran et al, 2009). The initial long primary RNA transcript (pri-miRNA) is capped (methyl-7-GpppG) and polyadenylated, and thus the transcription of most miRNA genes is mediated by RNA Polymerase II, although a few are transcribed by Polymerase III (Borchert et al, 2006; Cai et al, 2004; Kim, 2005; Lee et al, 2004b). Within the pri-miRNA a secondary structure forms comprising an ~80-110 nucleotide (nt) stem loop.

A protein complex called the microprocessor, composed of DGCR8 (DiGeorge syndrome critical region 8), Drosha, and p68/72 DEAD-box RNA helicase, binds to the stem loop and cleaves it to produce an ~70-100 nt precursor miRNA (pre-miRNA; Lee et al, 2003). Most pri-miRNA have a bulge or mismatched base at this site (Yi et al, 2003). The p68/72 helicases bind the dsRNA of the stem loop and present the site to Drosha (Fukuda et al, 2007). Drosha cleaves the phosphodiester bond leaving a 2-nt 3'overhang, and the resulting pre-miRNA is released into the nucleus (Lee et al, 2003). In some cases, other proteins are required for the interaction of Drosha with the pri-miRNA; for instance, KSRP (KH-type splicing regulatory protein) is an RNA-binding protein that binds to G-containing stretches in the terminal loop of the stem loop structure

and is necessary for Drosha: pri-miRNA interaction for a subset of miRNA's including miR-21 which is discussed below (Trabucchi et al, 2009). The pre-miRNA is exported from the nucleus by exportin 5 (Bohnsack et al, 2004; Lund et al, 2004).

In the cytoplasm, pre-miRNA is further cleaved by a second RNase III enzyme, Dicer, to form a transient ~19-23 nt RNA duplex (Hutvagner et al, 2001). Although Dicer is an RNase III enzyme like Drosha, it has an extra domain, the PAZ domain that allows it to act as a molecular ruler without the need for a cofactor. The PAZ domain binds to the 2 –nt 3'overhang at the base of the pre-miRNA, forcing the dsRNA-binding domain to bind to the stem (Tahbaz et al, 2004). Dicer's two RNAse III domains form an intramolecular dimer ~22 nt from the base of the pre-miRNA, and this dimer cleaves the pre-miRNA to form an ~19-23 nt RNA duplex that has a 2 nt overhang on its 3' ends (Tahbaz et al, 2004). The miRNA duplex has multiple mismatches and G:U wobble pairs, which causes the 5' end, of the duplex to be less stable at the and this 5' strand is preferentially loaded onto the RNA-induced silencing complex (RISC; Khvorova et al, 2003; Schwarz et al, 2003).

The RISC is composed of Dicer, TRBP (TAR RNA-binding protein), and one of several different Argonaute proteins (there are four in the human, AGO1-4; and five in the mouse, AGO1-5; Hutvagner and Simard, 2008). TRBP recruits Argonaute to the Dicer:miRNA complex, thus forming miRISC (Chendrimada et al, 2005). The 5' end of the miRNA tucks into a binding pocket on the Argonaute protein, and bases 2- 8 bind the target mRNA with perfect or close to perfect complementarity (Brennecke et al, 2005; Pratt and Macrae, 2009). These bases make up the 'seed sequence' of the miRNA, and

are especially important for conferring target specificity (Brennecke et al, 2005; Lai, 2002). The seed sequence is exposed on the outside of the Argonaute protein, allowing the sequence to probe for a target through the cellular milieu and passing mRNAs (Pratt and Macrae, 2009). In addition, many other proteins have been shown to interact with Argonaute proteins, but their exact roles in the miRISC are still being studied (Peters and Meister, 2007).

MicroRNA Mechanisms of Action

Binding of miRISC to target mRNAs are postulated to cause: 1) mRNA cleavage and degradation; 2) mRNA deadenylation and degradation; 3) inhibition of translation initiation of mRNAs; 4) mRNA inhibition of translation elongation; or 5) mRNA translational enhancement. The first mechanism appears to be the most prevalent in plants, but in vertebrates the other mechanisms are thought to be more frequently used. For mRNA cleavage, the miRISC must contain a catalytically active Argonaute (Liu et al, 2004), and the miRNA and mRNA must contain a near perfect complementarity match. The other Argonautes, however, can also mediate mRNA degradation, even without perfect complementarity by interaction of the Argonaute protein with GW182 (Rehwinkel et al, 2005). GW182 recruits the decapping enzymes DCP1 and DCP2, and decapping of the mRNA leads to exonuclease degradation of the transcript (Rehwinkel et al, 2005). In other cases, miRNA:mRNA binding causes translational repression. Binding of miRNA to mRNA blocks translation due to RISC interaction with translation initiation factors, such as eIF6, eIF4E, and

eIF4G, which prevents assembly of 80S ribosomes (Chendrimada et al, 2007). Other reports suggest that miRNA represses mRNA translation during the elongation step, a notion that is supported by the fact that translationally repressed mRNAs have been found to associate with polysomes (Jackson and Standart, 2007; Maroney et al, 2006; Nottrott et al, 2006; Petersen et al, 2006).

Naming MicroRNAs

To provide structure to the naming and characterization of the thousands of recently identified miRNA, the Sanger Institute miRNA Registry was established (http://microrna.sanger.ac.uk/ ; Griffiths-Jones et al, 2006). The names of miRNA consist of four components, each conveying a specialized piece of information about the given miRNA: species, form (precursor or mature), identification number, and origin of miRNA (either processing origin or chromosomal origin). Each component is separated by dashes and is represented by the following template: xxx-miR-#-suffix. The xxx signifies the species (i.e, hsa=human, mmu=mouse). To distinguish between the precursor and mature forms of miRNA, a small case 'r' ('mir') represents the precursor form of the miRNA, while an uppercase 'R' ('miR') represents the mature form of the miRNA. Generally, an identification number is assigned in the sequential order of discovery, thus more recently identified miRNAs has large numbers. The suffix identifier denotes either the processing origin or the chromosomal origin of the miRNA. For the processing origin, opposite arms from a single pre-miRNA are denoted '5p' and '3p'. After one arm is experimentally identified as the predominant

arm, the less predominant arm is labeled with an asterisk suffix. For genomic origin, miRNA that arise from different genomic loci but have identical mature sequences are labeled with numbered suffixes, and if they arise from paralogous genomic loci and have highly similar mature sequences, they are labeled with lettered suffixes. Rarely, miRNA genes have been identified at the same chromosomal location but on opposing DNA strands (sense versus antisense), and thus have unique mature miRNA sequences. The miRNA on the antisense chromosome is identified with an 'as' suffix. A given miRNA can have several suffixes. For example, hsa-miR-19b-1, which originates from chromosome 13, is identical to hsa-miR-19b-2 which originates from chromosome X, and both of these are paralogous (share 70% of mature sequence) to hsa-miR-19a which also originates from chromosome 13.

Regulation of microRNA Expression

Many miRNAs are expressed in specific cell types or at specific time periods, suggesting that, like protein-coding genes, miRNA expression is highly regulated (Lagos-Quintana et al, 2002; Landgraf et al, 2007). However, the sequence and functional elements of miRNA promoters is only just starting to be examined. The few miRNA promoters that have been examined, however, appear to be very similar to those of protein-coding genes. Pri-miR-375, for example, is a miRNA that is expressed only in pancreatic islets (Avnit-Sagi et al, 2009). Its promoter has transcription factor binding sites for pancreatic- enriched transcription factors, such as HNF1 and Ngn3, and chromatin immunoprecipitation (CHIP) studies confirmed interaction of Ngn3 with the

pri-miR-375 promoter (Avnit-Sagi et al, 2009; Keller et al, 2007). In addition, approximately 50% of miRNAs exist as genomic clusters, and thus are probably coordinately transcribed as one larger primary transcript that are subsequently processed into shorter pri-miRNAs. For example, the embryonic-stem cell (ES cell) specific miRNA gene cluster miR-290 to miR-295 is coordinately transcribed to help maintain ES cell pluripotency (Houbaviy et al, 2003). Finally, some miRNA genes lie within the intron or exon of a protein-coding gene, and thus are co-expressed with their host gene and rely on transcriptional activation of the host gene promoter for their expression (Kim and Kim, 2007; Raver-Shapira and Oren, 2007).

The processing of the primary or precursor forms of the miRNA can also be regulated, thus resulting in a form of post-transcriptional regulation. Indeed, Thomson et al. found a discreprancy between the primary/precursor levels of miRNAs and the corresponding mature miRNAs during mouse embryonic development (Thomson et al, 2006). Specific cases of miRNAs being post-transcriptionally regulated have also been reported. In pluripotent stem cells the RNA-binding protein Lin28-homolog (LIN28) selectively binds the pri-let-7 family members to prevent their interaction with Drosha, thus preventing let-7 mediated differentiation (Viswanathan et al, 2008). Another miRNA, miR-138, is found only in the brain, although its precursor forms are found ubiquitously in all tissues, suggesting that it is processed by Dicer only in the brain (Obernosterer et al, 2006). In another case, two RNA-binding proteins, nuclear factor 90 (NF90) and NF45, bind pri-miRNAs to prevent Drosha cleavage into pre-miRNA (Sakamoto et al, 2009). Interestingly, this binding appears to be differential, and in

human embryonic kidney 293T cells, NF90 and NF45 had a higher affinity for pri-let-7a than pri-miR-21(Sakamoto et al, 2009). It would be of interest to examine whether NF90 and NF45 bind distinct pri-miRNA in different cells or in response to different treatments.

MicroRNA Functions

MicroRNAs have been shown to be important in many biological processes, including cell proliferation, differentiation, and apoptosis (Asangani et al, 2008; Cloonan et al, 2008; Silber et al, 2008). For example, whole Dicer null mouse ES cells are viable, but they display major problems in growth and differentiation (Kanellopoulou et al, 2005). The ES cell-specific miR-290-295 cluster has been shown to play an important role in preventing ES cell self-renewal by indirectly decreasing the activation of Oct4 (Sinkkonen et al, 2008). In addition, another miRNA , miR-21 promotes differentiation by targeting Nanog and Sox2, important regulators of ES cell pluripotency and self-renewal (Singh et al, 2008). MicroRNAs have also been shown to be involved in the differentiation of adult stem cells during haematopoiesis, myogenesis, cardiogenesis, neurogenesis, and osteogenesis. During erythropoiesis, miR-221 and miR-222 decrease during erythroid differentiation, releasing their inhibition of C-kit protein translation, leading to the expansion of early erythroblasts (Fontana et al, 2008).

Due to their vital roles in cell growth and apoptosis, miRNAs have also been associated with many disease states. MicroRNA-133 and miR-1, for example, have been associated with the increase in cardiomyocyte hypertrophy that occurs during heart

failure (Care et al, 2007). Within the brain, several miRNAs are up regulated in the diseased temporal lobe neocortex of Alzheimer's patients, (Sethi and Lukiw, 2009), and a single nucleotide polymorphisms (SNP) affecting miRNA binding sites have been associated with Tourette's Syndrome and obsessive compulsive disorder (Abelson et al, 2005; Muinos-Gimeno et al, 2009). Within the female reproductive tract, miRNAs have been shown to play a role in endometriosis and uterine leiomyomas (Marsh et al, 2008; Wang et al, 2007; Peng et al, 2008; Pan et al, 2007).

Perhaps the most studied role for miRNAs is that in cancer development, progression, and prognosis. Many miRNA genes are located in genomic regions that are amplified, deleted, or rearranged in cancer (Fontana et al, 2008). Thus, it is not surprising that many of these miRNA have been shown to play a role in cancer, and have even been given the designation "oncomir". Three of the most studied oncomirs are miR-34, miR-17-5p, and miR-21 (Esquela-Kerscher and Slack, 2006). MicroRNA-34 is down regulated in cancer, and studies have indicated that it acts as a tumor supressor and induces cell growth arrest and apoptosis by activating the p53 pathway (Yamakuchi et al, 2008). MicroRNA-17-5p and miR-21 are both up regulated in many types of cancer and tumors, and have been demonstrated in many cases to prevent apoptosis, counter intuitively they have also been found to promote differentiation (Esquela-Kerscher and Slack, 2006). miR-21, a topic of this dissertation, will be discussed in greater detail below.

Methods for Studying MicroRNA

A first step in microRNA study is to determine the microRNAs that are expressed within a given tissue or at a given time period. These preliminary studies can include miRNA cloning analysis, miRNA microarray analysis or miRNA seq. Most microRNAs are identified first by cloning (Lagos-Quintana et al, 2001; Lau et al, 2001; Lee and Ambros, 2001), and cloning is especially useful in identifying miRNAs that are novel to a specific tissue and cloning frequency can give a general idea of abundance. (Ro et al, 2007a; Ro et al, 2007b).

With the establishment of a list of known miRNAs, miRNA microarrays were developed to allow for high throughput profiling of miRNA expression. MicroRNA microarrays provide a rapid means with which to establish the miRNAome of a cell, and can provide a fairly good idea of miRNA abundance and changes between treatment groups (Li and Ruan, 2009). However, novel miRNAs will not be detected in a miRNA microarray studies. MicroRNA sequencing (miRNA-seq), a type of RNA-Seq, is the use of next-generation sequencing or massively parallel high-throughput DNA sequencing to sequence miRNAs. miRNA-seq differs from other forms of RNA-seq in that input material is often enriched for small RNAs. miRNA-seq allows researchers to examine tissue-specific expression patterns, disease associations, isoforms of miRNAs, and to discover previously uncharacterized miRNAs. Once a miRNA is identified by one of these two methods, its expression pattern is typically confirmed using Northern blot analysis or quantitative real time PCR (qRT- PCR).

As new qRT-PCR methods have been developed that allow for increased specificity, qRT-PCR has become the method of choice for microRNA detection due to its higher sensitivity than Northern blotting and allows for the study of quantitative changes. While several qRT-PCR methods have been introduced that allow for amplification and quantification these extremely small miRNA, in this study I have chosen to use a stem loop RT PCR method to analyze miRNAs in my system due to ease of use and an its improved ability to normalize the data (Fiedler, 2009). The first strand synthesis in qRT-PCR is initiated with an miRNA-specific forward primer, and then a reverse primer specific to the universal tag completes the amplification.

After a miRNA has been shown to be expressed in a given tissue or at a given time period, the next question is, 'What is its function ?'. There are two ways to ascertain the function of a miRNA: 1) inhibit the miRNA; and 2) add exogenous miRNA. MicroRNAs function can be inhibited by using a modified synthetic antisense oligonucleotide that binds the miRNA and prevents its interaction with its target. Ideally, these synthetic oligonucleotides will have a high biostability and will bind the miRNA with high affinity. 2'O-methyl oligonucleotides are RNA molecules that have a methyl group at the 2' position of the ribose that increases nuclease resistance and thus makes the oligonucleotide more stable (Grunweller et al, 2003). The interaction of a 2'O-methyl oligonucleotide with its target miRNA has a similar affinity as a native RNA:RNA interaction. Another option, are the locked nucleic acids (LNA), which have a 2'-O,4'-C methylene bridge that locks the structure in a bicyclic formation. This bridge dramatically increases the biostability of the molecule, and, furthermore, increases the melting

temperature of the LNA:miRNA duplex by several degrees (Grunweller et al, 2003). Therefore, LNA molecules often have a more potent and lasting effect than 2'O-methyl oligonucleotides (Grunweller et al, 2003). The third option is to use miRIDIAN microRNA mimics or inhibitors, that are double-stranded RNA oligonucleotides, chemically enhanced with a proprietary design. MicroRNA mimics act by preferential programming of RISC-like complex with the active strand of the miRNA and exclusion of passenger strand through a proprietary chemical modification pattern. miRNA inhibitors compete with mRNAs for programmed RISC. The exogenous pre-miRNA is cleaved by Dicer and incorporated into RISC in the same manner as endogenous pre-miRNA.

Knockdown or over expression of a microRNA will cause a change in the expression of target proteins. To identify these target proteins, a two-dimensional gel approach can be used. For example, protein from cells treated with a nonspecific control LNA and protein from cells treated with an LNA to the specific miRNA can be isolated and labeled with either Cy3 (green) or Cy5 (red), respectively. The labeled protein is run on a single two-dimensional gel, that separates the protein by both size and by isoelectric point. Unchanged proteins are visualized as yellow spots, while changed proteins are visualized as either red or green spots. The spots can be excised from the gel and analyzed by mass spectrometry to determine the identity of the protein. The changed proteins can be analyzed using bioinformatics software to find miRNA-binding sites in their 3'UTR, and the presence of a site can suggest that the protein is a direct target of the miRNA. If a protein is predicted to be a direct target of the miRNA, luciferase assays

provide the best approach for confirming the interaction. The 3'UTR of the predicted target mRNA is ligated into a plasmid containing the luciferase gene. The plasmid is co-transfected into the cell with the LNA to the miRNA, and if luciferase activity increases, the miRNA may be directly interacting with that particular 3'UTR. To confirm the interaction, various mutations can be made within the 3'UTR so the miRNA binding sites are disrupted.

miRNA 21 and cancer

miRNA-21 and gastrointestinal, and colorectal cancer

Gastric cancer (GC) is an important malignant disease around the world and the second leading cause of death due to cancer worldwide. Abnormalities of miRNAs have been implicated in the carcinogenesis of GC. miRNA-21 is frequently up regulated in various cancers (Volinia et al, 2006; Xu et al.2012). PTEN (phosphatase and tensin homolog) is a tumor suppressor gene frequently mutated in cancer, which regulates cellular proliferation, growth, and apoptosis (Meng et al, 2007). miRNA-21 was demonstrated to regulate radiosensitivity in gastric carcinoma cells, possibly by direct modulation of PTEN expression (Lima et al, 2011). miRNA-21 expression was significantly associated with the degree of differentiation of the tumor tissues and showed that reduction in miRNA-21 expression has a remarkable effect on the biological behavior of gastric cancer cells. They also found that PTEN expression was remarkably increased after miRNA-21 inhibition, implying that miRNA-21 inhibition may upregulate PTEN expression. Finally Xu et al document a close association between the elevated miRNA-

21and lymph node metastasis. The overall survival rates in GC patients with low miRNA-21 levels were significantly higher than those with high miRNA-21 levels.

Colorectal cancer (CRC) is the third most common cancer in developed countries, while stomach and esophagus cancers are the third and the sixth common cancers, respectively in developing countries. Diet and other environmental factors play a important role in development of all these cancers (Ventura et al, 2009; Rossbach et al, 2012; Lee et al, 2009). Schetter et al published a landmark paper on miRNA 21 in colon using two cohorts of patients. In Maryland cohort, for those who received chemotherapy, high miRNA-21 expression in tumors predicted worse overall survival giving preliminary support to the notion that high miRNA-21 is associated with a poor therapeutic outcome. In the Hong Kong cohort, high miRNA-21 expression in tumors was associated with a poor response to therapy (Schetter et al, 2008; Xie et al, 2010). In order to shed light on the mechanism by which miR-21 might be associated with pro-cancer state Yu et al. reported that miRNA-21 plays an important role in regulating stemness by modulating transforming growth factor beta-receptor 2 (TGFbR2) signaling in colon cancer cells, down regulation of miRNA-21 enhances luciferase-TGFbR2-3' untranslated region (UTR activity) (Yu et al, 2012). Valeri et al. reported that miRNA-21 directly targets the 3' UTR of MSH2 and MSH6 mRNA, resulting in down regulation of protein expression. They also showed an inverse correlation between miRNA-21 over-expression and MSH2 expression in CRC tissues (Valeri et al, 2012), MSH2 and MSH6 are genes in misatch repair pathway (MMR) and inherited mutations in MMR genes lead to cancer. In other studies cells that over-express miRNA- 21 showed significantly reduced 5-fluorouracil

(5-FU)- induced G2/M damage arrest and apoptosis that is characteristic of defective mismatch repair (MMR). Because miRNA-21 expression increased in cell lines continuously exposed to 5-FU (drug commonly used to treat cancer), cancer cells may develop a secondary resistance to 5-FU through miR-21 over-expression. Thus, miR-21-dependent downregulation of MSH2-MSH6 may be responsible for both primary and secondary resistance to 5-FU (Rossi et al, 2007; Yamamoto et al, 2012). The analysis of normal and tumor tissues from 22 colorectal cancer patients demonstrated an inverse correlation between miRNA-21 and Pdcd4-protein, a tumor suppressor. Programmed cell death 4 (Pdcd4) is also shown to be negatively regulated by miRNA-21, which induces invasion, intravasation, or metastasis in Colo206f-cells (Corte et al, 2011). In addition, Asangani et al. (Asangani et al, 2008) studied the inhibition of Pdcd4 by miRNA-21 and found that over-expression of miRNA-21 causes tumor cells to invade, intravasate, and metastasize more aggressively when implanted into mouse models.

miRNA-21 and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a common cancer worldwide, especially in Japan and other East Asian countries, and the third most frequent cause of cancer- related deaths in the world (Bosch et al, 2004). Studies has shown that plasma miRNA-21 levels correlated significantly with miRNA-21 expression levels in tumoral tissues, though the correlation coefficient was relatively low while serum miRNAs are a very hot topic in cancer diagnosis, additional studies are needed to prove that the elevated levels of miRNA 21 in serum of HCC patients are due to tumor growth (Tomimaru et al, 2011). HCC is a highly heterogeneous, and aggressive cancer that has a poor prognosis. Up regulation of miR-21

reduces cell death and promotes angiogenesis and invasion of HCC (Negrini et al, 2011). Finally elevated serum miRNA 21 levels have been detected in patients with chronic hepatitis as well as HCC (Xu et al, 2011). Chronic hepatitis often leads to HCC

miRNA-21 and glioblastoma

In the first study exploring the role of miRNAs in glioblastomas, Chan et al found miRNA-21 to be upregulated and showed that knock- down of miRNA-21 in cultured glioblastoma cell lines triggered caspase activation and associated apoptotic cell death, suggesting an antiapoptotic function of miRNA-21 (Chan et al, 2005). An independent study that used microarray analysis to compare the expression of 245 miRNAs in glioblastoma versus normal tissues also identified miRNA-21 levels as being increased in glioblastoma tumors (Ciafre et al, 2005). Others found that knockdown of miRNA-21 in cultured glioblastoma cells resulted in a significant drop in cell number. This reduction was accompanied by increases in caspase-3 and -7 enzymatic activities and TUNEL staining (Corsten et al, 2007). miRNA-21 targets signaling pathways of tumor supressor p53, TGF-b and mitochondrial apoptotic pathway (Kim et al, 2009), inhibits translation of mRNAs RECK, TGFBR, DAXX, PDCD4, p63, JMY, TOPORS, HNRNPK, and TP53BP2 as these are all involved in apotopsis, hence miRNA-21 regulates apoptosis, cell cycle and translation in glioblastoma cells.

miRNA-21 and prostate cancer

Prostate cancer is considered to be the most diagnosed cancer and the second leading

cause of cancer death in men older than 40 years of age (Peng et al, 2011; Hao et al, 2011). miRNA-21 is up regulated in different solid tumors like prostate cancer and has been shown to act as oncogene in prostate cancer cells by targeting tumor suppressors such as PTEN and PDCD4. miRNA 21activation is thought to enhance general processes of tumor invasion and metastasis by promoting extracellular remodeling in the tumor environment (White et al, 2011; Kuner et al, 2012). More recently, elevated serum miRNA-21 has been detected in androgen-dependent prostate cancer (ADPC) and hormone refractory prostate cancer (HRPC) patients, especially in those who are resistant to docetaxel-based chemotherapy. This finding suggests that serum miRNA-21 might be a potential predictor of the efficacy of docetaxel-based chemotherapy of prostate cancer.

miRNA-21 and ovarian cancer

Ovarian cancer is the second-most common cancer in women and is the leading cause of death from gynecological cancer. It is also the fifth leading cause of cancer-related death in women, causing an estimated almost 5 % of all death (http://www.cancer.gov/cancertopics/pdq/genetics/breast-and ovarian/).Taylor and Gercel-Taylor have detected exosomal miRNAs in blood that were released from ovarian tumors ((Taylor et al, 2008). There were a total eight miRNAs including miRNA- 21, miRNA-141, miRNA-200a, miRNA-200c, miRNA- 200b, miRNA-203, miRNA-205, and miRNA-214, that were expressed at high levels in malignant ovarian cancer cells and exosomes, whereas those miRNAs from exosomes of benign ovarian disease and ovarian cancer patients were significantly different (Tie et al, 2009). Exosomes are 40 to 100 nm vesicles secreted by a wide range of mammalian cell types, and are released from the cell

when multivesicular bodies fuse with the plasma membrane. Moreover, in another cohort study of 28 ovarian cancer patients, a significant alteration in five upregulated miRNAs (miRNA-21, 92, 93, 126 and 29a) and three down regulated miRNAs (miRNA-155, 127 and 99b) in serum were identified in the cancer group compared with the control (Resnick et al, 2009).

Table 1.1: Confirmed human miRNA 21 targets based on online software prediction (miRECORDS)

Symbol
PTEN
TPM1
E2F1
TGFBR2
CDK6
TIMP3
PDCD4
SERPINB5
NFIB
PDCD4
CDKN1A
FAS
FAM3C
HIPK3
PRRG4
ACTA2
BTG2
BMPR2
SESN1
IL6R
SOCS5
GLCCI1

Role of MiR-21 in Drug Resistance

Although significant advances have been made in the treatment of various cancers, and molecular-targeting agents have been used to improve patient survival, both intrinsic and acquired drug resistance remains a major clinical obstacle to successful treatment and leads to tumor recurrence. Better understanding of drug resistance mechanisms is urgently needed to improve current chemotherapy regimens. MiRNA-mediated drug resistance is yet another mechanism of drug resistance. Recently, numerous studies have indicated that aberrant miR-21 expression is strongly related to chemoresistance. In a recent study, microarray analysis showed that eight miRNAs were significantly up regulated and seven miRNAs were markedly down regulated in the chemoresistance phenotype of tumor cells (Table 1.2), and its involvement in tumor cell responses to chemotherapeutic agents has also been confirmed by many studies (Table 1.2). miRNA 21 was one of the miRNA that is upregulated in different kinds of cancer and its fuctional role in developing chemoresistance has been confirmed by several studies.

miRNA 21 expression is upregulated in wide variety of cancers including including liver, lung, pancreas, colon, breast cancer. This seems to increase levels of miR 21 in serum that could be useful biomarker although additional studies are needed to discern their prognostic value. miRNA 21 inhibits the expression of several tumor suppressor genes as well as genes involved in apotopsis.This could explain the high levels of miRNA 21 in tumors and provide mechanism of action. miRNA 21 overexpression is alo associated with resistance to chemotherapeutic drugs and in pardueto dysregulation of mismatch repair gene.

Table 1.2: miRNA 21 expression in different types of cancer and its functional role in mediating chemoresistance.

Cancer type	miR-21 expression	Target	Anticancer agent
Breast cancer	Upregulation	PDCD4, PTEN4	4Hydroxytamoxifen,Faslodex,Topotecan
Glioblastoma	Upregulation	LRRFIP1	VM-26
Pancreatic cancer	Upregulation	PTEN, RECK	Gemcitabine
Prostate cancer	Upregulation	PDCD4, MARCKS	Docetaxel
Cholangiocarcinoma	Upregulation	PTEN	Gemcitabine
Lung cancer	Upregulation	FasL	Doxorubicin
Myelocytic leukemia	Upregulation	PDCD4	Arabinosylcytosine, Arsenic Trioxide

Anti-inflammatory macrophages and miR-21

Successful recruitment of a pro-inflammatory response and timely resolution of inflammation are both required for successful wound healing (Koh et al, 2011; Khanna et al, 2010). Macrophages are immune cells involved in various biological processes including tissue repair and host defense. The first evidence supporting the significance of miRNA in governing macrophage function was published in 2007, when profiling studies were performed to identify miRNAs induced in primary murine macrophages after exposure to pro-inflammatory conditions. miR-155 was recognized as a common target of a broad range of inflammatory mediators (Connell et al, 2007) Ever since, interest in understanding the role of miRNA in regulating the inflammatory response to injury has risen sharply (Roy et al, 2012).

In one of the first works that reported the anti-inflammatory properties of miR-21 in macrophages, it was noted that miR-21 silences pro-inflammatory interleukin (IL)-12 gene (Lu et al, 2009). The IL-12 family is composed of three heterodimeric cytokines with overlapping pro-inflammatory and immunoregulatory functions. In human airway epithelial cells, IL-13 induces miR-21 (Case et al, 2011). In mouse lungs, miR-21 inhibits toll-like receptor 2 (TLR2) agonist-induced lung inflammation (Case et al, 2011). Resolvins, including D and E series resolvins, are endogenous lipid mediators generated during the resolution phase of acute inflammation from the omega-3 polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid (Ji et al, 2011). miR-21 is induced by resolvin D1 and may play a role in resolving

acute inflammation (Recchiuti et al, 2011). PDCD4 has pro-inflammatory properties. Translational inhibition of PDCD4 by miR-21 therefore has anti-inflammatory consequences in the context of sepsis (Merline et al, 2011).

Beyond its direct effects on macrophages, miR-21 has numerous biological targets validated in a variety of cell types that support the notion that miRNA 21 incvolves additional anti-inflammatory mechanisms. Analysis of predicted target genes of miR-21 on the basis of resources available in TargetScan4.0, PicTar, and miRanda resulted in the identification of a total of 930 candidates (Gao et al, 2012). Additional characterization of these candidates through target-pathway analysis (Case et al, 2011) suggested two specific signalling pathways that are significantly regulated by miR-21: (i) Janus kinase (JAK) and signal transducer and activators of transcription (STAT) signalling pathway (target count 16; *CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, STAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9, JAK3, IL12A, IL13RA2*); and (ii) cytokine – cytokine receptor interaction (target count 20; *CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, STAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9,JAK3, IL12A, IL13RA2*) (Gao et al, 2012). Collectively, these pathways represent the core of the cytokine response system. Dysregulation of cytokine signalling is known to be a root cause of inflammation. From a therapeutic standpoint, targeting cytokine networks is viewed as a promising strategy to control chronic inflammatory diseases.

JAK/STAT signalling helps elicit transcriptional response to external stimuli (Mohr et al, 2012). Specifically, it directly links ligand binding to a membrane-bound receptor with the activation of a transcription factor. Cytokines specifically bind to

their corresponding receptors, leading to the activation of receptor-associated JAKs. The receptor-bound JAKs activate STAT transcription factors that translocate into the nucleus to induce target gene expression. JAK-STAT signalling is directly implicated in cytokine-mediated inflammation (Terrel et al, 2006; Campbell et al, 2005). Inhibition of this pathway by small compounds is recognized as a potential therapeutic target for various inflammatory diseases (Tamiya et al, 2011) The silencing effects of miR-21 on the JAK-STAT pathway may be utilized as an anti inflammatory strategy.

miR-21 may exercise anti-inflammatory effects also through silencing of PTEN. Myeloid PTEN is known to promote inflammation (Schabbauer et al, 2012). Furthermore, PTEN silencing strengthens PI3K-Akt signalling, which is known to suppress inflammation (Schabbauer et al, 2004; Oishi et al, 2012; Shimp et al, 2012). Akt is also known to coordinate macrophage transitions and resolution of inflammation during tissue repair (Perdiguero et al, 2011). DOCK (dedicator of cytokinesis) guanine nucleotide exchange factors activate the Rho-family GTPases Rac and Cdc42 to control phagocytosis. Phagocytosis, in addition to clearance of unwanted cells, may influence inflammatory outcomes (Rosas et al, 2008; Maderna et al, 2003). miR-21 may thus influence phagocytosis and inflammation through silencing of DOCK. MCP-1, a key driver of inflammation, is also a biologically validated target of miR-21(Yao et al, 2012). Silencing of MCP-1 represents a potentially influential mechanism that can be added to the anti-inflammatory mechanisms of miR-21.

In specific cases, pro-inflammatory stimuli are reported to induce miR-21,(Okayama et al, 2012) leading to the hypothesis that inflammation is initiated with a

built-in provision to resolve itself. In support of this hypothesis, it is noted that activation of NF-kB, a major driver of inflammation, leads to the induction of miR-21 (Ruan et al, 2011) which in turn may exert anti-inflammatory properties by suppressing NF-kB signalling (Marquez et al, 2010). Likewise miR-21 is also induced by TNFα, a landmark pro-inflammatory cytokine (Roggli et al, 2010). Further studies on miR-21 in macrophage and other cell types are likely to shed new light on the role of miRNA 21 in resolution of inflammation.

HNF4α and miRNAs

miRNA 122 - Among the miRNAs, miR-122 is a paradigm for the role of miRNAs in the liver (Girard et al, 2008). miR-122 was cloned as a liver-specific miRNA and constituted 70% of the total miRNA population in the liver (Lagos et al, 2002; Chang et al, 2004). Functional studies showed that miR-122 was involved in multiple metabolic processes including cholesterol biosynthesis (Krutzfeldt et al, 2005), fatty-acid synthesis, and oxidation (Esau et al, 2006). Moreover, the dependence of HCV replication on miR-122 expression in the liver (Jopling et al, 2005; Henke et al, 2008; Pfeffer et al, 2009) and the loss of miR-122 expression in liver cancer, correlating with the suppression of the hepatic phenotype and the gain of metastatic properties (Coulouam et al, 2009). This suggests that miR-122 is an important component of gene regulatory networks in the liver for normal hepatocyte function.

Li et al has demonstrated that HNF4α binds to the miR-122 promoter region through the conserved DR-1 element, and tranactivates miRNA- 122 promoter

through this element. They also show that, the activation could be further enhanced by the addition of co-activator PGC1α. Using over expression and knockdown strategies, it was also shown that HNF4α positively regulates miR122 expression in both human HCC cell line (Huh7 cells) and the mouse liver (Li et al, 2011).

miRNA 34a and miRNA 200 a, b, c, - Garibaldi et al has shown that HNF4α silencing in RLSCdH (resident liver stem cells derived hepatocytes) and in mouse models correlates with a strong down regulation of miRNA 34a and miRNA 200 a,b,c, expression. This is most likely due to a direct mechanism since endogenous HNF4α was found to be recruited on miR-200 a, b, c and 34a promoters in both RLSCdH and mouse liver. Notably, in HNF4α Knock out mouse models, the obove mentioned miRNAs down regulation correlates to a strong up regulation of the stemness markers SCA1 and FOXA1. Thus, HNF4α, first identified as a positive regulator of hepatocyte differentiation and is recently located at the crossroad of other cellular functional categories (i.e. cell cycle, apoptosis, stress response) appears to also participate in the active repression of stemness via induction of miRNAs (Garibaldi et.al, 2012).

miRNA 124, miRNA 24, and miRNA 629- Hatziapostolou et al group has performed a screen for miRNAs possessing an HNF4α binding site, and identified miR-124 as a preferential HNF4α target. They found that miR-124 was, in fact, an integral part of ta feedback loop, because inhibition of miR-124 by an antisense as-miR-124, or following HNF4α silencing, enhanced STAT3 phosphorylation which was coupled to an induction

of IL-6 secretion and expression of IL-6R, a direct target of miR-124.

The transient inhibition of hepatocyte nuclear factor-4α (HNF4α) following miR-24 and miR-629 induction promotes stable repression of the pro-tumorigenic HNF4α feedback circuit. This results in miR-124 inhibition, followed by an increase in interleukin (IL)-6 secretion and IL-6R expression. These two events lead to hyper phosphorylation of signal transducer and activator of transcription 3 (STAT3), which auto-amplifies the process through the re-induction of miR-24 and miR-629 leading to hepatocellular carcinoma. (Hatziapostolou et al, 2012).

miRNA 34a, miRNA 34c-5p and miRNA 449a – Wang et al has screened for inhibitory effects of microRNAs (miRNA) on the 3′-untranslated region (3′-UTR) of *HNF4α* mRNA. The potential recognition elements of a set of miRNAs were identified utilizing bioinformatics analysis. The family members of miR-34 and miR-449, including miR-34a, miR-34c-5p and miR-449a, share the same target elements located at two distinct locations within the 3′-UTR of *HNF4α.* The over-expression of miR-34a, miR-34c-5p or miR-449a in HepG2 cells led to a significant decrease in the activity of luciferase reporter carrying 3′-UTR of HNF4α. The repressive effect on reporter activity was partially or fully eliminated when one or two of the binding site(s) for miR-34a/miR-34c-5p/miR-449a were deleted within the 3′-UTR. The protein level of HNF4α was dramatically reduced by over-expression of miR-34a, miR-34c-5p and miR-449a, which correlates with a decrease in the binding activity of HNF4α and transactivation of HNF4α target genes. These results suggest that the recognition sites of miR-34a, miR-34c-5p and miR-

449a within 3′-UTR of *HNF4A* is functional. The mechanism of down-regulation of the binding activity and transactivation of HNF4α by the miRNAs involves the decrease in HNF4α protein level via miRNAs selectively targeting *HNF4A* 3′-UTR, leading to the translational repression of HNF4α expression. This miRNA dependent regulation of HNF4α expression affects transactivation and the activity of downstream HNF4α regulated genes that may have important impact on liver phenotype (Wang et al, 2012).

miRNA 24 and miRNA 34 -. Takagi et al identified potential recognition elements for miR-24 (MRE24) in the coding region and the 3′-untranslated region (3′-UTR), and those for miR-34a (MRE34a) were in the 3′-UTR in *HNF4α* mRNA. The HNF4α protein levels in HepG2 cells was markedly decreased by the over expression of miR-24 and miR-34a while its mRNA levels was significantly decreased by the over expression of miR-24 but not by miR-34a. Luciferase analysis in HEK293 cells revealed, that the reporter activity of the plasmid containing the 3′-UTR of HNF4α was significantly decreased by miR-34a while the activity of plasmid containing the HNF4α coding region was significantly decreased by miR-24. These results suggest that the binding sites for miR-24 in the coding region and miR-34a in the 3′-UTR of *HNF4A* gene is functional in the negative regulation by mRNA degradation and translational repression, respectively. The down-regulation of HNF4α by these microRNAs resulted in the decrease of various target genes such as cytochrome P450 7A1 and 8B1 as well as morphological changes and the decrease of the S phase population in HepG2 cells. In conclusion, it was found that human HNF4α was downregulated by miR-24 and miR-34a, the expression of which are

regulated by cellular stress, affecting the metabolism and cellular biology (Takagi et al, 2010).

My goal is to understand transcriptional regulation of miRNA-21 by isoform-specific HNF4α, and decipher its potential functional significance in colon cancer and mouse model of chemical (DSS) induced colitis.

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Chapter 2

Isoform-specific regulation of miRNA 21 by HNF4α in human cancer cell lines.

Abstract

Hepatocyte nuclear factor 4α (HNF4α/NR2A1) is a member of the nuclear receptor superfamily of transcription factors that is important for the development of the liver, intestine/colon, pancreas and kidney. Based on usage of alternative promoters (P1 and P2) and alternate splicing, it is estimated that nine different splice variants of HNF4α exist. The P1- and P2-driven variants differ by 16 amino acids in their N-terminal AB domain that encodes an activation function (AF-1). It has been shown previously that expression of P1-driven HNF4α protein is decreased in different human cancers such as hepatocellular, gastric, renal and colorectal carcinomas, while the P2-driven HNF4α is either unchanged or upregulated. The functional consequences of the up regulation of the P2-driven HNF4α in human cancer are not known. miRNA 21, an oncogenic miRNA has been implicated in the development, growth, progression and metastasis of different types of cancers including colorectal, hepatocellular and pancreatic cancers. Here we investigated a potential link between levels of specific splice variants of HNF4α and miRNA 21 by performing a series of *in vivo* assays. Our results show that ectopic expression of P2-driven HNF4α preferentially regulates miRNA 21 in human embryonic kidney and human colon cancer cell lines. Knockdown of P2-driven HNF4α resulted in preferential decrease of miRNA 21 expression levels in human colorectal cancer cells (Caco-2). In order to understand potential functional significance of miRNA-21 regulation by HNF4α, Knockdown experiments using siRNA targeting both P1- and P2-driven HNF4α were performed that results in downregulation of miRNA 21 levels and

reduces cell proliferation in human colorectal cancer cells (Caco-2). Furthermore ectopic expression of a miRNA 21 mimic in cells in which HNF4α was knockdown restored cell proliferation. In exploring the molecular mechanism by which HNF4α regulates miRNA 21, we observed that HNF4α binds the promoter region of miRNA 21 *in vitro* and up regulates its promoter activity in *vivo*, furthermore transient transfection assay using a Luciferase (LUC) reporter suggests that P2-driven HNF4α preferentially activates the human miRNA 21 promoter compared to P1-driven HNF4α. Taken together, our observations lead us to propose that P2-driven HNF4α preferentially activates miRNA 21, an oncogenic miRNA, thus suggesting a functional significance of continued P2-driven HNF4α expression in colon cancer.

Introduction

Hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear receptor superfamily, has been shown to play major roles in hepatic homeostasis, epithelial differentiation in the colon and pancreatic β cell development (Garrison et al, 2006; Gupta et al, 2007; Gupta et al, 2005; Miura et al, 2006). Transcription from alternate promoters (referred to as P1 and P2) and alternate splicing predict at least nine different variants of HNF4α (Drewes et al, 1996; Thomas et al, 200). Expression analysis has revealed tissue-specific expression of different HNF4α isoforms. The P1-driven HNF4α isoforms are expressed in adult liver, proximal tubular cells of kidney, intestine, colon and fetal pancreas. On the other hand, the expression of P2-driven isoforms of HNF4α is detected in fetal liver, adult pancreas, stomach, intestine, colon, ovary, and mammary tissue (Harries et al, 2008; Ishikawa et al, 2008; Jiang et al, 2003; Kanazawa et al, 2008; Torres-Padilla et al, 2001). In addition to the major role of HNF4α as a central metabolic regulator, there is strong evidence exists for its involvement in regulating cell proliferation (Chiba et al, 2005; Erdmann et al, 2007; Hwang-Verslues and Sladek, 2008; Lucas et al, 2005; Yin et al, 2008b), apoptosis (Erdmann et al, 2007) and maintenance of the epithelial morphology exists (Lazarevich and Fleishman, 2008; Spagnoli et al, 2000). A few of these studies have also pointed to the functional differences between P1- and P2-driven HNF4α in regulating cell proliferation and apoptosis (Erdmann et al, 2007; Gupta et al, 2007; Niehof and Borlak, 2008; Algamas et.al, 2013; Rieck et.al, 2012). Taken together, these

studies suggest that P1-driven HNF4α is anti-proliferative, while P2-driven HNF4α may be pro-proliferative.

The dichotomy in the function of HNF4α isoforms is consistent with other reports indicating that expression of P1-driven HNF4α isoforms are down regulated in different human cancers such as hepatocellular, gastric, renal and colorectal carcinomas, while the levels of P2-driven isoforms remain unchanged or upregulated (Oshima et al, 2007; Tanaka et al, 2006; Chellappa et al 2012). Both P1- and P2-driven HNF4α are expressed in the normal adult gut. Three studies have examined the expression pattern of HNF4α isoforms in colon cancer. The first study reported the expression of P2-driven HNF4α in all the normal and cancerous tissues examined, while P1-driven HNF4α was expressed in only 5 out of 18 primary colorectal carcinomas. Furthermore, in cases where P1-driven HNF4α expression was detected, the nuclear accumulation of HNF4α was found to be much less than in the normal tissues. A second study revealed that only 26 out of 43 tumors stained positive for a P1-HNF4α specific antibody (P1), while all 43 tumors showed staining with an antibody that recognizes both P1- and P2-driven HNF4α (P1/P2), suggesting that P2-driven HNF4α remains unchanged or up regulated. Finally a third study from our lab examined a larger set of tumor samples revealed that approximately 80% of tumor samples showed P1-HNF4α staining that was not exclusively nuclear, this was in contrast to normal tissues where HNF4α staining was observed exclusively in the nucleus (Chellappa et.al, 2012). Whereas many intriguing links exist between HNF4α isoforms and cancer, the functional differences between the

P1- and P2-driven HNF4α proteins have not been thoroughly examined and the spectrum of the target genes regulated by these two isoforms has not been well defined.

Two colon cancer studies have shown that high miRNA-21 expression in tumors predicts a worse overall survival suggesting a correlation between higher level of miRNA 21 and poor therapeutic outcome (Schetter et al, 2008; Xie et.al, 2010). Yang et al. showed that overexpression of miRNA 21 is associated with worse prognosis and poorer response to chemotherapeutics in colorectal cancer (Yang et al, 2009). A third study reported that miRNA 21 directly targets the 3′ untranslated region (UTR) of MSH2 and MSH6 mRNA (genes in misatch repair pathway (MMR) and inherited mutations in MMR genes lead to cancer, and also chemoresitance), resulting in down regulation of protein expression, an inverse correlation between miRNA 21 overexpression and MSH2 expression was shown in colorectal carcinoma tissues (CRC) (Valeri et.al, 2010), in the same study it was also shown that cells that overexpress miRNA 21 showed significantly reduced 5-fluorouracil (5-FU)- induced G2/M damage arrest and apoptosis that is characteristic of defective Mismatch repair (MMR). 5-fluorouracil is an anti-cancer ("antineoplastic" or "cytotoxic") chemotherapy drug used to treat several types of cancer including colon, rectum, head and neck cancer. Thus, miRNA 21-dependent downregulation of MSH2 and MSH6 may be responsible for both primary (resistance during treatment) and secondary resistance (due to metastatis of primary tumors) to 5-FU.

In this study, we investigated a potential link between HNF4α and miRNA-21 and its role in cancer using two colon cancer cell lines. Our work reveals that P2-driven HNF4α activates miRNA 21 levels more readily than P1- driven HNF4α,

suggesting a potential function of P2- driven HNF4α in promoting proliferation, and tumor growth in cancer via activation of miRNA 21.

MATERIALS AND METHODS

Cell culture

Human colon cancer cell lines (HCT116 and Caco-2) and human embryonic kidney cell (293T) were purchased from ATCC (Manassas, VA). Caco-2 and 293T cells were cultured in Dulbecco's Modified Eagle Medium from GIBCO (Grand Island, NY) supplemented with 10% fetal bovine serum (Cellgro, Mediatech Inc, Herndon, VA) and 1% pencillin/streptomycyin. HCT116 cells were cultured in McCoy's 5A media from GIBCO (Grand Island, NY) supplemented with 10% fetal bovine serum (Cellgro, Mediatech Inc, Herndon, VA) and 1% pencillin/streptomycyin. All cells were maintained at 37^0C at 5% CO2.

Construction and Generation of the Tet-On HNF4α Inducible cell lines of the Tet-On HNF4α Inducible plasmids

Doxycyclin-inducible HNF4α2 and HNF4α8 HCT116 cell lines were constructed by Linh voung in the sladek lab. pTRE.hHNF4α2 and pTRE.hHNF4α8 were made by sub cloning the human HNF4α2 (NM_000457) and HNF4α8 (NM_175914.3) cDNAS from the pcDNA3.1 (+) vector (generous gifts from Dr. Christophe Rachez), into the pTRE.Tight vector (Clontech) at the respective EcoRI/BamHI sites. The reverse tetracycline-

controlled transcriptional activator (pCAG.rtTA.IR.PURO) plasmid was a gift from Dr. Chee-Gee Liew.

Human HCT116 colorectal cancer cells were cultured as described above but with TET free serum. For transfection, HCT116 cells were seeded at $3x10^6$ cells/well in a 6-well plate. The next day, 1 μg of linearized pCAG.rtTA.IR.PURO DNA was transfected into the cells via Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were trypsinized and cells from 1 well of a 6-well plate were transferred to a 150 mm plate and selected for puromycin resistant colonies, starting with 0.50μg/mL of the antibiotic. Colonies were picked and tested for rtTA. The chosen clone (cl11) went through a second transfection and selection. This time, linearized pTRE.hHNF4α2 or pTRE.hHNF4α8 plus a fragmented Neo^R gene, at a 10:1 ratio respectively, were transfected into the cells via Lipofectamine 2000. The Neo^R gene was cut out from the pTet-on vector (Clontech) at the XhoI sites. The selection process started with 50 μg/mL of the G418 antibotic and increased to 70 μg/mL. All picked clones were expanded and analyzed for inducible expression of HNF4α2 or HNF4α8 via immunoblotting.

Immunoblotting for HNF4α

Samples were harvested using RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25 % sodium deoxycholate, 0.5 mM EDTA, 150 mM sodium chloride), plus inhibitors 1μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate plus1 mM sodium fluoride, 1 mM PMSF, phosphatase inhibitor cocktail I & II (1:100) (Catalog# P2850, Catalog# P5726, Sigma-aldrich), and protease inhibitor cocktail

(Catalog# P8340, Sigma-aldrich) (1:10). Protein concentrations of cell lysates were measured using the Bradford reagent. Samples analyzed by 10% SDS-PAGE were transferred onto PVDF membrane (Millipore). Transferred blots were blocked with 5% milk in 1xTBST (10 mM Tris pH 8.0, 150 mM sodium chloride, 0.05% Tween 20) for 15 min at room temperature. Blots were then incubated at 4^0C overnight with primary antibody (1.10000) (P1/P2-driven HNF4α, Clone H1415 Catalog # PP- H1415- 00, R & D Systems). The blots were washed three times with 1xTBST and incubated with goat anti –mouse Ig conjugated to HRP (1:5000) for 2 hrs at room temperature. Blots were then washed three times with 1xTBST and three times with 1xTBS (10 mM Tris pH 8.0, 150 mM sodium chloride) and developed by electro chemiluminesence (Dura kit, Pierce) Gels were stained with Coomassie after immunoblotting to ensure equal protein loading.

Immunoblotting for Cyclin D1

Samples analyzed on 10% SDS-PAGE were transferred onto PVDF membrane. Transferred blots were then blocked with 5% milk (in 1xTBS) for 25 min at room temperature, and washed three times with 1xTBST. Blots were then incubated with cyclin D1 antibody (1.1000 in 1xTBST & 5% milk) at 4^0C overnight, washed three times with 1xTBST (Catalog #2922S, Cell Signaling). Blots were then incubated with goat anti-Ig rabbit conjugated to HRP (1:20,000 in 1xTBST) for 2 hrs at room temperature, washed three times with 1xTBST and 1xTBS and developed with the Dura reagent.

Stem loop RT-PCR for analyzing miRNA level

Total RNA was extracted from mice distal colon by using Qiagen miRNEASY kit. In brief, the reverse transcription reaction was performed in 15 μL containing 1μg of total RNA, 1 μL 10 mM dNTPs, 0.5 μL reverse transcriptase (Invitrogen Superscript 111 RT kit), 1 μL stem-loop reverse transcriptase primer, 4 μL 10× reverse transcription buffer, 2 μL 1mM DTT, 1 μL RNAase out, and 10.5 μL DEPC water. For synthesis of cDNA, the reaction mixtures were incubated at 16°C for 30 min, followed by pulsed Reverse transcription of 60 cycles at 30°C for 30 sec, at 42°C for 30 sec and then 50°C for 1 sec. The end point PCR reaction was performed with a final volume of 20 μL, containing 10 μL cDNA, 0.5 μL Taq polymerase, 0.4 μL forward primer, 0.4 μL reverse primer, 0.4 μL 10 mmol dNTP mix, 2 μL 10× PCR buffer and 6 μL DEPC water (1 cycle of 95°C for 5 min, followed by 25-35 cycles of 95°C for 15 sec and 60°C for 1 min). All reactions were performed in triplicates. miRNA levels were normalized using miRNA 192 or U6 RNA as internal reference genes. The sequences of primers used for amplification are listed in TABLE 2.1. The reaction products were analyzed on a 4% agarose gel in 1xTBE and visualized by UV illuminometer of ethidium bromide stained gels.

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Construction of human miRNA 21 promoter

The miRNA 21 promoter region was amplified from human genomic DNA by using PCR. The 460-bp fragment containing two putative HNF4α binding sites was amplified

using the forward primer-5′ TGTATTCTGGGTAAGAAGGA3′ and the reverse primer-5′-TACTCTGGTATGGCACAAAGAA3′. The promoter was first cloned into PCR-Blunt 11 Topo vector that was further digested by restriction enzymes KpnI and EcoRV. Finally the digested 460-bp fragment was cloned into the luciferase reporter plasmid, pGL4.10 (luc) (Promega). The sequence fidelity of PCR amplified fragment was verified by sequencing in ucr genomics core.

Transient transfection: Luciferase assay

293T cells were plated (0.2 $X10^6$ cells) in 6-well plates. After 24 hours, the cells were transfected using Lipofectamine reagent according to the manufacturer's protocol. Different amounts of pcDNA3.1 expression vectors containing either human HNF4α2 (NM_000457), or human HNF4α8 (NM_175914.3) or empty vector (pcDNA 3.1), 500 ng of human miRNA 21 promoter luciferase reporter and 200 ng of CMV.βgal were used. Cells were harvested after 24 hours using Triton lysis buffer (1% Triton X-100, 25 mM Gly-Gly pH 7.8, 15 mM magnesium sulfate, 4 mM EGTA, 1 mM DTT). Luciferase and β-gal activity were measured as described previously (Maeda et al, 2002a). Significant differences between pcDNA 3.1 and human HNF4α2 or human HNF4α8 were determined by student's t-test.

Transfection of miRNA 21 mimic or HNF4α siRNA in Caco-2 cells:

Chemically modified miRNA 21 mimic designed to increase miRNA 21 levels activity and miRNA mimic-NC (based on *C elegans* miRNA with minimal sequence identity in

human and mouse) were purchased from Dharmacon (Cat # 300492-03-0005). The miRNA 21 mimic sequence used was 5′UAGCUUAUCAGACUGAUGUUGA3′. siRNAs complementary to either the P1-driven HNF4α or P2-driven HNF4α custom synthesized by Dr chellappa from sladek lab and siRNAs complementary to both P1 and P2- driven HNF4α and to inhibit endogenous HNF4α expression and siRNA for negative control were obtained from Dharmacon. Cells were transfected with the miRNA 21 mimic or the siRNAs against HNF4α using RNAi/MAX (Invitrogen) according to the manufacturer's instructions. Briefly, the day before transfection, the cells were seeded into 6-well plates. After 24 hours, 30 – 50 nmoles of siRNA/ HNF4α, and or 200 nmoles of the miRNA 21 mimic were transfected using RNAi/MAX. After incubation for 90 hrs, the cells were harvested, and whole cell lysates and total RNA were prepared and analyzed as described above. All the siRNA sequences are listed in TABLE 2.1.

Electrophoretic mobility shift assay (EMSA).

Briefly, 293T cells (2×10^6) were plated in 100-mm tissue culture dishes. After 24 h cells were transfected with 10 μg of wild type or mutant human HNF4α2 using Lipofectamine 2000. Nuclear extracts were prepared from transfected 293T cells. and immunoblot analysis was carried out to determine the amount of wild type or mutant HNF4α2 expressed. Two sets of double stranded DNA oligos of approximately 30 mer that has potential HNF4α binding site on human miRNA 21 promoter is ordered from IDT. The sequence of oligo DNA top strand for first probe is a) 5’AAAAGCTCCGAGTACATAAATTTATCAAAGATCACTATCCCAAT is 3’ The

sequence of oligo DNA top strand for second probe is (b) 5'AAAAGCTCCGAGTACATAAATTTATCAAAGATCACTATCCCAAT 3'. The sequence for bottom strand used to anneal the top strand is the reverse complement of one of the top strands given above. A standard gel shift reaction of 15 μL was set up with 1 to 2 μg total nuclear protein (normalized to HNF4α2 protein level with mock transfected nuclear extract), 0. 5 ng of 32 P-labeled double stranded oligonucleotide corresponding to −322 to −335 and -401 to -413 of the human miRNA 21promoter and 1 μg of either HNF4α anti body α445) or bovine serum albumin (BSA) and run on a 8% native polyacrylamide gel. Dried gels were then subjected to autoradiography

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Results

Isoform-specific regulation of miRNA 21 by HNF4α

In a physiologically normal colon both P1- and P2-driven HNF4α (HNF4α2 and HNF4α8) isoforms are present. In order to dissect isoform-specific functions of HNF4α, human cancer cell lines that do not express endogenous HNF4α were used so that we could ectopically express either human HNF4α2 or human HNF4α8, and independent study their effects. However initially we employed human embryonic kidney cells (293T) due to their superior transfection efficiency, which is approximately 90%.

Transient over expression of P2-driven HNF4α preferentially activates endogenous miRNA 21 expression levels compared to P1-driven HNF4α and control plasmid (pcDNA3.1) in 293T cells (Fig 2.1). Since the aim of this study is to dissect the function of isoform-specific HNF4α in the colon, we used a colon cancer cell line

(HCT116 cells) that does not express endogenous HNF4α. Ectopic expression of P2-driven HNF4α preferentially activated endogenous miRNA 21 expression compared to P1-driven HNF4α, and control plasmid (pcDNA 3.1) in HCT116 cells (Fig 2.2). Next we used stable and inducible HCT116 cell lines that express either HNF4α2 or HNF4α8 upon treatment with doxycyclin (Doxy). Stable induction of HNF4α8 by using 1 ug/ml doxy preferentially activated endogenous miRNA 21 compared to HNF4α2 and the parental clone (Fig 2.3). In both transient and stable assays done on HCT116 cells, the increase in miRNA 21 expression correlated with an increase in cyclin D1 protein levels (Fig 2.2, 2.3). Recently, Raymond et.al reported that miR-21 enables rapid hepatocyte proliferation during liver regeneration by accelerating cyclin D1 translation by relieving Akt1/mTOR complex 1 signaling (and thus eIF-4F–mediated translation initiation) from Rhob-mediated suppression (Raymond et.al; 2012). This findings, along with our results, suggest that HNF4α may regulate Cyclin D1 through miRNA 21, and that P2- driven HNF4α may do so in a more effective fashion than P1- driven HNF4α.

Functional consequence of HNF4α gene silencing in Caco-2 cells

The studies in 293T and HCT116 cells show that ectopic expression of HNF4α activates the expression of the endogenous miRNA 21 gene. Inorder to examine the role of the HNF4α isoforms in a some what more physiological setting, another human colon cancer cell line Caco-2 which expresses both endogenous P1- and P2- driven HNF4α was used. Caco-2 cells were treated with siRNAs targeting both P1- and P2-driven HNF4α (si P1/P2) which resulted not only in decreased HNF4α protein but also miRNA 21 levels

(Fig 2.4A), interestingly it also resulted in a decrease in cell content (Fig 2.4B compare lane 5 to 3 and 2). However the miRNA 21 mimic also increased the cell count above that of untransfected control (Fig 2.5 compare lane 4 to lane 1), suggesting that HNF4α increases cell number at least partially via activation of miRNA 21, further to dissect the differential activation of miRNA 21, by HNF4α, Caco-2 cells were transfected with siRNA for negative control (siC), or P1-driven HNF4α (siP1), or P2-driven HNF4α (siP2) or both P1- and P2-driven HNF4α (siP1/P2). Immunoblot and stem loop RT-PCR analysis revealed that knockdown of P2-driven HNF4α led to greater decrease of miRNA 21 levels than did knockdown of P1-driven HNF4α (Fig 2.6). Intrestingly, the siP2 also lead to an increase in P1-driven HNF4α but these was not sufficient to increase miRNA 21 back to control levels. Taken together these results indicate that while P1-driven HNF4α regulates the expression of miRNA 21 in Caco-2 cells, but P2-driven HNF4α preferentially regulates it more than P1-driven HNF4α.

HNF4α and miRNA 21 expression in different colon cancer cell lines

Three human colon cancer cell lines expressing different levels of endogenous HNF4α protein were examined for expression of miRNA 21. The high HNF4α expressing cell lines, such as Caco-2, exhibited higher miRNA 21 levels than SW480 and HCT 116 cells which exhibit very low or undetectable levels of HNF4α (Fig 2.7).

miRNA-21 is a direct transcriptional target of HNF4α

The functional correlation that was observed between HNF4α and miRNA 21 necessitates the elucidation of the mechanism by which HNF4α regulates miRNA 21. HNF4α may regulate miRNA 21 through direct transcriptional control or indirectly by

activating some undefined intermediate. To examine the possibility of HNF4α binding directly to the regulatory region of the miRNA 21 promoter, the human miRNA 21 promoter was analyzed for HNF4α binding sites by using HNF4α motif finder (Bolotin et al, 2010). In order to improve the prediction of potential HNF4α target genes, the Sladek lab analyzed HNF4α2 in the protein binding microarray (PBM), in *vitro* high throughput DNA binding assay. They also used Support Vector Machine (SVM), a powerful machine learning model to predict additional HNF4α binding sequences with high accuracy. Both the PBM and SVM binding sites were combined and integrated into HNF4α motif finder (http://nrmotif.ucr.edu/fuzzhtmlform.html). The HNF4 motif finder was used to identify two potential HNF4α binding sites in the human miRNA 21promoter (Fig2.8 A, 2.8 B).

An earlier study by Fujita et. al delineated the human miRNA 21 promoter region as being from approximately -460 to +1 (Fujita et.al; 2008). Transient transfection of HNF4α8 into 293T cells at low concentrations (12.5 ng, and 25 ng) activated the human miRNA 21 promoter approximately two-fold compared to HNF4α2 or to the control plasmid (pcDNA 3.1), in contrast transfection of HNF4α2 at low doses did not activate miRNA-21 luc reporter however at high concentrations (50 ng) both HNF4α2 and HNF4α8 activated the miRNA 21 promoter by approximately three-fold compared to control plasmid. Immunoblot analysis revealed equal amounts of HNF4α2 and HNF4α8 protein levels. Taken together, luciferase assays suggest that human HNF4α8 activates miRNA 21 promoter more robustly than human HNF4α2 at lower concentrations (Fig

2.8) despite the fact that HNF4α2 typically activates gene expression more robustly than HNF4α8.

Both P1- and P2-driven HNF4α bind to the sequences on human miRNA 21 promoter

In order to determine the DNA–protein interaction between HNF4α and predicted binding sites on miRNA 21 promoter, gel shift assays were performed with oligonucleotides corresponding to -322 to -335 bp and −401 to −414 bp region of miRNA 21 Promoter. The radiolabeled oligonucleotide probe formed DNA–protein complexes upon incubating with nuclear extracts of both HNF4α2 and HNF4α8 transfected cells. The specificity of DNA–protein complexes was further confirmed by the supershift when incubated with HNF4α antibody (α445) (Fig 2.9).

Table 2.1: List of primers

has-mir-21 RT primer	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA 3'
has-mir-21 F primer	5' GCGGCGGTAGCTTATCAGACTGA 3'
Universal Reverse primer	5' GTGCAGGGTCCGAGGT 3'
has-mir-194-2 RT primer	5' GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA 3'
has-mir-194 F primer	5' GCGGCGG TGTAACAGCAACTCCA 3'
U6 RT primer	5' CGCTTCACGAATTTGCGTGTCAT 3'
U6: Forward	5' GCTTCGGCAGCACATATACTAAAAT 3'
U6: Reverse	5' CGCTTCACGAATTTGCGTGTCAT 3'
has-mir-192 RT primer	5' TGGATACGACTCAACA GTGGTATCCAGTGCAGGGTCC 3'
has-mir-192 F primer	5' GCGGCGGCTGACCTATGAATT 3'
mmu-miR-21 promoter F	5' CTCGCCAGCAGTAATGCATT3'
mmu-miR-21 promoter R	5' TGGTAGCTTCAGTTGCCAGA 3'
hsa miRNA 21 promoter F -	5' TGTATTCTGGGTAAGAAGGA 3'
hsa miRNA 21 promoter R –	5' TA CTCTGGTATGGCACAAAGAA 3'
siP1-HNF4a Sense	5' UUGAGAA UGUGCAGGUGUUUU 3'
siP1-HNF4a Antisense	3' UUA A CUCUUACA CGUCACAA (5'-P) 5'
siP2-HNF4a Sense	5' GUGGAGAGUUCUUACGACAUU 3'
siP2-HNF4a Antisense	3' UUCACCUCUCAAGA AUGCUGU (5'-P) 5'

Figure 2.1: hHNF4α8 preferentially activates endogenous miRNA 21 expression in in 293T cells. Whole cell lysates and total RNA were isolated from 293T cells transfected with pcDNA3.1, HNF4α2 or HNF4α8 for 24 hrs. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. The miRNA-21 levels were determined by stem loop RT-PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coomassie staining verified equal loading of protein extracts. This experiment has been repeated three times with similar results. Shown is the representative experiment of a total of four experiments done.

.

Fig 2.1

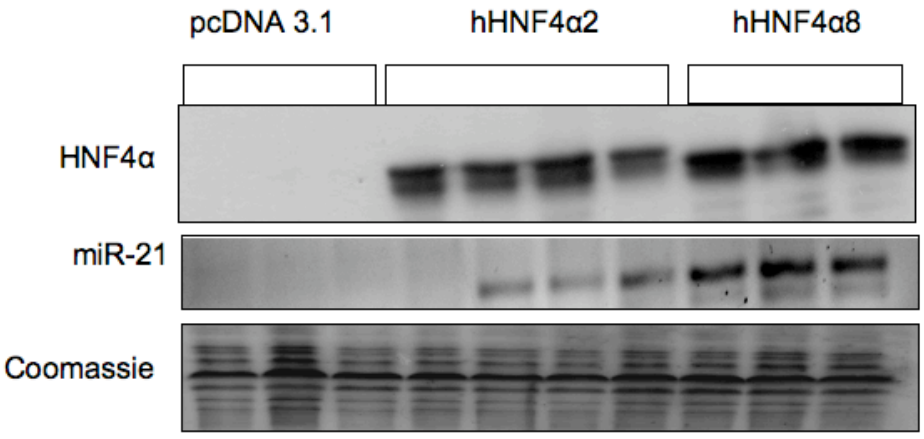


Figure 2.2: HNF4α8 preferentially activates endogenous miRNA 21 expression levels by transient transfection in HCT116 cells. Whole cell lysates and total RNA were isolated from HCT116 cells transfected either with pcDNA3.1, HNF4α2 or HNF4α8 for 24hrs. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. The miRNA-21 levels were determined by stem loop RT-PCR and normalized with the U6 small nuclear RNA levels. The PCR reaction products were analyzed by electrophoresis on a 4% agarose gel in 1 X TBE. Shown is representative experiment of a total of two experiments done.

Fig 2.2

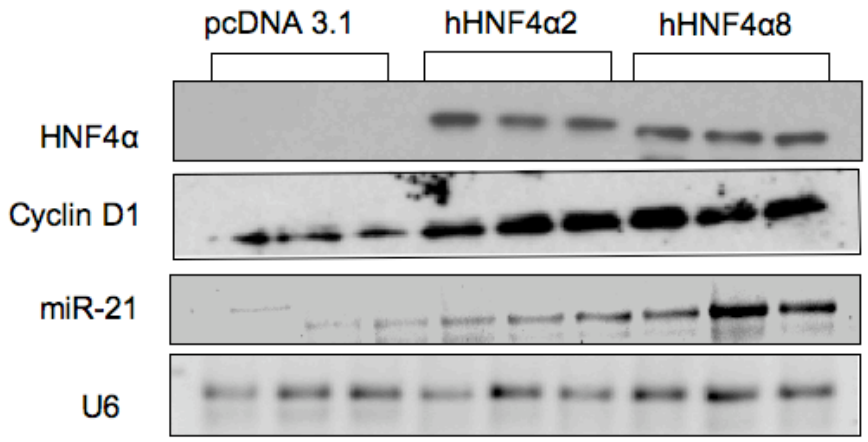


Figure 2.3: Human HNF4α8 preferentially activates endogenous miRNA 21 expression levels in stable inducible HCT116 cells. Whole cell lysates and total RNA were isolated from stable inducible HCT116 cells expressing human HNF4α2 or HNF4α8. Cells were induced with doxycyclin (1 μg/ml conc) for 36 hrs. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. The miRNA-21 levels were determined by stem loop RT-PCR and normalized to the U6 small nuclear RNA levels. The PCR reaction products were analyzed by electrophoresis on a 4% agarose gel in 1 X TBE. Shown is representative experiment of a total of five experiments done.

Fig 2.3

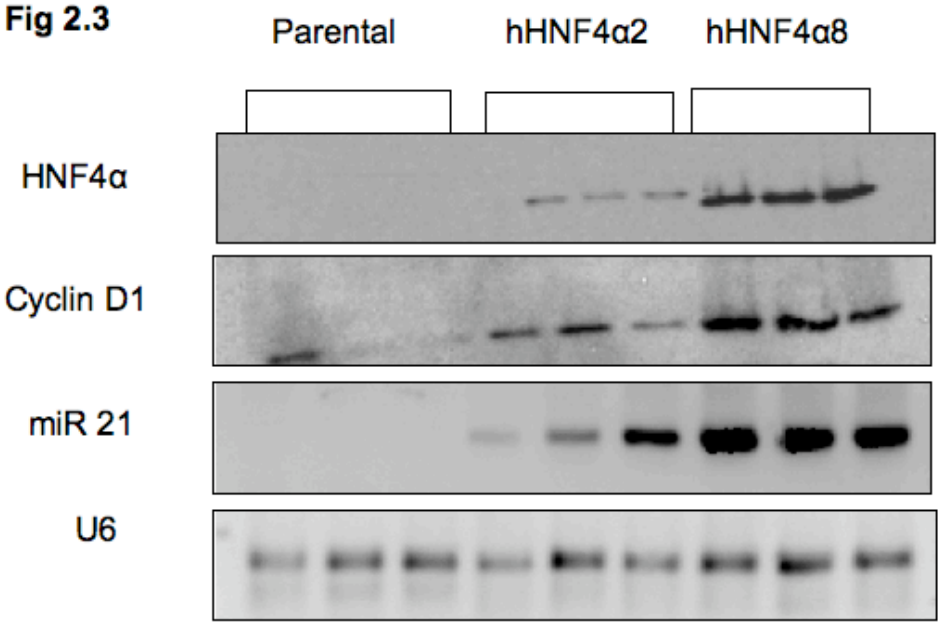
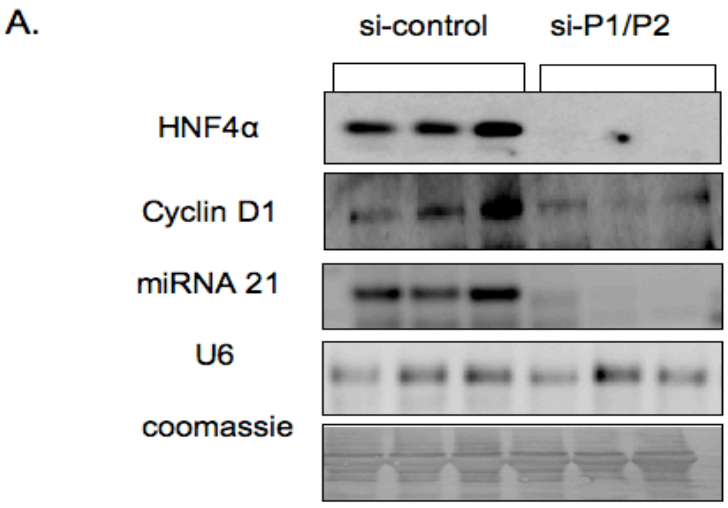


Figure 2.4: A. Knockdown of HNF4α leads to down regulation of miRNA-21 and Cyclin D1 expression levels in Caco-2 cells. Whole cell lysates and total RNA were isolated from Caco-2 cell lines transfected with siRNAs for HNF4α (si P1/P2) for 90 hrs. HNF4α and cyclin D1 protein levels were determined by immunoblot using 30 μg of whole cell extracts. The miRNA 21 levels was determined by stem loop RT-PCR and normalized with the U6 small nuclear RNA levels. Shown is representative experiment of a total of three experiments done.

B. Knockdown of HNF4α decreases cell number in Caco-2 cells. Cells were collected 90 hrs after transfection with 30 nmoles of siRNA for HNF4α (si P1/P2), and the cell number is counted by using automatic cell counter. Shown is representative experiment of a total of three experiments done.

Fig 2.4



B.

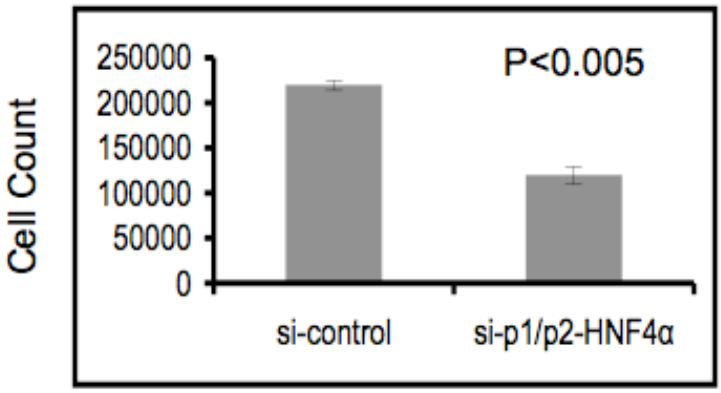
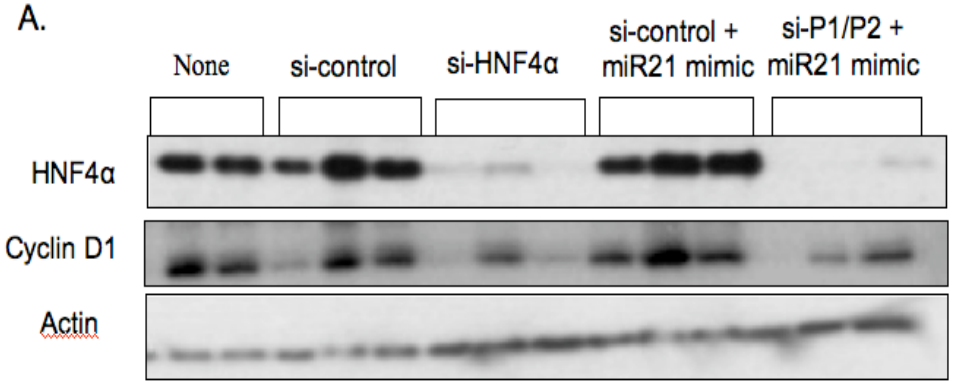

Cell Count
250000
200000
150000
100000
50000
0
P<0.005
si-control
si-p1/p2-HNF4α

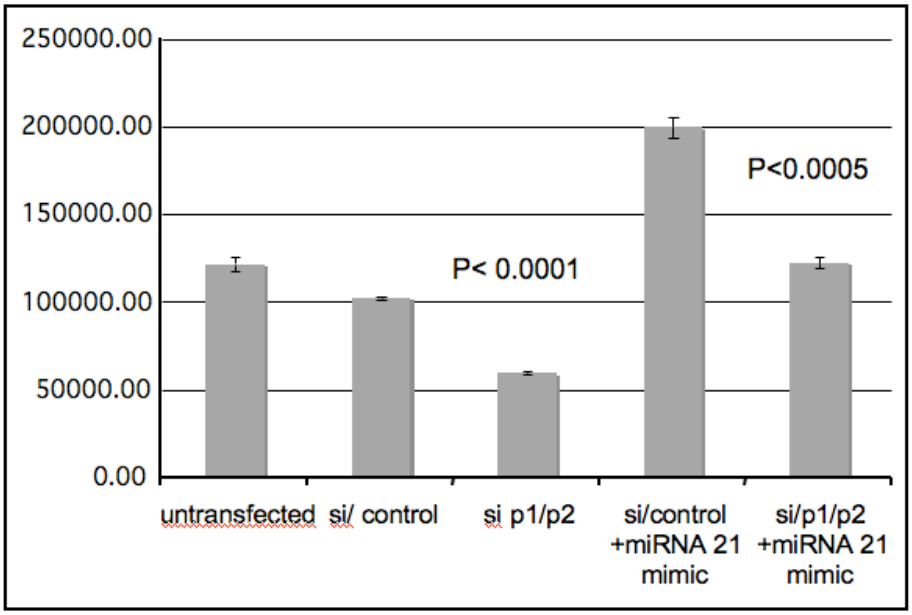
Figure 2.5: miRNA 21 mimic restores cell proliferation in HNF4α knockdown cells. **(**Cells were collected 90 hrs after transfection with either siRNA targeting P1- and P2-driven HNF4α (siP1/P2), or miRNA 21 mimics (200 nanomoles) as indicated in figure. Cell counting was done using an automatic cell counter. This experiment was performed in triplicate once. Shown is representative experiment of a total of one experiment done.

Fig 2.5

A.


None
si-control
si-HNF4α
si-control + miR21 mimic
si-P1/P2 + miR21 mimic
HNF4α
Cyclin D1
Actin

B.


250000.00
200000.00
150000.00
100000.00
50000.00
0.00
P<0.0005
P< 0.0001
untransfected
si/ control
si p1/p2
si/control +miRNA 21 mimic
si/p1/p2 +miRNA 21 mimic

.

Figure 2.6: Knockdown assays suggest that HNF4α8 preferentially activates miRNA 21 in Caco-2 cells. Whole cell lysates and total RNA were isolated from Caco-2 cells, transfected with siRNAs for different isoforms of HNF4α for 90 hrs as indicated. HNF4α protein levels were determined by immunoblot by using 30 μg of whole cell extracts. The miRNA 21 levels was determined by stem loop RT-PCR and normalized with the U6 small nuclear RNA levels. Shown is representative experiment of a total of two experiments done.

Fig 2.6

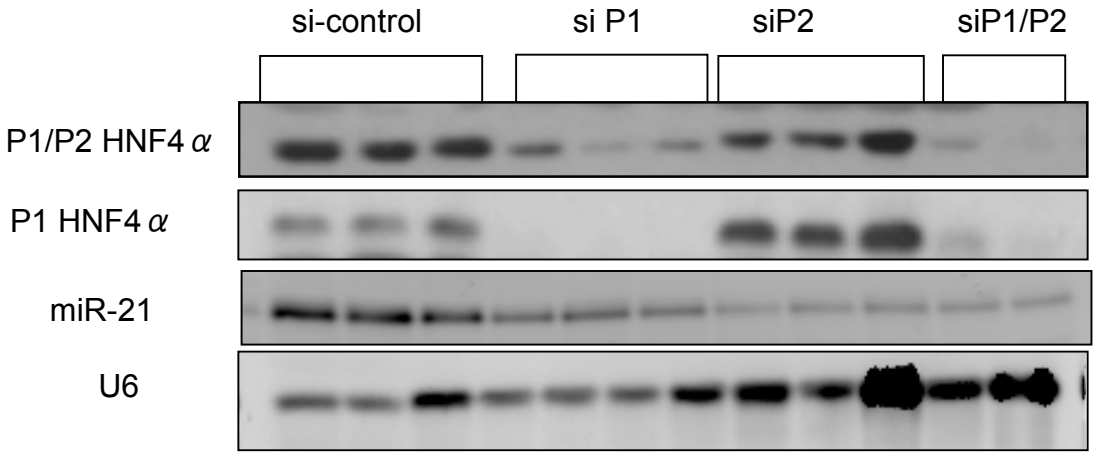


Fig 2.7: Corelation between endogenous HNF4α and miRNA 21 expression in different colon cancer cell lines. Whole cell lysates and total RNA were isolated from Caco-2, SW480 and HCT116 cells. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. miRNA-21 levels were determined by stem loop RT-PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coommasie staining allowed for normalization of protein loading. Shown is representative experiment of a total of two experiments done.

Fig 2.7

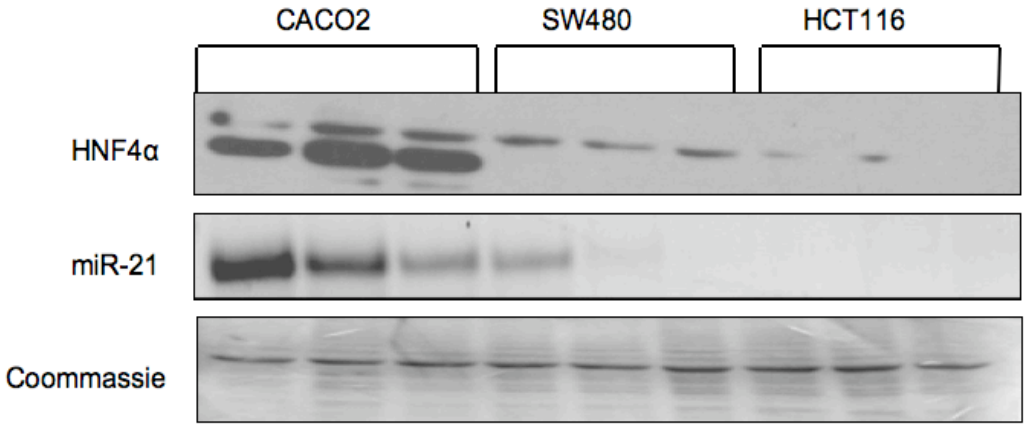


Figure 2.8: miR-21 is a transcriptional target of HNF4α.
A) Human miRNA 21 promoter sequence.
B) Schematic diagram of the human miR 21 promoter with the two potential HNF4α binding sites
C) 293T cells were transfected with indicated amounts of either HNF4α8 or HNF4α2 (**6.5, 12.5 or 25 ng**), 500 ng of miRNA 21 promoter luciferase reporter and 200 ng CMV.β-galactosidase. Cells were harvested after 24 hrs and luciferase and β-galactosidase activity were determined. Each treatment group was performed in triplicate; data are shown as the mean +/- standard deviation, shown is one of the two representative experiments performed in duplicate.
D) HNF4α protein levels from tranfected 293T were determined by immunoblot analysis, using 30 μg of whole cell extracts

Fig 2.8

A.

•1 TGTATTCTGG **GTAAGAAGGA GCTCCGAGTA CATAAA TTTA TCAAAGATCA**
•51 **CTATCCCAAT CATCTCAGAA CAAGCTGTTA CTAATGTACT GGAGTTTCTG**
•101 **TGCAAACTGT CTACCATAAA CCATGAAAG G ATTCAAAGTT CA TAGTTCCT**
•151 **TCTTTGTTCC TTTGTTAATC ACTGACTTCT GACTAGTGGG AGGTGCCTCC**
•201 **CAAGTTTGCT AATGCATTCT TTTTGaA GGATGACGCA CAGATTGTCC**
•251 **TAATAAGGAC TTAGATTGAG AAGACCGCC CCCTCTGAGA AGAGGGGACA**
•300 **AGTCAGAGAG AGGGCGGGCA GTTTCTTTTT TAACTAGGGA TGACACAAGC**
•301 **ATAAGTCATT TCCTTATTAA TTGGTTCAAA CCAGTTCTTA CAGGAACTAG**
•351 **TGGTGATAAA TGTGGGACTT CTGAGAAGTC ATTCATTTTA TTCTTTGTGC**
•401 **CATACCAGAG TA**

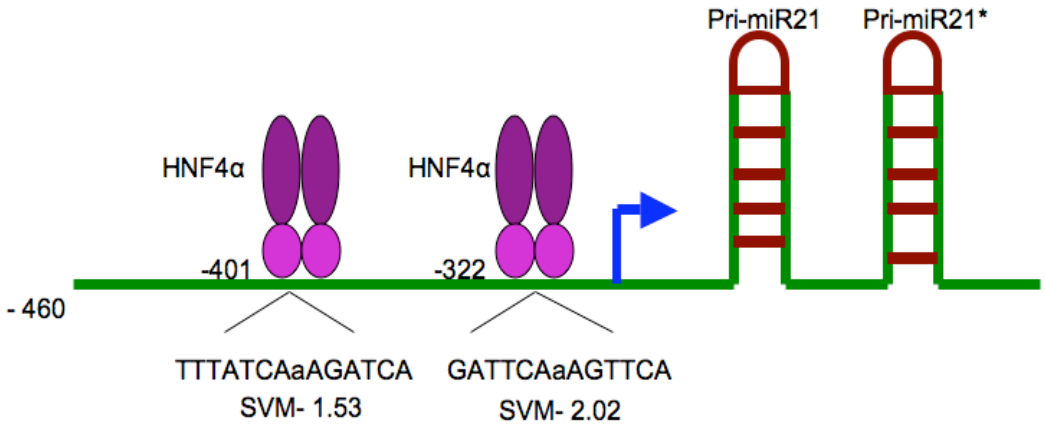
HNF4a binding sites by SVM

GC BOX

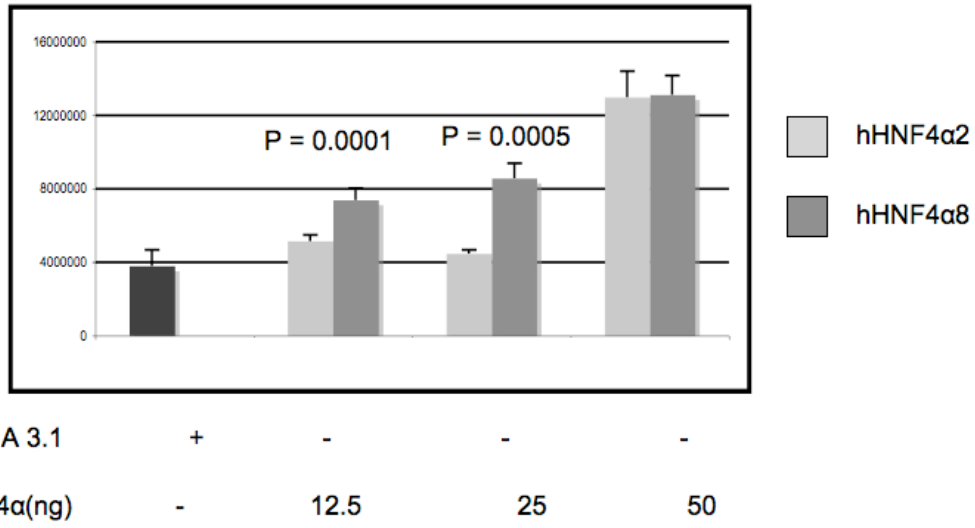
TATA BOX

STAT 3 binding sites

B.


Pri-miR21
Pri-miR21*
HNF4α
HNF4α
-401
-322
- 460
TTTATCAaAGATCA
SVM- 1.53
GATTCAaAGTTCA
SVM- 2.02

C.


16000000
12000000
8000000
4000000
0
P = 0.0001
P = 0.0005
hHNF4α2
hHNF4α8
PCDNA 3.1 + - - -
hHNF4α(ng) - 12.5 25 50

D.

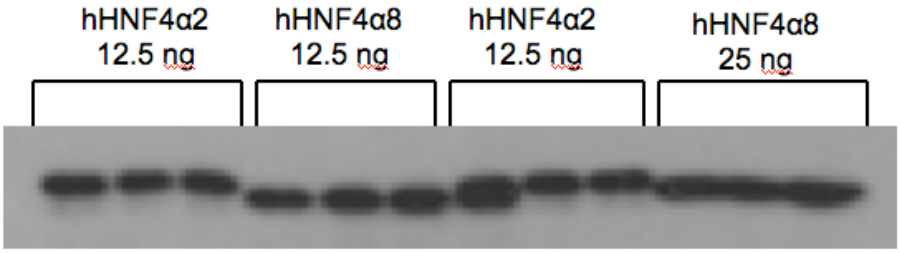
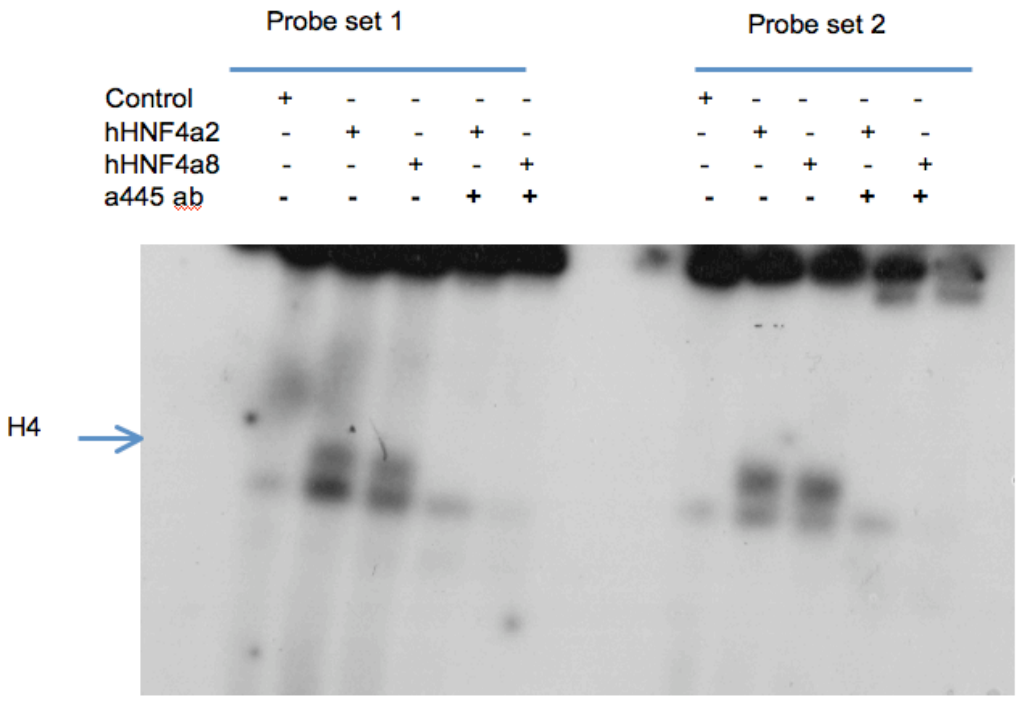


Fig 2.9: P1- and P2-driven HNF4α bind to the sequences on human miRNA 21 promoter. EMSA was performed with nuclear extracts from 293T cells expressing either control plasmid, or hHNF4α2 or hHNF4α8. Antibody to HNF4 (a445) is added for supershift analysis. EMSA was performed using the -322 to -335 and -401 to -414 predicted HNF4α binding sites from the miR-21 promoter. HNF4:DNA complexes are indicated. This experiment is repeated once

Fig 2.9



Discussion

There are two promoters in *HNF4A* gene that drive the expression of HNF4α transcripts that vary by 16-30 amino acids in their N-terminis. These HNF4α isoforms (P1-and P2-driven) have been found to be differentially regulated in human colon cancer but their specific roles are unknown. Here we show that the HNF4α isoform that is maintained in many colon cancers (HNF4α8) preferentially upregulates the expression of miRNA 21, a pro-oncogenic small RNA that is overexpressed in many cancers, including colon cancer. We observed up regulation of the endogenous miRNA 21 in both colon (HCT116) and non colon (293T) cells as well as under transient and stable conditions. Both P1- and P2-driven HNF4α upregulate miRNA 21 although the effect was more pronounced with P2-driven HNF4α (HNF4α8). Knockdown of HNF4α isoforms in a colon cancer line that expresses both isoforms (Caco-2) confirmed a preferential role for P2- HNF4α in miRNA 21 expression. We also show that expression of Cyclin D1 a know target of miRNA 21 is dependent on HNF4α expression. Finally we identify two putative HNF4α binding sites in human miRNA 21 promoter and show that the promoter is a direct target of HNF4α in luciferase assay. Again HNF4α8 acted in a preferential fashion with a more robust activation of luciferase construct.

Recently, several studies have described findings of aberrant expression of miRNAs in human tumors and have investigated their potential role as oncogenes or tumor suppressor genes, depending on the targets they regulate Among them, miR-21 has

been identified as one of the most highly expressed miRNA in various tumors, including colorectal cancers, and increase in miR-21 expression in these tumors (CRC) is associated with an increase in cell proliferation, migration, invasion and metastasis suggesting that miR-21 is a key regulatory molecule in cancer initiation and/or progression. Studies have also shown that over expression of miRNA 21 in colon cancer is associated with chemoresistance and poor therapeutic outcome. Like wise few studies suggest that P2-driven HNF4α promotes proliferation in β cells, and is also considered as embryonic isoform regulating embryonic gene expression (during cancer tissues become more embryonic like). Studies have also shown that P1-HNF4α expression is reduced in human and mouse HCC tissues and CRC tissues while P2-HNF4α expression is aberrantly activated. Taken together, these findings suggest that P1-HNF4α acts as a tumor suppressor in hepatocytes. However, the precise role of P2-HNF4α in colon cancer remains to be determined.

This finding, led us to hypothesize that HNF4α may play a key role in the regulation of pathways driving proliferation in the large intestine. Recent work done by a post doc from our lab has showed that P2-driven HNF4α is expressed in bottom half of colonic crypts (proliferative compartments) while P1-driven HNF4α is expressed only in top half of colonic crypts (differentiated compartments) (Dr.Chellappa, unpublished results). This positive correlation between P2-driven HNF4α expression and colonic proliferation suggests its role in promoting proliferation. We surmise that the direct links between HNF4α and the proliferation and malignant-associated genes miRNA 21 may be related to stress response such as colon cancer. In summary, all our observations imply

that the P2-driven HNF4α has the potential to function as an oncogene by assisting tumor initiation and progression via activation of miRNA 21. A comprehensive analysis of the genes regulated by P2-driven HNF4α in different cell types will be required to obtain a complete understanding of its role in cancer manifestation. In contrast recent reports show that conditional intestinal HNF4α knockout (P1-and P2- driven HNF4α) facilitates tumor progression concomitant to a reduced expression of the Cdx2 tumor suppressor gene (Saandi et al, 2012). Although the difference between published and our in vitro data, remains to be resolved, it should be noted that this HNF4α knockout data did not differentiate between P1-and P2- driven HNF4α.

Preferential activation of miRNA 21 by P2-driven HNF4α might be due to higher binding specificity of P2-driven HNF4α to miRNA 21 promoter. EMSA assays in the presence of P1-driven and P2-driven HNF4α, would reveal that weather these sites are responsible for activation of miRNA 21.

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Chapter 3

Isoform-specific regulation of miRNA 21 by HNF4α in Mice model of colitis

Abstract

Several studies have implicated HNF4α in regulating intestinal epithelial function by stimulating intestinal epithelial cell differentiation, which results in the formation of a tight epithelial barrier. It is also known to play an important role during early embryonic colon development where it regulates genes related to epithelial functions. In addition, HNF4α has been shown to function in protecting the epithelium during experimental colitis in young adult mice, as suggested by studies showing reduction of HNF4α level in Inflammatory Bowel Disease (IBD). However the roles of the different HNF4α isoforms and miRNA 21 not been established in colitis.

In order to investigate the differential roles of P1- and P2-derived isoforms of HNF4α in colitis, isoform-specific mice generated by reciprocal swapping of first exons of P1- and P2- promoters (α7-HMZ and WT) were subjected to an experimental model of chemically induced colitis. Both P1- and P2-driven HNF4α are expressed in distal colon of the WT mice while only P2-driven HNF4α is expressed in α7-HMZ mice. We show that the α7-HMZ mice are more susceptible to DSS-induced colitis than the WT mice as assessed by mortality, rectal bleeding, colon length, and spleen/body weight ratios. Immunoblotting and stem loop RT PCR performed on colonic tissue isolated from the DSS-treated mice show that the level of expression of HNF4α and miRNA 21 correlate with better recovery from DSS induced colitis. These findings suggest that HNF4α-mediated regulation of miRNA 21 may play an important role in the etiology of IBD and it can be used as a potential marker for better prognosis of IBD.

Introduction

In humans, the most common Inflammatory Bowel diseases (IBD) are ulcerative colitis (UC) and Crohn's disease (CD). The less prevalent forms of IBDs include collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, infective colitis and indeterminate colitis (Geboes, 2008). Patients with CD possess a patchy inflammation in any part of the gastrointestinal tract starting from mouth to anus. In contrast, UC presents as a diffuse mucosal inflammation restricted to the colon and the rectum (Carter et al, 2004; Bernstein et al, 2006). Another difference between the disease is that UC is restricted to the mucosa whereas CD affects the whole bowel wall (Goodman et al, 1976). Also IBD is associated with a variety of extra-intestinal manifestations such as liver malfunctions, arthritis, skin manifestations and eye problems. These problems may result in greater morbidity than the underlying intestinal disease and may even be the initial presenting symptoms of IBD (Ardizzone et al, 2008). Though the etiology of IBD is not fully understood, nuclear receptors have been shown to play a critical role in the pathogenesis of IBD. The vitamin D receptor (VDR) (Froicu et al, 2003; lim et al 2005), peroxisome proliferator-activated receptor gamma (PPAR γ) (Dubuquoy et al, 2003; Adachi et al, 2006) PPARα (Cuzzocrea et al, 2004) PPARδ (Bassaganya et al, 2004), pregnane X receptor (PXR) (Langmann et al, 2004), and glucocorticoid receptor (GR) (Schottelius et al, 2000, Stange et al, 2000) have all been shown to influence the severity of IBD symptoms in experimental models.

Numerous studies have used Dextran Sodium sulfate (DSS) induced

colitis model to investigate the pathogenesis of colitis and different factors affecting colitis. In the DSS model, colitis is induced by the addition of DSS to the drinking water (Yan et al, 2009; Yao et al, 2010; Solomon et al, 2010). In general, acute colitis is induced using a relatively higher concentration of DSS (2-5%) administered continuously for shorter periods (4-14 days). Chronic colitis is induced by continuous administration of a low concentration of DSS (1%) or by cyclical administration of relatively higher doses of DSS separated by several days of untreated drinking water. For the purpose of this study, we have focused on the acute model of DSS-induced colitis.

The clinical features of DSS-induced colitis in mice reflect those seen in human UC. They include weight loss, watery diarrhea, presence of stool blood, decrease in food and water intake, decrease in the activity levels, and even death in extreme cases (Yan et al, 2009; Solomon et al, 2010). Usually watery diarrhea and occult blood are the earliest features of DSS-induced colitis and may occur as early as day 2 of DSS administration (Kullmann et al, 2001). The disease symptoms tend to worsen as DSS administration proceeds, and inflammation may be fully established within 7-10 days depending on the animal species and the concentration of DSS (Mahler et al, 1998). However, animals tend to recover upon withdrawal of the DSS administration. Crypt loss and crypt shortening are the earliest histological features of DSS-induced colitis and occur around day 2 or 3 of DSS intake (Kitajima et al, 1998). By the end of DSS cycle, the mucosa is characterized by extensive erosion and inflammation.

miRNAs are a recently discovered class of short (18-24 nucleotides in length) non-coding single-stranded RNAs that regulate gene expression by

controlling the stability and translation of protein coding mRNAs (Esteller et al, 2011; Guo et al, 2010). A number of biological processes are regulated by miRNAs, including cell differentiation, proliferation, apoptosis, and cell cycle control (Kenna et al, 2010; Miska et al, 2005; Schickel et al, 2008). For example, miR-21 has been classified as an "oncomiR" (Esquela et al, 2006; Pan et al, 2011). The almost omnipresent over expression of miR-21 in human cancers (Pan et al, 2011; si et al, 2007; Iorio et al, 2005), including colorectal cancer, provides new options for cancer therapy. Furthermore miR-21 is the only miRNA commonly found to be up regulated in inflamed tissue or sera of IBD patients (Wu et al, 2008; Takagi et al, 2010; Fasseu et al, 2010; Zahm et al, 2011). In contrast, it is also known that miR-21 silences pro-inflammatory interleukin IL-12 (Lu et al, 2009) and in the lungs, miR-21 inhibits toll-like receptor 2 agonist-induced lung inflammation in mice (Case et al, 2011). miR-21 may exercise anti-inflammatory effects also through silencing of PTEN. PTEN is known to promote inflammation in cetain cell types (Schabbauer et al, 2011). All the above observations lead us to hypothesize that the purpose of the up regulation of miRNA 21 during IBD, is to promote intestinal tisssue healing via the anti-inflammatory actions of miR-21. However, level of expression of miRNA 21 during recovery from IBD has not been determined.

HNF4α was originally identified as an endoderm- specific transcriptional regulator present in the liver, pancreas and intestine and during embryonic development (Chen et al, 1994). Recently HNF4α, has emerged as a potential regulator of intestinal epithelial function. *In vitro studies* indicate that it stimulates intestinal epithelial cell differentiation resulting in the formation of a tight epithelial barrier (Lussier et at, 2008).

HNF4α has been shown to regulate ion selectivity, impairment of which contributes to the manifestation of IBD (Resta et al, 2009; Montrose et al, 2003). It is also known to play an important role during early embryonic colon development where it regulates genes related to epithelial function (Garrison et al, 2006). In addition, HNF4α is required to protect the epithelium during experimental colitis in young adult mice (Ahn et al, 2008). However there are two different major isoforms of HNF4α that are driven by two different promoters (P1 and P2). The role of these isoforms in colitis has not been examined.

In order to investigate a potential differential role of P1- and P2-derived isoforms of HNF4α in the distal colon, we employed isoform-specific mice generated by the weiss group (α7-HMZ) (Briancon et al, 2006). Both P1- and P2-driven HNF4α are expressed in the distal colon of WT mice while only P2-driven HNF4α is expressed in the α7-HMZ mice. Although recent studies indicate that P1- and P2-driven HNF4α are differentially regulated during hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC), (Niehof and Borlak, 2008; Tanaka et al, 2006; Chellapa et al, 2012), the isoform-specific role of P1- and P2-driven HNF4α during DSS-induced colitis has never been investigated. In this study, we investigated a potential functional role for HNF4α and miRNA 21 during recovery from DSS induced colitis. We also show that P1- and P2 driven isoforms could play distinct roles.

MATERIALS AND METHODS

Isoform-specific mice

HNF4α isoform-specific mice generated by reciprocal swapping of the first exons, from the two promoters (P1 and P2) were a gift from the weiss group at the Pasteur institute (Briancon et al, 2006). These mice are from a mixed background that includes C57BL and 129/sV. The mice were maintained at a controlled temperature (25°C) on a 12 hr light/dark cycle and fed ad libitum, with a standard lab chow and drinking water. All mice were euthanized by exposure to CO2 gas in mid morning. Distal colons were removed (3cm from rectum) and snap frozen in liquid nitrogen or placed in RNA Later (Qiagen) and stored at -80 degrees C. Care and treatment of experimental animals was in accordance with guidelines from the University of California, Riverside, Institutional Animal Care and Use Committee (IACUC).

Induction and assessment DSS-induced colitis**:** Young adult (8-12 week old) WT type and α7 HMZ female mice (n= total of 30 for each group) were administered 3% DSS in the drinking water for 6 days followed by 3 days of untreated water. Daily changes in body weight and clinical signs of colitis, such as rectal bleeding, diarrhea, and bloody stool, were assessed. At day nine after onset of DSS treatment and recovery, mice were sacrificed, and the colonic tissues were collected for immunoblotting and miRNA analysis. The last sement of the colon approximately 3 cm before the start of rectum was collected and designated as distal colon. One part of distal colon is stored in RNA later, second part is snapfrozen in liquid nitrogen for potein analysis, and third part is fixed in

formalin were fixed in 10% formalin and embedded in OTC. 5-µm sections were stained with Hematoxylin and Eosin (H&E).

Expression Profiling

Afyymetrix arrays were done by Dr Chellappa (Sladek lab, unpublished results) from isoform-specific mice and their corresponding wild type controls. Mouse Exon 1.0 ST Arrays from Affymetrix were hybridized at the Genomics Core in the UCR Institute of Integrated Genome Biology with RNA extracted using Trizol Reagent and Qiagen RNA Extraction kit from the distal colon of young adult male mice (12-16 weeks old) on a standard lab chow fed ad libitum. RNA was pooled from two to three mice per genotype per array; results from duplicate arrays were averaged. Isoform-specific mice (α1 HMZ and α7 HMZ) were compared to their appropriate WT littermate controls. The data were analyzed using Robust Multi-array Average (RMA) background adjustment and quantile normalization on probe-level data sets with Bioconductor packages, Exonmap, and Affy (www.bioconductor.org). To determine the differentially expressed transcripts only the probes with p-value < 0.05 (Student t-test) and fold change more than 2-fold were considered. Gene Ontology overrepresentation analysis was conducted using DAVID (http://david.abcc.ncifcrf.gov/).

Immunoblotting for HNF4α

Samples were harvested using RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25 % sodium deoxycholate, 0.5 mM EDTA, 150 mM sodium chloride), plus inhibitors 1µg/ml aprotonin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM sodium orthovanadate

plus1 mM sodium fluoride, 1 mM PMSF, phosphatase inhibitor cocktail I & II (1:100) (Catalog# P2850, Catalog# P5726, Sigma-aldrich), and protease inhibitor cocktail (Catalog# P8340, Sigma-aldrich) (1:10). Protein concentrations of cell lysates were measured using the Bradford reagent. Samples analyzed by 10% SDS-PAGE were transferred onto PVDF membrane (Millipore). Transferred blots were blocked with 5% milk in 1xTBST (10 mM Tris pH 8.0, 150 mM sodium chloride, 0.05% Tween 20) for 15 min at room temperature. Blots were then incubated at 4^0C overnight with primary antibody (1.10000) (P1/P2-driven HNF4α, Clone H1415 Catalog # PP- H1415- 00, R & D Systems). The blots were washed three times with 1xTBST and incubated with goat anti –mouse Ig conjugated to HRP (1:5000) for 2 hrs at room temperature. Blots were then washed three times with 1xTBST and three times with 1xTBS (10 mM Tris pH 8.0, 150 mM sodium chloride) and developed by electro chemiluminesence (Dura kit, Pierce) Gels were stained with Coomassie after immunoblotting to ensure equal protein loading.

Immunoblotting for Cyclin D1

Samples analyzed on 10% SDS-PAGE were transferred onto PVDF membrane. Transferred blots were then blocked with 5% milk (in 1xTBS) for 25 min at room temperature, and washed three times with 1xTBST. Blots were then incubated with cyclin D1 antibody (1.1000 in 1xTBST & 5% milk) at 4^0C overnight, washed three times with 1xTBST (Catalog #2922S, Cell Signaling). Blots were then incubated with goat anti-Ig rabbit conjugated to HRP (1:20,000 in 1xTBST) for 2 hrs at room temperature, washed three times with 1xTBST and 1xTBS and developed with the Dura reagent.

Stem loop RT-PCR for analyzing miRNA level

Total RNA was extracted from mice distal colon by using Qiagen miRNEASY kit. In brief, the reverse transcription reaction was performed in 15 μL containing 1μg of total RNA, 1 μL 10 mM dNTPs, 0.5 μL reverse transcriptase (Invitrogen Superscript 111 RT kit), 1 μL stem-loop reverse transcriptase primer, 4 μL 10× reverse transcription buffer, 2 μL 1mM DTT, 1 μL RNAase out, and 10.5 μL DEPC water. For synthesis of cDNA, the reaction mixtures were incubated at 16°C for 30 min, followed by pulsed Reverse transcription of 60 cycles at 30°C for 30 sec, at 42°C for 30 sec and then 50°C for 1 sec. The end point PCR reaction was performed with a final volume of 20 μL, containing 10 μL cDNA, 0.5 μL Taq polymerase, 0.4 μL forward primer, 0.4 μL reverse primer, 0.4 μL 10 mmol dNTP mix, 2 μL 10× PCR buffer and 6 μL DEPC water (1 cycle of 95°C for 5 min, followed by 25-35 cycles of 95°C for 15 sec and 60°C for 1 min). All reactions were performed in triplicates. miRNA levels were normalized using miRNA 192 or U6 RNA as internal reference genes. The sequences of primers used for amplification are listed in TABLE 2.1. The reaction products were analyzed on a 4% agarose gel in 1xTBE and visualized by UV illuminometer of ethidium bromide stained gels.

Construction of mouse miRNA 21 promoter

The miRNA 21 promoter region was amplified from mouse genomic DNA (C57/BL6 liver) using PCR. The 607- bp fragment containing two putative HNF4α binding sites was amplified using the forward primer- 5'CTCGCCAGCAGTAATGCATT3' and the reverse

primer- 5'TGGTAGCTTC AGTTGCCAGA 3' and cloned into PCR-Blunt 11 Topo vector. The vector was digested with restriction enzymes KpnI and EcoRV to release 607 bp fragment, which was subsequently cloned into the luciferase reporter plasmid pGL4.10 (Promega). The sequence fidelity of final product was verified by sequencing.

Transient transfection assays

Human embryonic kidney cells (293T) cells were plated (0.2 $X10^6$ cells/well) in 6-well plates. After 24 hrs, the cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, with different amounts of expression vectors containing either human HNF4α2 (NM_000457), or human HNF4α8 (NM_175914.3) or empty vector (pcDNA 3.1), 500 ng of the mouse miRNA 21promoter luciferase reporter and 200 ng of CMV.βgal. Cells were harvested after 24 hrs using Triton lysis buffer (1% Triton X-100, 25 mM Gly-Gly pH 7.8, 15 mM magnesium sulfate, 4 mM EGTA, 1 mM DTT). Luciferase and β-gal activities were measured as described previously (Maeda et al, 2002) by luminometer. Significant differences between pcDNA 3.1 and HNF4α2 or HNF4α8 were determined by student's T-test that is indicated either by single asterisk ($p<0.05$) or double asterisks ($p<0.01$).

Results

Isoform-specific regulation of miRNA 21 by HNF4α in distal colon

The two promoters in the *HNF4A* gene drive the expression of transcripts that vary by 16-30 amino acids at their N terminus. These HNF4α isoforms are differentially regulated

in colon cancer but their specific roles remain unknown. As shown in Chapter 2, through a series of transient over expression and knockdown studies, we show that the P2-driven HNF4α preferentially activates miRNA 21 compared to P1-driven HNF4α in the human colon cancer cell lines. In order to determine whether HNF4α regulates miRNA 21 *in vivo*, WT and α7-HMZ mice were examined. We first examined basal levels of HNF4α and miR-21 in untreated young female adult mice. Females were used for our studies as they were only mice available to use and both females and males are equally susceptible to colitis. Whole cell extracts and total RNA isolated from the distal colon of WT and α7-HMZ mice were examined by immunoblotting and stem loop RT PCR analysis. No significant differences in total HNF4α protein levels between the WT and α7-HMZ mice are observed (Fig 3.1). However we did find that miRNA 21 levels were up regulated in the distal colon of both 8-week-old and 16-week-old α7-HMZ mice compared to the WT (Fig 3.2). Furthermore miRNA 21 expression was down regulated in the distal colon of 16-week-old WT compared to α1-HMZ mice (Fig 3.3). These results indicate that miRNA 21 expression correlates with P2-driven HNF4α in vivo.

In order to explore further isoform-specific differential expression of miRNA 21 expression, expression profiling of the distal colons of WT, α1-HMZ and α7-HMZ mice, were performed using affymetrix exon arrays (Dr. Chellappa, unpublished results). Some of these targets include tumor suppressors, genes involved in apoptosis and genes in DNA repair pathways (Table 3.1, 3.2). Taken together these results suggest that P2- driven HNF4α may preferentially regulate miRNA-21 in mouse colon and that this regulation is responsible for differential expression of protein-coding genes in the

isoform-specific mice.

Susceptibility of α7-HMZ mice to experimental DSS-induced colitis

A previous study showed that HNF4α transcript and proteins levels were markedly decreased in mice exposed to DSS treatment for 7 days, similar to what has been observed in human IBD patients (Ahn et al, 2008). This study further showed that an intestine specific HNF4α knockout mouse leads to an increased susceptibility to DSS-induced colitis as assessed by body weight, colon length, and histological analysis. This study suggests a protective role for HNF4α in inflammatory bowel disease (IBD) but did not explore the roles of the different HNF4α isoforms.

To investigate the roles of P1- and P2- driven HNF4α and miRNA-21 in colitis, we employed chemical (DSS)-induced model of colitis. Wild type and α7-HMZ mice were exposed to 3% DSS for 6 days and then allowed to recover for 3 days (Fig 3.4). During recovery from colitis, we observed that the α7-HMZ female mice are much more susceptible to DSS-induced colitis compared to WT mice (n=30 for each group). Colon shortening, used as a morphometric measure for the degree of inflammation, with colons being significantly shorter by approximately by 20% compared to the WT mice after DSS treatment (Fig 3.5A). Approximately 60% of α7-HMZ mice showed severe clinical symptoms such as rectal bleeding compared to WT mice during recovery from DSS induced colitis (Fig 3.5B). We also observed an increase in spleen/body weight ratio by 40% in the α7-HMZ mice compared to WT suggesting, an increase in inflammation (Fig 3.5C). Perphaps most remarkable was a 50% systemic mortality rate in α7-HMZ

mice compared to no deaths in WT mice during recovery from DSS treatment (Fig 3.5D). These findings indicate that α7-HMZ mice are more susceptible to DSS induced colitis.

During recovery from colitis, we also observed that the α7-HMZ male mice are much more susceptible to DSS-induced colitis compared to WT mice (n=10 for each group). As in female mice, the most remarkable effect was a 30% systemic mortality rate during recovery in α7-HMZ mice compared to no deaths in WT mice (Fig 3.7A). We also observed an increase of spleen/body weight ratio by 30% in the α7-HMZ mice compared to WT mice suggesting, an increase in inflammation (Fig 3.7B). The colon length of α7-HMZ mice was also considerably than WT mice after DSS treatment (Fig 3.7C). These findings indicate that α7-HMZ male mice are more susceptible to DSS induced colitis.

HNF4α, Cyclin D1, and miRNA 21 expression levels in isoform-specific DSS treated mice and their potential functional significance

Unpublished results from our lab show that HNF4α is expressed in proliferating and differentiated cells of colon (Dr. Chellappa, personal communication). Along similar lines, it has also been shown that the cell cycle regulator Cyclin D1 is expressed in proliferating and differentiated cells in normal colon (Yang et al, 2006). Cyclin D1 is an important regulator of cell cycle progression and can function as a transcriptionl co-regulator. The over expression of cyclin D1 has been linked to the development and progression of cancer. Recently, it has been shown that miRNA 21 expressions facilitates

cyclin D1 translation in the early phase of liver regeneration by relieving Akt1/mTOR complex 1 signaling from Rhob-mediated suppression (Raymond et al, 2012). Taken together these observations suggest that during stress (such as cancer, or IBD) HNF4α may activate miRNA 21, which in turn could elevate cyclin D1 levels.

In order to explore the mechanism responsible for the difference in susceptibility of α7-HMZ mice and WT mice to DSS-induced colitis, whole cell extracts were prepared from distal colonic tissue and analyzed for HNF4α and Cyclin D1 protein levels and miRNA 21 levels. HNF4α and cyclin D1 levels were elevated in colonic tissue of WT mice compared to the α7-HMZ mice, three days after recovery from DSS induced colitis. miRNA-21 levels were also elevated in distal colon of the WT mice compared to α7 HMZ mice (Fig 3.7). These results show that HNF4α protein levels correlates with miRNA 21 and Cyclin D1 levels, and that the levels of all three correlate with better recovery from DSS. This suggests that HNF4α-mediated miRNA21 regulation and hence cyclin D1 might be important for recovery from DSS induced colitis.

Mouse miR-21 is a transcriptional target of HNF4α

The correlation that we observed between the expression of HNF4α and miRNA 21 necessitates the elucidation of the mechanism by which HNF4α regulates miRNA 21. HNF4α may regulate miRNA 21 through direct transcriptional control or indirectly by activating an intermediate factor. To eliminate one of these possibilities, we first tested whether HNF4α binds directly to the regulatory region of the mouse miRNA 21 promoter. An earlier study showed that the regulatory regions required for miRNA 21 expression

reside within the promoter region spanning from – 607 bps to +1 bps (Fujita et.al, 2008). We analyzed -607 bp to +1 bp region of the mouse miRNA 21 promoter for possible HNF4α binding sites by using HNF4α Binding Site Scanner. In order to improve the predictability of potential HNF4α target genes, the Sladek lab has adapted the protein binding microarray (PBM) technology to rank thousands of putative HNF4α response elements based on their relative binding affinities using full length protein expressed in mammalian cells (bolotin et al, 2010). Furthermore, Support Vector Machine (SVM), a powerful machine-learning model was used to predict additional HNF4α binding sequences with high accuracy. Finally, both the PBM and SVM binding sites were combined and integrated into HNF4α Binding Site Scanner. The HNF4 Binding Site Scanner was used to identify two potential HNF4α binding sites on the mouse miRNA 21 promoter. The first sequence is 5' CAGCCAGAAGTCA 3', and second sequence is 5' CCAACCAAGGTCC 3'. To investigate whether these binding sites are required to activate miRNA 21 expression, transient transfection assays were performed using a luciferase reporter system. The pGL4.10 reporter construct containing the mouse miRNA 21 promoter (-607bp to +1 bp) was transfected into 293T cells along with human HNF4α2 or HNF4α8 expression vectors. Transient transfection of HNF4α2 and HNF4α8 at low concentrations (6.5 ng and 12.5 ng) activated mouse miRNA 21 promoter by one and half fold and two fold respectively, compared to control plasmid (pcDNA 3.1). Both HNF4α2 and HNF4α8 activated mouse miRNA 21 promoter compared to control, although HNF4α8 activates the miRNA 21 promoter luciferase construct by 40% more than

HNF4α2. These results are consistent with mouse tissues (Fig 3.2) where expression of HNF4α8 corelates more closely with the expression of miR-21.

Human HNF4α expression vectors were used for mouse studies as the DNA binding domain of rat and human (and mouse) HNF4α is 100% identical and hence these species are anticipated to have very similar if not identical DNA binding specificities. Protein binding microarrays (PBMs) from our lab showed no differences in DNA-binding characteristics of two human and rat HNF4α species. We have no reason to think there will be any species difference in DNA binding domain and this way we could compare results from Chapter 2.

Figure 3.1: HNF4α protein levels in distal colon of α7-HMZ vs WT mice Whole cell lysates were prepared from the distal colon of 16-week-old untreated female WT vs α7-HMZ mice. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. Coommasie staining allowed for normalization of protein loading. Each lane in the blot represents samples pooled from distal colon of two different animals. This experiment has been repeated once. A total of 15 mice were examined in each group.

Fig 3.1

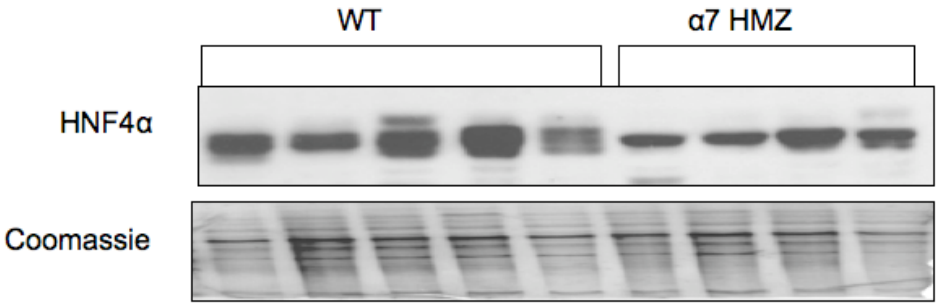
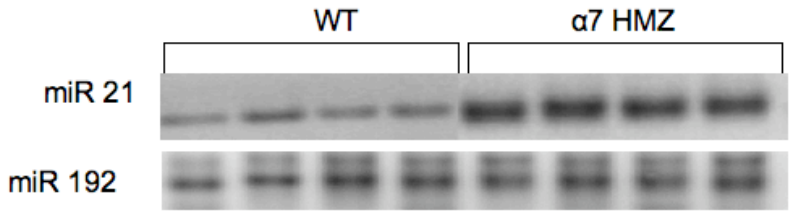


Fig 3.2: miRNA 21 expression levels are up regulated in the distal colon of α7-HMZ mice compared to WT mice. Total RNA was isolated from distal colon of both 8-week and 16-week-old untreated female HNF4α isoform-specific mice in females .The miRNA 21 levels were determined by stem loop RT-PCR and miRNA 192 was used as loading control. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE.
The images for miRNA 21 for WT and α7-HMZ are from the same gel, irrelavent lanes between the two groups were cropped out.

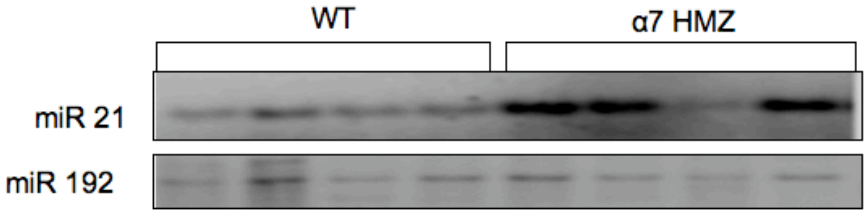
Fig 3.2

A.



Females 16 wk

B.



Females 8 wk

Table 3.1: Predicted and confirmed miRNA 21 targets which are down regulated in the distal colon of α7-HMZ vs WT mice.

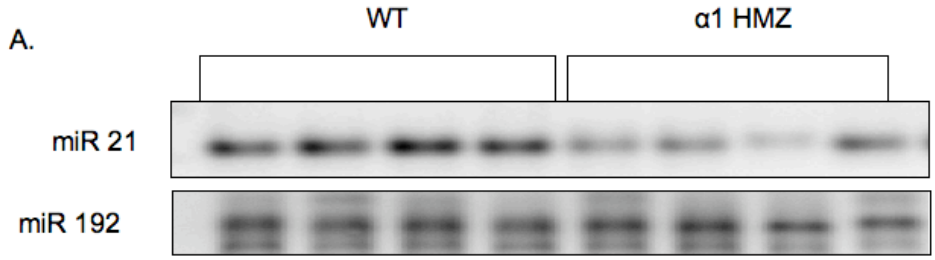
Confirmed miRNA 21 targets	**Fold change**	**Function**
Il12a	2.5	Antitumor response
Slc12a1	2.4	Tumor supressor
Pitx2	2	Tumor supressor
Mtap	2	Apoptopsis related genes
Apaf1	2.09	Mitochondrial apotopsis genes
Predicted miRNA 21 targets	**Fold change**	**Function**
Prkg2	3	Pro apotoptic gene
Sodtdc1	2.2	Tumor Supressor
Smug1	2.1	DNA repair (BER) pathway
Eml5	4.3	Microtubule associated protein
Ndst3	3.2	Development
Cep63	2.1	Tumor Supressor

Fig 3.3: HNF4α and miRNA 21 expression levels in distal colon of α1-HMZ mice compared to WT.

A: miRNA 21 levels are decreased in the distal colon of α1-HMZ mice compared to WT. Total RNA was isolated from distal colon of 16-week-old untreated female mice. The miRNA 21 levels were determined by stem loop RT-PCR and miRNA 192 was used as loading control. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Each lane in the blot represents samples pooled from distal colon of two different animals

B: HNF4α protein levels in distal colon of α1-HMZ vs WT mice Whole cell lysates were prepared from the distal colon of 16-week-old untreated female mice. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. Coommasie staining allowed for normalization of protein loading. Each lane in the blot represents samples pooled from distal colon of two different animals.

Fig 3.3



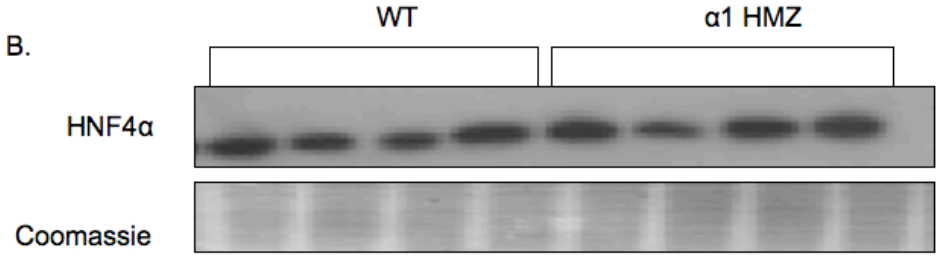


Table 3.2: Confirmed miRNA 21 targets that are up regulated in the distal colon of α1-HMZ compared to WT mice.

Confirmed miRNA 21 targets	Fold change	Function
Pten	3	Tumor supressor
Reck	2.07	Tumor supressor
CDK6	3.1	
TIMP3	3.5	ECM homeostatis
Prrg4	3.5	
Bmpr2	2.5	Tumor supressor
Apaf1	2.3	Pro apoptopic
SLC16a10	2.05	Amino acid transport
Mtap	2.9	polyamine metabolism
Sox5	2.6	Embryonic development

Fig 3.4: Schematic Representation of DSS treatment in mice. Young adult mice were subjected to DSS in their drinking water (3% DSS for 6 days in females, 2.5% DSS for 6 days in males) after which they were switched to untreated water for an additional 3 days. The mice were then sacrificed on the 10th day. Mice were fed ad libitum with standard lab chow throughout the entire treatment

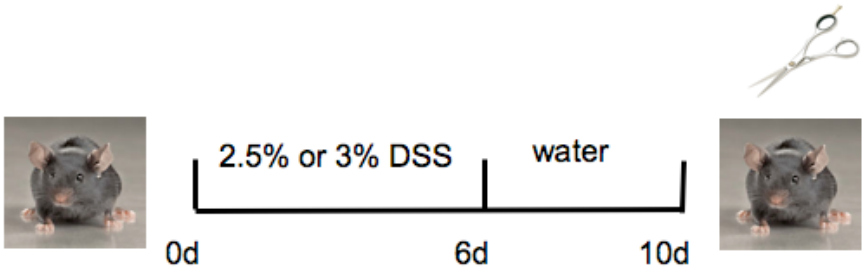


Fig 3.5: The α7-HMZ mice are more susceptible to colitis than WT mice in females (8-14 weeks).
Clinical assessment of DSS-induced colitis in the WT and 'α7' only mice treated with 3% DSS for 6 days and then allowed to recover for 3 days. (A) Mortality is observed only in α7-HMZ mice, but not in WT mice. (B) Rectal bleeding is observed in α7-HMZ mice, but not in WT mice. (C) Spleen/body WT ratio is significantly increased in α7-HMZ mice compared to wild type. (D) Distal colon is significantly shorter in the α7-HMZ mice compared to WT mice, during recovery from DSS induced colitis. Twenty mice in first group (WT, n=10, α7 HMZ, n=10) and forty mice (WT, n=20, α7 HMZ, n=20) in second group were analyzed in two independent experiments (total 60). Graphs include data from all 60 mice.

Fig 3.5

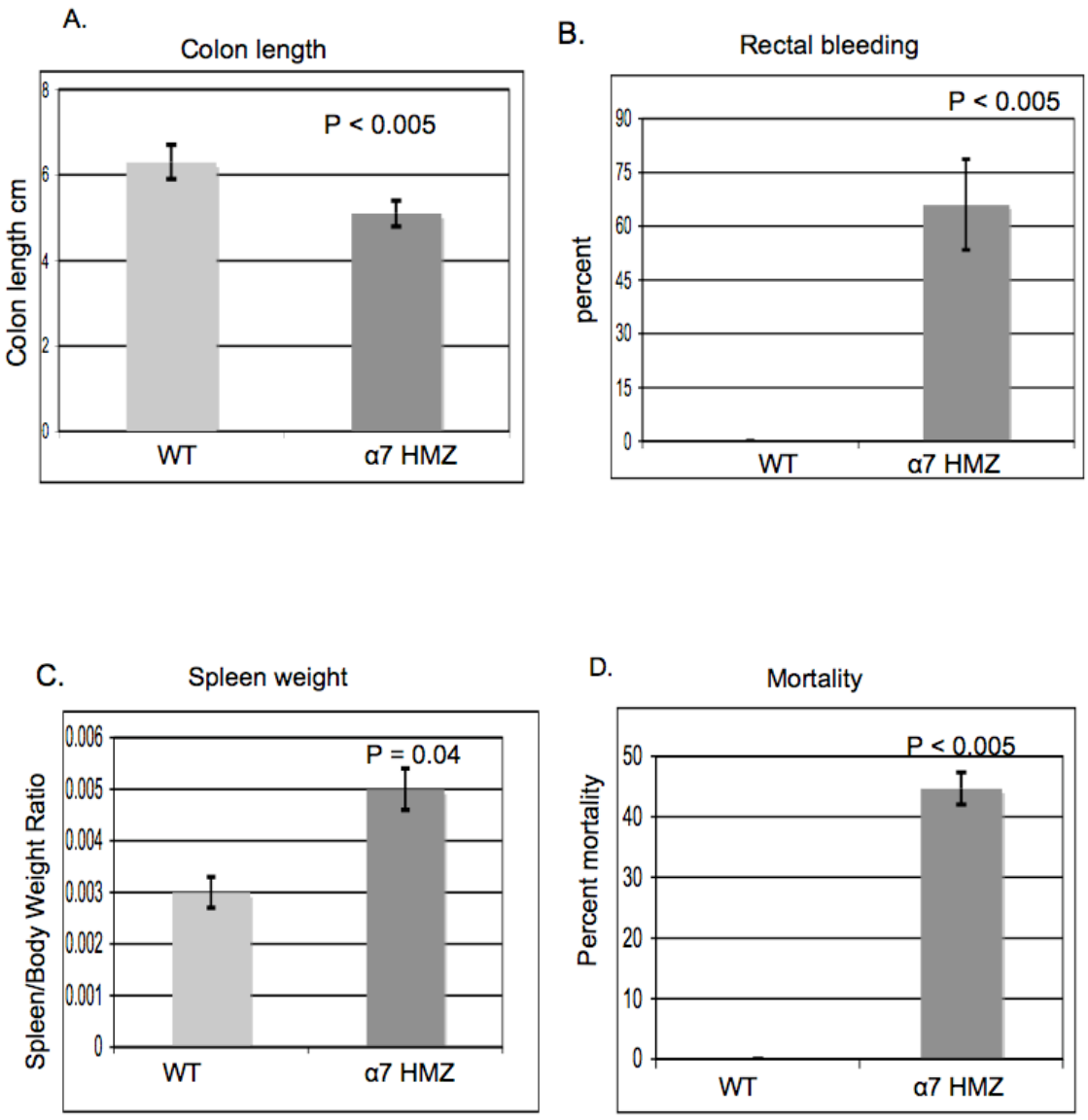
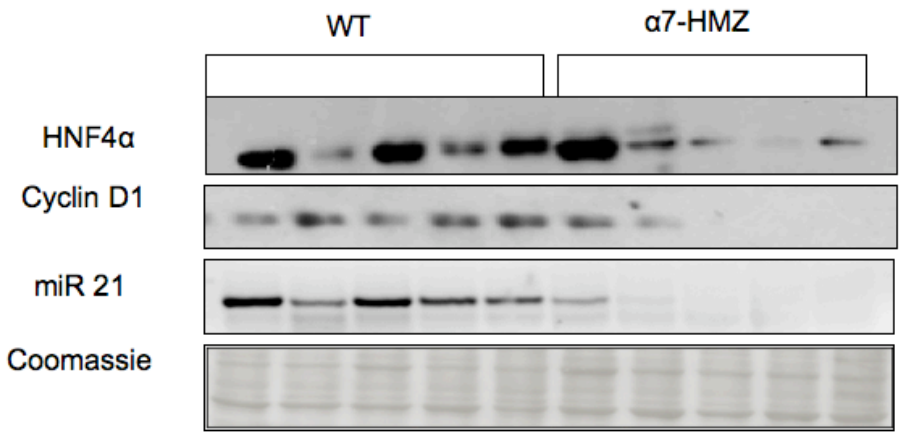

A.
Colon length
Colon length cm
P < 0.005
WT
α7 HMZ
B.
Rectal bleeding
percent
P < 0.005
WT
α7 HMZ
C.
Spleen weight
Spleen/Body Weight Ratio
P = 0.04
WT
α7 HMZ
D.
Mortality
Percent mortality
P < 0.005
WT
α7 HMZ

Figure 3.6:
A. Increased expression of HNF4α, Cyclin D1 and miRNA 21 levels in distal colon, of WT mice compared to α7-HMZ mice during recovery from DSS-induced colitis. Whole cell lysates and total RNA were isolated from distal colon of the DSS treated 8-14 –week-old female mice. HNF4α and Cyclin D1 protein levels were analyzed from distal colon by immunoblot analysis using 30 μg of whole cell extracts. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coomassie staining ensured equal loading of the protein extracts. Each lane represents distal colon (protein or RNA) isolated from a single animal.

B. Microscopic analysis of colon tissues. Colons were removed from isoform-specific mice three days after recovery from DSS treatment, fixed with formalin, and stained with hematoxylin and eosin. Representative results from 4 mice out of 2 independent experiments are shown.

Fig 3.6

A.



B.

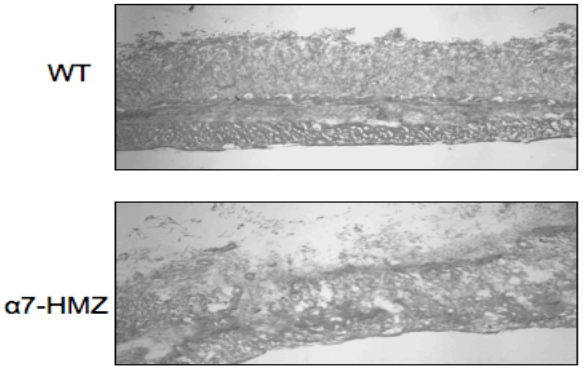


Fig 3.7: The α7-HMZ mice are more susceptible to colitis than WT mice in males (8-10 weeks).

Clinical assessment of DSS-induced colitis in the WT and 'α7' only mice treated with 2.5% DSS for 6 days and then allowed to recover for 3 days. (A) Mortality is observed only in α7-HMZ mice, but not in WT mice. (B) Spleen/ body wt ratio is significantly increased in α7-HMZ mice compared to wild type. (C) Distal colon is significantly shorter in the α7-HMZ mice compared to WT mice, during recovery from DSS induced colitis. Twenty mice in a group (WT, n=10, α7-HMZ, n=10) were analyzed. Graphs include data from all 20 mice.

Fig 3.7

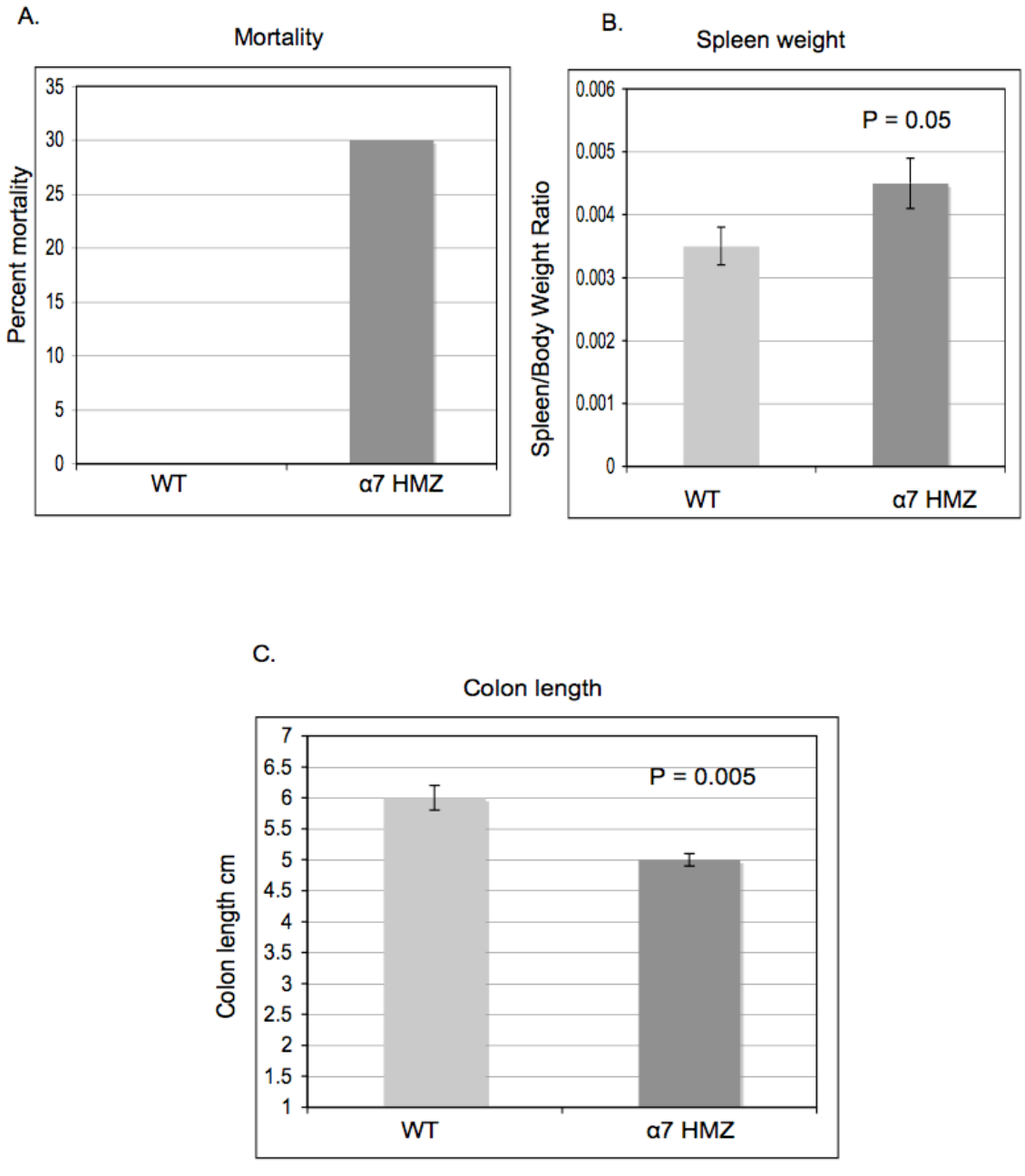

A.
Mortality
Percent mortality
35
30
25
20
15
10
5
0
WT
α7 HMZ
B.
Spleen weight
Spleen/Body Weight Ratio
0.006
0.005
0.004
0.003
0.002
0.001
0
P = 0.05
WT
α7 HMZ
C.
Colon length
Colon length cm
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
P = 0.005
WT
α7 HMZ

Figure 3.8: Increased expression of HNF4α, Cyclin D1 and in distal colon, of WT mice compared to α7-HMZ mice during recovery from DSS-induced colitis. Whole cell lysates and total RNA were isolated from distal colon of the DSS treated 8-10 – week-old male mice. HNF4α protein levels were analyzed from distal colon by immunoblot analysis using 30 μg of whole cell extracts. miRNA 21 levels were analyzed by stem loop RT PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coomassie staining ensured equal loading of the protein extracts. Each lane represents distal colon (protein or RNA) isolated from a single animal.

Fig 3.8

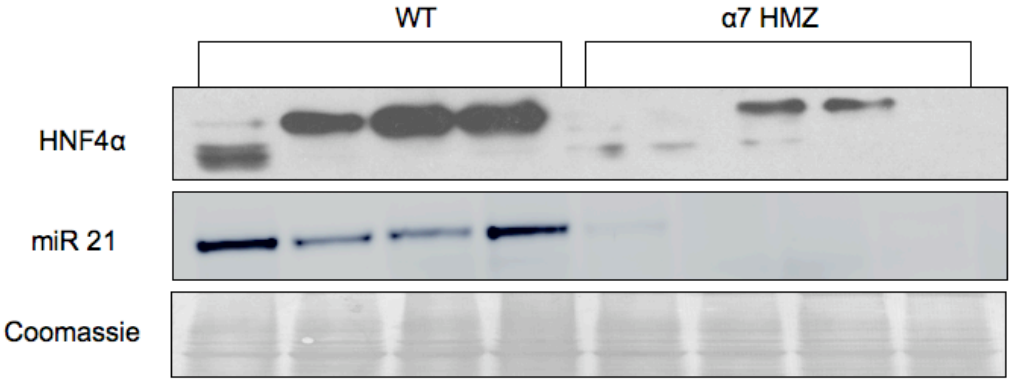
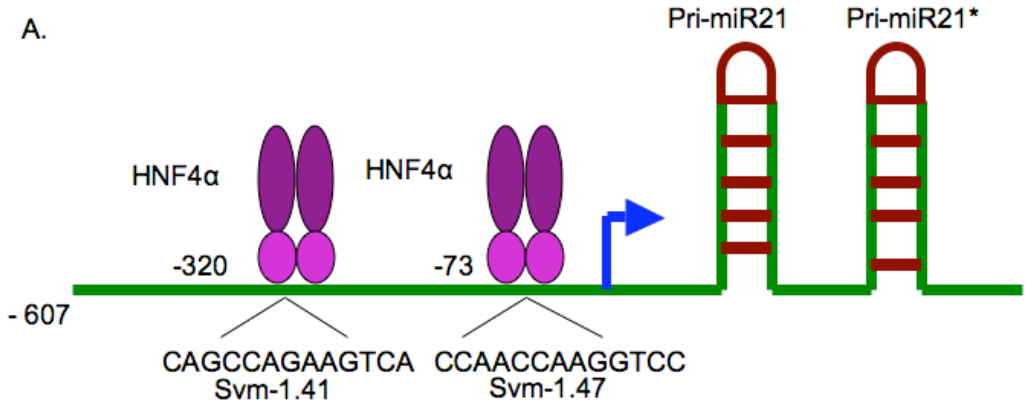


Figure 3.9: Mouse miRNA 21 is a direct transcriptional target of HNF4α.

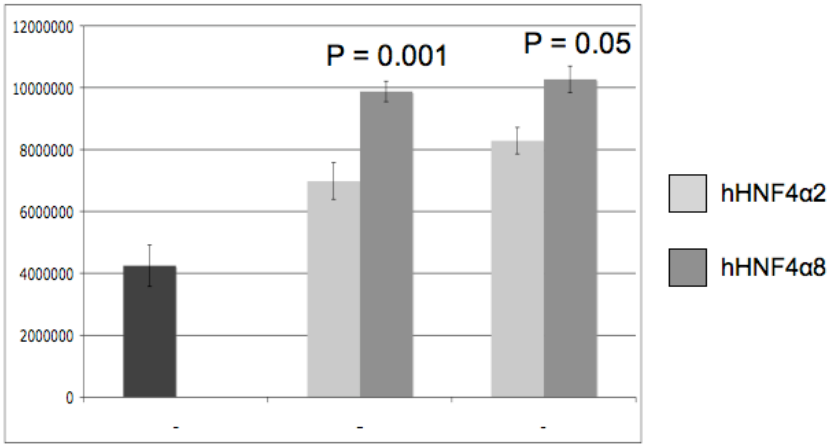
A) The promoter region of mouse miR-21 containing two predicted HNF4α binding sites as determined by HNF4 binding site scanner

B) 293T cells were transfected with different amounts of either hHNF4α8 or hHNF4α2 (**6.5, 12.5 ng**), 500 ng of the mouse miRNA 21 promoter luciferase reporter and 200 ng CMV.β-galactosidase. Cells were harvested after 24 hrs, luciferase activity was measured and mormalized with β-galactosidase as described. Each condition was performed in triplicate; data are shown as the mean +/- standard deviation, Shown is one of two representative experiments. P values are calculated between HNF4α2 and HNF4α8 at given amount of cDNA.

Fig 3.9


A.
Pri-miR21
Pri-miR21*
HNF4α
HNF4α
-320
-73
- 607
CAGCCAGAAGTCA
Svm-1.41
CCAACCAAGGTCC
Svm-1.47

B.



PCDNA 3.1	+	-	-
hHNF4α(ng)	-	6.5	12.5

Discussion

Inflammation is the primary response to infection or injury that functions to clear the injurious material or agent and promote tissue repair, it is characterized by the sequential release of a battery of mediators including; bioactive amines, eicosanoids, cytokines, chemokines and growth factors that regulate increased vascular permeability and recruitment of blood borne leukocytes. Inflammation is essentially a salutary response that normally resolves with the restoration of normal tissue structure and function, however when inflammation persists (chronic inflammation) it can cause tissue damage and loss of function. Persistent inflammation is linked with the incidence and progression of cancer. An important aspect of colitis-associated colon cancer is the inflammation that creates the colitis. Indeed inflammation is increasingly being seen as a major driver of many different kinds of cancer.

As discussed in Chapter 2 HNF4α isoform that is maintained in many colon cancers (HNF4α8) preferentially up regulates the expression of miRNA 21, a pro-oncogenic small RNA that is overexpressed in many cancers, including colon cancer. We observed up regulation of the endogenous miRNA 21 in both colon (HCT116) and non colon (293T) cancer cells in both transient and stable conditions. Both P1- and P2-driven HNF4α upregulate miRNA 21 although the effect was more pronounced with P2-driven HNF4α (HNF4α8). This helped us to hypothesize that during stress (cancer) α7-HMZ mice may be more susceptible to cancer compared to WT, and α1-HMZ may be less susceptible. Indeed a parallel study done on isoform-specific mice by showed that the

α7-HMZ mice are more prone to colitis induced colon cancer compared to WT, and α1-HMZ mice are less susceptible (Dr. Chellappa, Sladek lab unpublished data). This and miRNA

21 results presented in Chapter 2 and 3 are consistent with the notion that during colon cancer P1-driven HNF4α expression is down regulated while P2-driven HNF4α expression remains unchanged or up regulated (Tanaka et al, 2006; Chellappa et al, 2012), Thus, since miR-21 is considered as oncomir (si et al, 2007; Takagi et al, 2010) it is possible that the elevated expression of miRNA 21 in α7-HMZ mice may lead to enhanced proliferation during tumor growth.

In order to dissect the function of P1- and P2-driven HNF4α and miRNA 21 *in vivo*, distal colons from isoform-specific mice (α1-HMZ, α7-HMZ & WT controls) were used. The isoform-specific mice did not differ significantly in overall HNF4α protein levels but miRNA 21 levels were higher in distal colon of both 8-week-old and 16-week-old α7-HMZ mice compared with WT, likewise miRNA 21 expression levels were higher in distal colon of both 8-week old WT mice compared to α1-HMZ mice, suggesting that P2-driven HNF4α preferentially activates miRNA 21 compared to P1-driven HNF4α. Furthermore, several confirmed and predicted miRNA 21 targets that were down regulated in the α7-HMZ mice and upregulated in α1-HMZ mice were identified in expression profiling arrays. Some of these identified targets are tumor suppresser genes or genes involved in apoptosis or in DNA repair pathway.

To examine the role of P1- and P2-driven HNF4α and miRNA 21 during inflammation, a DSS-induced colitis model was employed. When the isoform-

specific mice (males and females) were exposed to either 2.5% DSS or 3% DSS respectively for 6 days and then allowed to recover for 3 days, we found that the α7-HMZ mice were more susceptible to DSS-induced colitis compared to the WT mice as assessed by body weight, colon length, rectal bleeding, and histological analysis (Fig 3.5, Fig 3.7). Immunoblot and microRNA analysis revealed that HNF4α and miRNA 21 levels are elevated in the distal colon of WT mice compared to the α7-HMZ mice during the recovery, suggesting that both HNF4α and miRNA 21 might play a important role during intestinal recovery (Fig 3.6, Fig 3.8). Then through a series of molecular experiments, we tested the nature of HNF4α-mediated regulation of miRNA 21 and found that HNF4α may bind to two predicted sites on the mouse miRNA 21 promoter, In addition, by using Luciferase-based transient transfection assay, we found that hHNF4α8 preferentially activated mouse miRNA 21 promoter than hHNF4α2. These experiments need to be repeated once more.

The first reported works of the anti-inflammatory properties of miR-21 was in macrophages, where miRNA 21 was shown to silence pro-inflammatory interleukin IL-12 (Lu et al, 2009). Subsequently in the lungs, miR-21 was found to inhibits toll-like receptor 2 agonist-induced lung inflammation in mice (Case et al, 2011). miR-21 may also exercise anti-inflammatory effects through the silencing of myleoid PTEN which is known to promote inflammation (Schabbauer et al, 2011). Analysis of predicted target genes of miRNA 21 on the basis of resources available in TargetScan4.0, PicTar, and miRanda resulted in the identification of a total of 930 candidates. Many of these

candidates are involved in the inflammatory response: (i) Janus kinase (JAK) and signal transducer and activators of transcription (STAT) signalling pathway (target count 16: *CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, TAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9, JAK3, IL12A, IL13RA2):* (ii) cytokine – cytokine receptor interaction (target count 20*): CSF3R, SPRY2, IL23R, CNTFR, IL15, IL7, IL13RA1, STAT3, SOS2, SPRY1, PIK3R1, LIFR, IL9, JAK3, IL12A, IL13RA2*) (Gao et al, 2011). Collectively, these pathways represent the core of the cytokine response system responsibe for inflammation underscoring the role of miRNA 21 in limiting the inflammatory response.

It is also known that during DSS induced colitis there is an increase in pro-inflammatory cytokines. Yutao et al showed that DSS significantly increased the production of pro inflammatory cytokines TNF-α, IL-1β, IL-6, IL-10, IL-12 (Yutao et al, 2009) while Kim et al has showed that IL-12p40 delivery (antagonist for IL-12 and IL-23) ameliorates DSS-induced colitis by suppressing IL-17A and inflammation in the intestinal mucosa. These observations along with our findings suggest that miRNA 21 may exert anti-inflammatory affects by down regulating pro-inflammatory cytokines, during DSS-induced colitis. An anti-inflammatory role of miRNA 21 is consistent with the DSS results presented in this Chapter. The α7-HMZ mice are more susceptible to DSS ,and on the 4^{th} day of recovery show no signs of miRNA 21 in their distal colon.This could mean that there is extensive inflammation in α7-HMZ mice which could cause death. In fact α7-HMZ mice have a significanty larger spleen to body wt ratio, a classical sign of infection and inflammation, as well as shoter colon crypt another sign of

inflammation. It would be very interesting to look at inflammatory and antiinflmmatory cytokines in α7-HMZ mice during recovery from DSS induced colitis.

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Chapter 4
Isoform-specific regulation of miRNA 194-2 by HNF4α

Abstract

Hepatocyte nuclear factor 4α (HNF4α/NR2A1) is a member of the nuclear receptor superfamily of transcription factors that is important for the development of the liver, intestine/colon, pancreas and kidney. HNF4α has been implicated in promoting intestinal epithelial differentiation, and the inhibition of epithelial mesenchymal transition (EMT). Likewise, miRNA 194-2 has been implicated in intestinal epithelial differentiation cell invasion and inhibition of EMT. Here, we investigate a potential link between specific splice variants of HNF4α with altered N-terminus P1-driven (HNF4α1/α2), and P2-driven (HNF4α7/α8) and miRNA 194-2 by performing a series of *in vivo and in vitro* assays. The results show that ectopic expression of P2-driven human HNF4α preferentially regulates miRNA 194-2 in human embryonic kidney and human colon cancer cell lines. We also show that compared to the WT mice that expresses both P1- and P2 driven HNF4α, miRNA 194-2 levels are up regulated in the distal colon of 16-week-old transgenic mice that expresses just P2- driven HNF4α. This suggests that P2-driven HNF4α preferentially activates miRNA 194-2 invivo and invitro. Further investigation is required to address the potential functional significance of this regulation.

Introduction

Hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear receptor superfamily, has been shown to play major roles in hepatic homeostasis, epithelial cell differentiation in the colon and pancreatic β cell development (Garrison et al, 2006; Gupta et al, 2007; Gupta et al, 2005; Miura et al, 2006). Transcription from alternate promoters (referred to as P1 and P2) and alternate splicing predict at least nine different variants of HNF4α (Drewes et al, 1996; Thomas et al, 20001). The P1-driven HNF4α isoforms are expressed in adult liver, proximal tubular cells of kidney, intestine, colon and fetal pancreas. On the other hand, the expression of P2-driven isoforms of HNF4α are detected in fetal liver, adult pancreas, stomach, intestine, colon, ovary, lymphatic organs and mammary tissue (Harries et al, 2008; Ishikawa et al, 2008; Jiang et al, 2003; Kanazawa et al, 2008; Torres-Padilla et al, 2001).

In addition to the major role of HNF4α as a central metabolic regulator in the liver, there is also strong evidence for its involvement in regulating proliferation (Chiba et al, 2005; Erdmann et al, 2007; Hwang-Verslues and Sladek, 2008; Lucas et al, 2005; Yin et al, 2008b), apoptosis (Erdmann et al, 2007) and maintenance of the epithelial morphology (Lazarevich and Fleishman, 2008; Spagnoli et al, 2000). A few of these studies have also pointed to the functional differences between P1- and P2-driven HNF4α in regulating cell proliferation and apoptosis (Erdmann et al, 2007; Gupta et al, 2007; Niehof and Borlak, 2008; Algamas et.al, 2013; Rieck et.al, 2012). Taken together, these

studies suggest that P1-driven HNF4α is anti-proliferative, while P2-driven HNF4α may be pro-proliferative.

The dichotomy in the function of HNF4α isoforms is consistent with other reports indicating that expression of P1-driven HNF4α isoforms are down regulated in different human cancers such as hepatocellular, gastric, renal and colorectal carcinomas, while the levels of P2-driven isoforms remain unchanged or upregulated (Oshima et al, 2007; Tanaka et al, 2006; Chellappa et al 2012). Both P1- and P2-driven HNF4α are expressed in the normal adult gut (Tanaka et al, 2006). HNF4α was originally identified as an endoderm-specific transcriptional regulator detectable in the liver, pancreas and intestine and during embryonic development (Sladek et al, 1990; Chen et al, 1994; Duncan et al, 1994). Recently HNF4α has emerged as a potential regulator of intestinal epithelial function. *In vitro* studies suggest that it stimulates intestinal epithelial cell differentiation resulting in the formation of a tight epithelial barrier (Lussier et at, 2008). HNF4α has been shown to regulate ion selectivity, the impairment of which contributes to the manifestation of inflammatory bowel disease (IBD) (Resta et al, 2009; Montrose et al, 2003). HNF4α is also known to play an important role during early embryonic colon development and to regulate genes related to epithelial functions (Garrison et al, 2006). In addition, HNF4α is required to protect the epithelium during experimental colitis in young adult mice (Ahn et al, 2008). None of these above studies, however differentiated between P1- and P2-driven HNF4α.

The regulatory role of miR-194 was first studied in normal and malignant cells of the gastrointestinal tract. High levels of miR-194 are expressed in the intestine and liver

(Lu et a, 2005; Barad et al, 2004). HNF1α, a transcriptional target of HNF4α can induce miR-194 expression during intestinal epithelial cell differentiation (Hino et al, 2008). miR-194 suppresses invasion and migration of liver mesenchymal-like cancer cells (Meng et al, 2010). miR-194 expression is also elevated in normal colon tissues and low in colon cancers (Braun et al, 2008). Low miR-194 expression has been associated with large tumor size and advanced stage in gastric cancer (Song et al, 2011). Activation of tumor suppressor p53 can induce miR-194 and two microRNAs clustered nearby miR-192 and miR-215 (Braun et al, 2008; Georges et al, 2008). miR-192 and miR-215 can induce p21 (Cyclin dependent kinase inhibitor p21) and cell cycle arrest in colon cancer cells (Braun et al, 2008). In endometrial cancer cells, miR-194 has been reported to inhibit self-renewal factor BMI-1, reduce cell invasion and inhibit epithelial mesenchymal transition (EMT) (Dong et al, 2011). It has also been shown that miR-194 can inhibit breast cancer cell migration, and that miR-194 and migration can be regulated by trastuzumab (humanized monoclonal antibody against HER-2 oncoprotein) (Feng et al, 2012). Finally miRNA 194-2 is expressed at higher levels by in Colon Cancer stem cells (SW1116csc) compared to human colon cancer cells (SW1116), thus suggesting a potential role in development and replication of Cancer stem cells (Yu et al, 2011).

In this study, we investigated a potential link between HNF4α and miRNA-194-2 using human colon cancer cell lines and mouse colonic tissue. Our work suggests that P2- driven HNF4α may activate the expression of miRNA 194-2 more efficiently than P1- driven HNF4α.

MATERIALS AND METHODS

Cell culture

Human colon cancer cells (SW480) and human embryonic kidney cell (293T) were purchased from ATCC (Manassas, VA). SW480 and 293T cells were cultured in Dulbecco's Modified Eagle Medium from GIBCO (Grand Island, NY) supplemented with 5% fetal bovine serum (Cellgro, Mediatech Inc, Herndon, VA) and 1% pencillin/streptomycyin. All cells were maintained at 37^0C at 5% CO2 and 95% air.

Isoform-specific mice

HNF4α isoform-specific mice generated by reciprocal swapping of the first exons, from the two promoters (P1 and P2) were a gift from the weiss group at the Pasteur institute (Briancon et al, 2006). These mice are from a mixed background that includes C57BL and 129/sV. The mice were maintained at a controlled temperature (25°C) on a 12 hr light/dark cycle and fed ad libitum, with a standard lab chow and drinking water. All mice were euthanized by exposure to CO2 gas in mid morning. Distal colons were removed (3cm from rectum) and snap frozen in liquid nitrogen or placed in RNA Later (Qiagen) and stored at -80 degrees C. Care and treatment of experimental animals was in accordance with guidelines from the University of California, Riverside, Institutional Animal Care and Use Committee (IACUC).

Transient transfection

293T and SW480 cells cells were plated (0.2 $X10^6$ cells) in 6-well plates. After 24 hours, the cells were transfected with different amounts of expression vectors containing either human HNF4α2 (NM_000457), or human HNF4α8 (NM_175914.3) or empty vector (pcDNA 3.1). 24 hours later cells were harvested using Triton lysis buffer (1% Triton X-100, 25 mM Gly-Gly pH 7.8, 15 mM magnesium sulfate, 4 mM EGTA, 1 mM DTT

Immunoblotting for HNF4α

Samples were harvested using RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.25 % sodium deoxycholate, 0.5 mM EDTA, 150 mM sodium chloride), plus inhibitors 1μg/ml aprotonin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate plus1 mM sodium fluoride, 1 mM PMSF, phosphatase inhibitor cocktail I & II (1:100) (Catalog# P2850, Catalog# P5726, Sigma-aldrich), and protease inhibitor cocktail (Catalog# P8340, Sigma-aldrich) (1:10). Protein concentrations of cell lysates were measured using the Bradford reagent. Samples analyzed by 10% SDS-PAGE were transferred onto PVDF membrane (Millipore). Transferred blots were blocked with 5% milk in 1xTBST (10 mM Tris pH 8.0, 150 mM sodium chloride, 0.05% Tween 20) for 15 min at room temperature. Blots were then incubated at 4^0C overnight with primary antibody (1.10000) (P1/P2-driven HNF4α, Clone H1415 Catalog # PP- H1415- 00, R & D Systems). The blots were washed three times with 1xTBST and incubated with goat

anti –mouse Ig conjugated to HRP (1:5000) for 2 hrs at room temperature. Blots were then washed three times with 1xTBST and three times with 1xTBS (10 mM Tris pH 8.0, 150 mM sodium chloride) and developed by electro chemiluminesence (Dura kit, Pierce) Gels were stained with Coomassie after immunoblotting to ensure equal protein loading.

Stem loop RT-PCR for analyzing miRNA level

Total RNA was extracted from mice distal colon by using Qiagen miRNEASY kit. In brief, the reverse transcription reaction was performed in 15 μL containing 1μg of total RNA, 1 μL 10 mM dNTPs, 0.5 μL reverse transcriptase (Invitrogen Superscript 111 RT kit), 1 μL stem-loop reverse transcriptase primer, 4 μL 10× reverse transcription buffer, 2 μL 1mM DTT, 1 μL RNAase out, and 10.5 μL DEPC water. For synthesis of cDNA, the reaction mixtures were incubated at 16°C for 30 min, followed by pulsed Reverse transcription of 60 cycles at 30°C for 30 sec, at 42°C for 30 sec and then 50°C for 1 sec. The end point PCR reaction was performed with a final volume of 20 μL, containing 10 μL cDNA, 0.5 μL Taq polymerase, 0.4 μL forward primer, 0.4 μL reverse primer, 0.4 μL 10 mmol dNTP mix, 2 μL 10× PCR buffer and 6 μL DEPC water (1 cycle of 95°C for 5 min, followed by 25-35 cycles of 95°C for 15 sec and 60°C for 1 min). All reactions were performed in triplicates. miRNA levels were normalized using miRNA 192 or U6 RNA as internal reference genes. The sequences of primers used for amplification are listed in TABLE 2.1. The reaction products were analyzed on a 4% agarose gel in 1xTBE and visualized by UV illuminometer of ethidium bromide stained gels.

Results

Isoform- specific regulation of miRNA 194-2 by HNF4α

In a physiologically normal colon both P1- and P2-driven HNF4α (HNF4α2 and HNF4α8) isoforms are present. In order to dissect isoform- specific functions of HNF4α, human cancer cell lines that do not express endogenous HNF4α were used so that we could ectopically express either human HNF4α2 or human HNF4α8, in an independent fashion. However, initially we employed human embryonic kidney cells (293T) due to their superior transfection efficiency that is approximately 90%.

Transient ectopic expression of P2-driven HNF4α preferentially activates endogenous miRNA 194-2 expression compared to P1-driven HNF4α and control plasmid (pcDNA3.1) in 293T cells (Fig 4.1). Since the aim of this study is to dissect the function of the different HNF4α isoforms in the colon, we next used a colon cancer cell line (SW480 cells) that does not express endogenous HNF4α. Ectopic expression of both P1-driven and P2-driven HNF4α increased endogenous miRNA 194-2 levels compared to the control plasmid (pcDNA 3.1) in SW480 cells (Fig 4.3).

HNF4α and miRNA 194-2 expression in different colon cancer cell lines

Three human colon cancer cell lines expressing different levels of endogenous HNF4α protein were examined for the expression of miRNA 194-2. The high HNF4α expressing cell line, such as Caco-2, exhibited higher miRNA 194-2 levels compared to SW480, and HCT 116, that exhibit very low or undetectable levels of HNF4α (Fig 4.2).

Isoform- specific regulation of miRNA 194-2 by HNF4α in distal colon

In order to determine whether HNF4α regulates miRNA 194-2 *in vivo*, the distal colonic tissues from the isoform-specific mice (α7-HMZ and WT controls) were analyzed. We first examined basal levels of HNF4α and miR-194-2 in untreated young adult female mice. No significant differences in total HNF4α protein levels between the WT and α7 -HMZ mice were observed (Fig 4.4A). However, we did find that miRNA 194-2 levels were higher in the distal colon of 16-week-old α7-HMZ mice compared to the WT, suggesting that invivo P2-driven HNF4α preferentially activates miRNA 194-2 expression compared to P1-driven HNF4α (Fig 4.4B).

Fig 4.1: HNF4α8 preferentially activates endogenous miRNA 194-2 expression levels in 293T cells. Whole cell lysates and total RNA were isolated from 293T cells transfected with pcDNA3.1 (1 μg), HNF4α2 (1 μg) or HNF4α8 (1 μg), for 24 hrs. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. The miRNA-194-2 levels were determined by stem loop RT-PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coommasie staining allowed for normalization of protein loading. Shown are the results from two of five independent experiments.

Fig 4.1

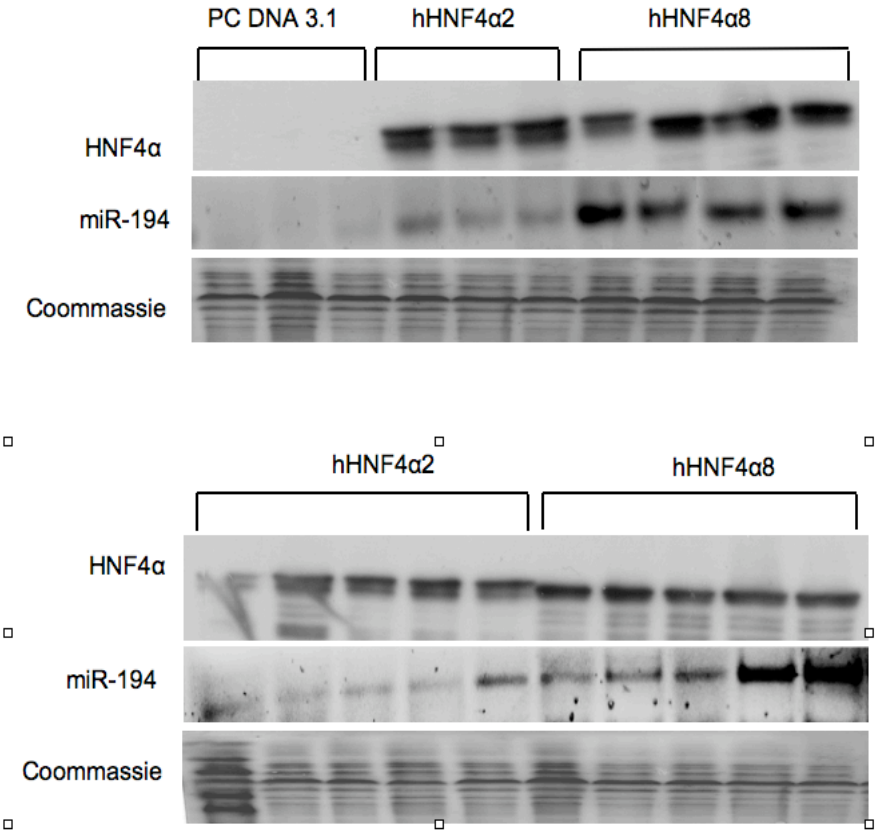

PC DNA 3.1
hHNF4α2
hHNF4α8
HNF4α
miR-194
Coommassie
hHNF4α2
hHNF4α8
HNF4α
miR-194
Coommassie

Fig 4.2: Both HNF4α2 and HNF4α8 can activate endogenous miRNA 194-2 expression in SW480 cells. Whole cell lysates and total RNA were isolated from 293T cells transfected with pcDNA3.1 (empty vector), HNF4α2 or HNF4α8 for 24 hrs. HNF4α protein levels were determined by immunoblot analysis using 30 μg, of whole cell extracts. miRNA-194-2 levels were determined by stem loop RT-PCR and normalized with the miRNA 21 levels. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Coommasie staining allowed for normalization of protein loading. This experiment is done once.

Fig 4.2

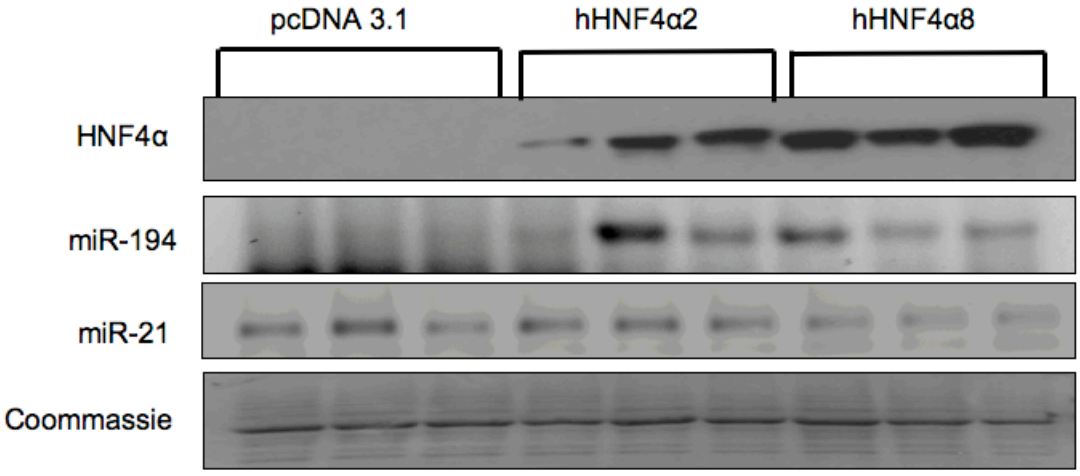


Fig 4.3: **Corelation between endogenous HNF4α and miRNA 194-2 expression in different colon cancer cell lines.** Whole cell lysates and total RNA were isolated from Caco-2, SW480 and HCT116 cells. HNF4α protein levels were determined by immunoblot analysis using 30 µg, of whole cell extracts. miRNA-194-2 levels were determined by stem loop RT-PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE. Each lane in blot or gel represents samples pooled from three different wells in a six well plate. Coommasie staining allowed for normalization of protein loading. This experiment was repeated once with similar results.

Fig 4.3

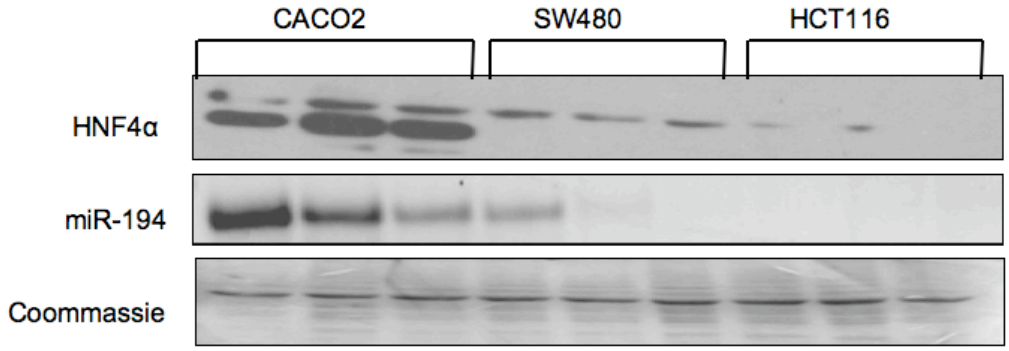
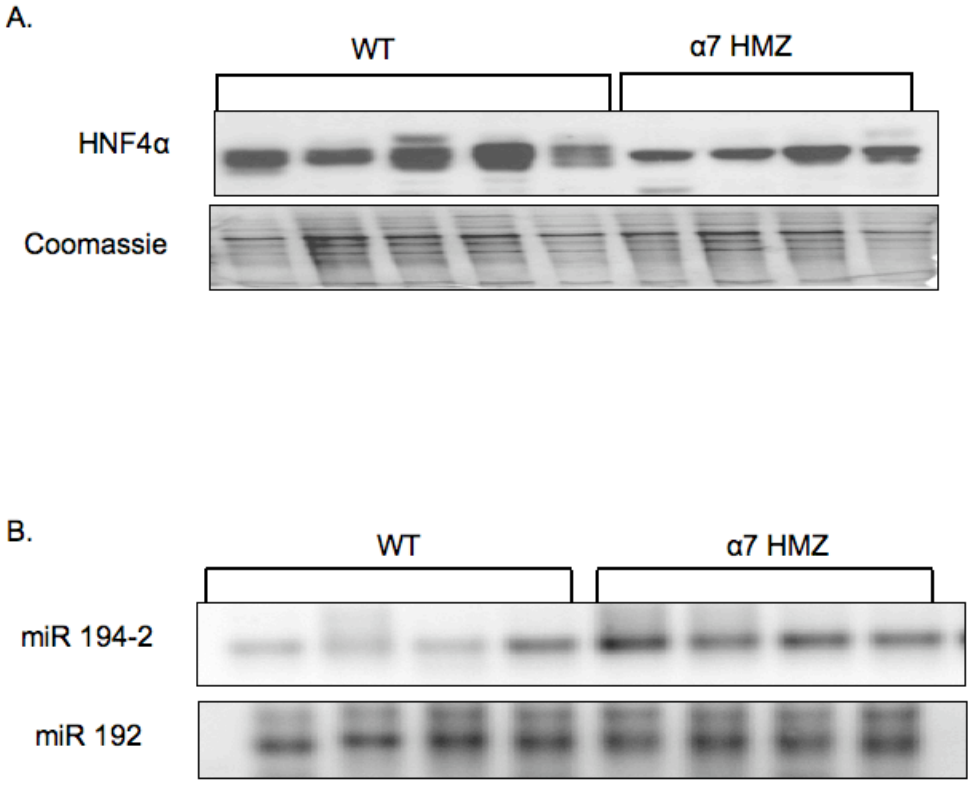


Figure 4.4 A: HNF4α protein levels in distal colon of α7-HMZ vs WT mice Whole cell lysates were prepared from the distal colon of 16-week-old untreated female mice. HNF4α protein levels were determined by immunoblot analysis using 30 μg of whole cell extracts. Coommasie staining allowed for normalization of protein loading. Each lane in the blot represents samples pooled from the distal colon of two different animals.

Fig 4.4 B.: miRNA 194-2 expression levels are up regulated in the distal colon of α7 HMZ mice compared to WT mice. Total RNA was isolated from distal colon of 16-week old untreated female mice. The miRNA 194-2 levels were determined by stem loop RT-PCR and miRNA 192 was used as loading control. Each lane in the blot gel represents samples pooled from the distal colon of two different animals. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE.

Fig 4.4



Discussion.

There are two promoters in a *HNF4A* gene that drive the expression of transcripts that vary by 16-30 amino acids in their N-terminus. These HNF4α isoforms have been found to be differentially regulated in human colon cancer but their specific roles remain unknown. Here we show that the isoform that is maintained in many colon cancers (HNF4α8) preferentially up regulates the expression of miRNA 194-2, a small RNA that is over expressed in colon cancer stem cells compared to colon cancer cells. We observed up regulation of the endogenous miRNA 194-2 in both colon (SW480) and non colon (293T) cells under conditions of transient expression by HNF4α.

In order to dissect the function of P1- and P2-driven HNF4α and miRNA 194-2 *in vivo*, distal colons from isoform-specific mice (WT and α7- HMZ) were used. Interestingly miRNA 194-2 expression levels were higher in distal colon of 16-week-old α7-HMZ mice compared to WT, suggesting that P2-driven HNF4α may preferentially activate miRNA 194-2 compared to P1-driven HNF4α.

An epithelial-mesenchymal transition (EMT) is a biological process that includes loss of cell-cell adhesion mediated by E-cadherin repression and an increase in cell mobility. This allows a polarized epithelial cell, which normally interacts with basement membrane, to undergo multiple biochemical changes that enables it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness,

elevated resistance to apoptosis, and greatly increased production of ECM components. EMT is also a characteristic feature of cells undergoing proliferation.

There are several studies that suggest that HNF4α plays an important role in cell adhesion, and that loss of HNF4α leads to metastasis. Two independent studies have shown that the loss of HNF4α and E-cadherin (an HNF4α target gene) (Weiss et al, 1998) is a requirement for EMT to occur (Cicchini et al, 2006; Cicchini et al, 2008). In addition, a positive feed back loop exists between HNF4α and E-cadherin as E-cadherin positively regulates HNF4α protein level in the nucleus (Peignon et al, 2006). Furthermore, ectopic expression of HNF4α was sufficient to induce mesenchymal to epithelial transition (MET) in NIH 3T3 fibroblasts (Parviz et al, 2003) and epithelial morphology in F9 embryonal carcinoma cells (Chiba et al, 2003). Loss of HNF4α in colon cancer predicts poor prognosis and is associated with liver metastasis (Oshima et al, 2007), taken together these reports suggests, that HNF4α functions as a suppressor of metastasis although the exact mechanisms and relevant target genes remain to be elucidated. HNF4α plays a major role during epithelial morphogenesis by regulating the expression of cell adhesion molecules (Cavallaro and Christofori, 2004a, b; Chiba et al, 2003), since the loss of cell adhesion is an early event in metastasis, this may be one link between HNF4α and metastasis. Similarly, it has also been shown that ectopic expression of miR-194 in Endometrial cancer cells induced MET by restoring E-cadherin expression, reducing vimentin expression, and inhibiting cell invasion *in vitro* (Dong et al, 2011). It has also been shown that miR-194 can inhibit breast cancer cell migration,

and that miR-194 and migration can be regulated by trastuzumab (Feng et al, 2012). So maybe HNF4α regulates miRNA 194-2 levels therby promoting cell adhesion.

In contrast, to the protective or anticancer role of miRNA 194-2, recently it was shown that the expression of miR-192 and miR-194 is up regulated in human breast cancer cell lines compared with non-cancer cells and manipulation of the miR-194 expression level using a synthetic inhibiting RNA produced a small but significant suppression of cell proliferation and up regulation in the expression of several genes that are thought to act as tumor suppressors in MCF-7 and T47D breast cancer cells (Daisuke et al, 2013). miR-194 and miR-210, were significantly up regulated in sera of both early and advanced-stage diffuse gastric carcinoma-bearing mice, tissues and lymph node metastasis tissues, compared with in corresponding controls (Rotkrua et al, 2013). Moreover, miR-194 is over expressed in oesophageal cancer (Song and Meltzer, 2012) as well as in pancreatic ductal adenocarcinomas (Mees et al, 2010). It has also been proven that miR-194 regulates metastasis formation through targeting of the metastasis-suppressor gene, EP300 (Mees et al, 2010). miRNA 194-2 is expressed at high levels by approximately seven fold in colon cancer stem cells (SW1116csc) compared to human colon cancer cells (SW1116), thus suggesting its potential role in development and replication of Cancer stem cells (yu et al, 2011). Earlier literature has also shown that during colon cancer P1- driven HNF4α expression is down regulated while P2-driven HNF4α expression remains unchanged or up regulated, thereby suggesting the possibility that P2-driven HNF4α elevates the expression of miRNA 194-2 leading to proliferation and invasion of cells during tumor growth (Tanaka et al, 2006; Chellappa et al, 2012).

Based on the above observations it is not clear whether miRNA 194-2 is an oncogene or tumor suppressor. Further investigations are needed to understand the role of HNF4α and miRNA 194-2 in colon.

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Chapter 5
Conclusions and Future Directions

HNF4α and miRNAs in general are known to play a critical role in the intestine (Garrison et al, 2006; Mckenna et, al 2010). HNF4α is also well known for its regulation of protein coding genes, but it has not been extensively investigated for its control of non-coding RNA genes. Furthermore, there are two promoters (P1 and P2) that drive the expression of the *HNF4A* gene. However the functional significance of the different proteins expressed by those promoters is not known. It is not known whether they exhibit differential roles in regulating miRNA gene expression.

In Chapters 2, 3, and 4, we show for the first time that 1) the levels of two miRNAs (miRNA 21 and miRNA 194-2) correlate with the levels of P2-driven but not P1-driven HNF4α in distal colon of the mouse; 2) this correlation is maintained during mouse model of colitis where the expression of P2-driven HNF4α, miRNA 194-2 and miRNA 21 are completely lost; 3) mice expressing just P2-driven HNF4α (α7-HMZ) are more sensitive to DSS; 4) P2-driven HNF4α preferentially activates the expression of the endogenous miRNA 21 gene as well as a heterologous construct in several cell-based systems. In this Chapter we present additional preliminary results for the first time that demonstrate, that expression of HNF4α and miRNA 21 and miRNA 194-2 corelates with recovery from DSS treatment (Fig 5.1). Taken together, these results indicate that HNF4α activates miRNA 21 and miRNA 194-2 expression in distal colon and that the expression is lost during DSS-induced colitis.

However, both miRNA 21 and miRNA 194-2 have been shown to have pleiotropic effects. miRNA 21 promotes cell proliferation and tumor growth and yet it also has a anti-inflammatory role. While the pro-cancer effects have been shown in the colon, the anti-inflammatory effects , which one would expect to have anti-cancer affect only in tissues other than colon. Similarly, miRNA 194-2 has been shown to inhibit cell migration, cell invasion, and to promote cell adhesion and differentiation ("anti cancer") but it is is also upregulated in gastric cancer, oesophageal cancer as well as in pancreatic ductal adenocarcinomas ("pro cancer"). None of miRNA 194-2 effects effects have been investigated in colon tissue according to our knowledge.

HNF4α, miRNA 21 and miRNA 194-2 in colitis

In Chapter 5 we show that there is complete knockdown of miRNA 21 and miRNA 194-2 levels in both α7-HMZ and WT mice treated with DSS, suggesting that there might be a global down regulation of the miRNA processing machinery, during DSS-induced colitis (Fig 5.1). There have been few studies looking at the expression of various miRNAs in ulcerative colitis (UC) crohns disease in humans. To our knowledge, an involvement of RNAi in DSS-induced colitis has not been noted. It would be of interest to determine whether the levels of major players in RNAi machinery levels of (DICER, DROSHA, and EXPORTIN) are down regulated in DSS-treated mice.

In Chapter 3 we also show that when the isoform-specific mice (males and females) are exposed to DSS for 6 days and then allowed to recover for 3 days, the α7-HMZ mice are more susceptible to DSS-induced colitis than WT mice as assessed by

body weight, colon length, rectal bleeding, and histological analysis. In Chapters 3, 4, and 5 immunoblot and microRNA analysis revealed that HNF4α and miRNA 21 levels and miRNA 194-2 levels are elevated in the distal colon of WT mice compared to the α7-HMZ mice during the recovery, suggesting HNF4α as well as miRNA 21 & miRNA 194-2 might play an important role during intestinal recovery from DSS. Others have shown that HNF4α knockout mice are more susceptible to DSS-induced colitis, although the mechanism was not investigated (Ahn et al, 2008). Colitis is typically associated with inflammation and miRNA 21 has also been shown to play a anti-inflammatory role in other tissues (Lu et al, 2009). Therefore, it would of interest to determine whether miRNA 21 also plays an anti-inflammatory role during colitis by examining the levels of pro- and anti-inflammatory cytokines in distal colon of WT and α7-HMZ mice.

Additional experiments need to be performed in order to establish a functional role for miRNA 21 and miRNA 194-2 in the distal colon and colitis. First one would use tail vein injection of antagomirs to downregulate either miRNA 21 or miRNA 194-2 (singly or together) and then examine the of overall morphology of the colon, as well as the length of colonic crypts. These mice could also be examined for sensitivity to DSS as done in Chapter 3. Since knockdown in the colon via tail vein injection is not well established and is technically difficult, an alternate approach would be to examine miRNA 21 knockout mice, which are commercially available. The final set of experiments would be to determine the consequences of the miRNA knockdown/knockout mice in the colon, by expression profiling via next generation sequencing identify targets and pathways.

Interestingly, miRNA 21 has also been shown to target the 3' UTR of HNF4α mRNA in a heterologous system (Wirsing et al, 2011) suggesting a potential feed back loop. However the effect of miRNA 21 on the endogenous *HNF4A* gene either at transcriptional or at translational level, was not examined; specific effects on the different HNF4α isoforms were also not investigated. In the future it would be of interest to study the role of miRNA 21 regulation on endogenous *HNF4A* gene in both normal colon and colitis.

HNF4α, miRNA 21 and miRNA 194-2 in cancer

Recently, several studies have described findings of aberrant expression of miRNAs in human tumors and investigated their potential role as oncogenes or tumor suppressor genes, depending on the targets they regulate. Among them, miR-21 was identified as one of the most highly expressed miRNA in a variety of tumors, including colorectal cancer (CRC). Furthermore, increase in miR-21 expression in tumors has been associated with an increase in cell proliferation, migration, invasion and metastasis, suggesting that miR-21 is a key regulatory molecule in cancer initiation and/or progression (Krichevsky et al, 2009). Similarly, it was been shown that the expression of miR-192 and miR-194 is up regulated in human breast cancer cells, pancreatic ductal carcinoma, sera of patients with both early and advanced-stage gastric cancer. miRNA 194-2 is expressed at higher levels colon cancer stem cells (SW1116csc) compared to human colon cancer cells (SW1116), thus suggesting an unique role for this miRNA in Cancer (Yu et al, 2011).

Like miR21, miR194-2 has anti-cancer as well as pro-cancer effects. While miR194-2 is ectopically expressed in endometrial cancer cells, it induces mesenchymal-to-epithelial transition (MET) by restoring E-cadherin expression, reducing vimentin expression, and inhibiting cell invasion (Dong et al, 2011). It has also been shown that miR-194 can inhibit breast cancer cell migration and be regulated by the anti-cancer drug Trastuzumab (Feng et al, 2012).

Like miRNA 21 and miRNA 194-2, HNF4α is highly expressed in intestine and distal colon. However, its role in colon cancer is even more obscure than that of the miRNAs. The expression of one isoform (P1-HNF4α) is reduced in human and mouse hepatocellular carcinoma (HCC) and CRC tissues while the other isoform (P2-HNF4α) is aberrantly activated (Niehof and Borlak, 2008; Tanaka et al., 2006; Chellappa et al, 2012). The findings presented in this dissertation indicating that P2-driven HNF4α preferentially activates the expression of miRNA 21 and miRNA 194-2 are consistent with a pro-cancer role for this isoform. However, while we have shown that P2-driven HNF4a upregulates miR1 and miR194-2 in human colon cancer cell lines, we have not proven that it does so in tumors in either humans or mice.

In addition to the caveats noted above, there is one final unresolved issue raised by our current results. As discussed in Chapter 2, miRNA 21 is expressed at higher levels in the distal colon of untreated α7-HMZ mice compared to WT, but α7-HMZ mice are also more susceptible to DSS-induced colitis. This sensitivity could be due to the complete loss of HNF4α protein in α7-HMZ mice during recovery by some as yet unexplained mechanism. Such a loss would lead to the downregulation of miRNA 21, as

we observed, and consequently a loss of its anti-inflammatory effects, which remains to be verified. The fact that the higher levels of miRNA 21 *before* DSS treatment do not protect the α7-HMZ mice against DSS-induced colitis could be explained if the miR21 in the untreated (i.e., healthy) tissue is not as functional as that in the stressed tissue. This hypothesis is in fact supported by a recent study that showed that miRNA 21 exhibits reduced binding and silencing activity in healthy mouse liver compared to cancer cells (John et al, 2012).

While many aspects of the role of the HNF4α isoforms, miR21 and miR194-2 in colitis and colon cancer remain to resolved, we have at least provided evidence that this is an important network that merits further investigation.

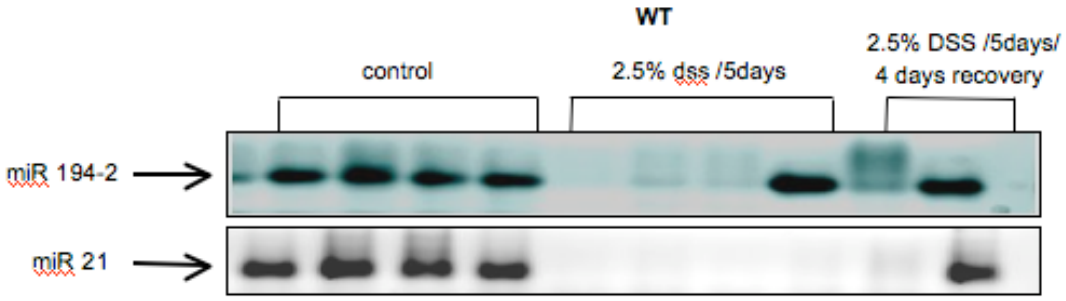
Fig 5.1: miRNA 21 and miRNA 194-2 levels in distal colon of DSS treated mice.

A) miRNA 21 and miRNA 194-2 levels levels are downregulated in DSS treated but upregulated during recovery from DSS treatment in wild type mice. Total RNA was isolated from distal colon of both 8-10-week-old male wild type mice. The miRNA 21 and miRNA 194-2 levels were determined by stem loop RT-PCR. Each lane in the gel represents distal colon isolated from individual mice. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE.

B) Fig 5.2: miRNA 21 and miRNA 194-2 levels levels are downregulated after DSS treatment and also during recovery from DSS in α7-HMZ mice. Total RNA was isolated from distal colon of both 8-10-week -old male α7-HMZ mice. Each lane in the gel represents distal colon isolated from individual mice. The miRNA 21 and miRNA 194-2 levels were determined by stem loop RT-PCR. The PCR reaction products were analyzed by electrophoresis on 4% agarose gel in 1 X TBE.

Fig 5.1

A)


WT
control
2.5% dss /5days
2.5% DSS /5days/
4 days recovery
miR 194-2
miR 21

B)

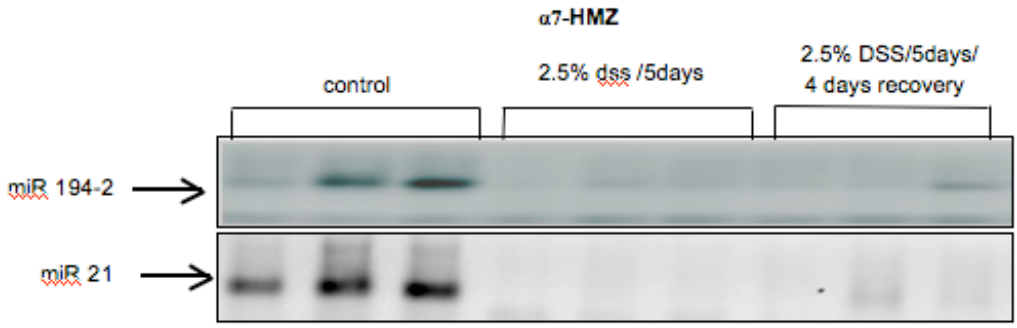
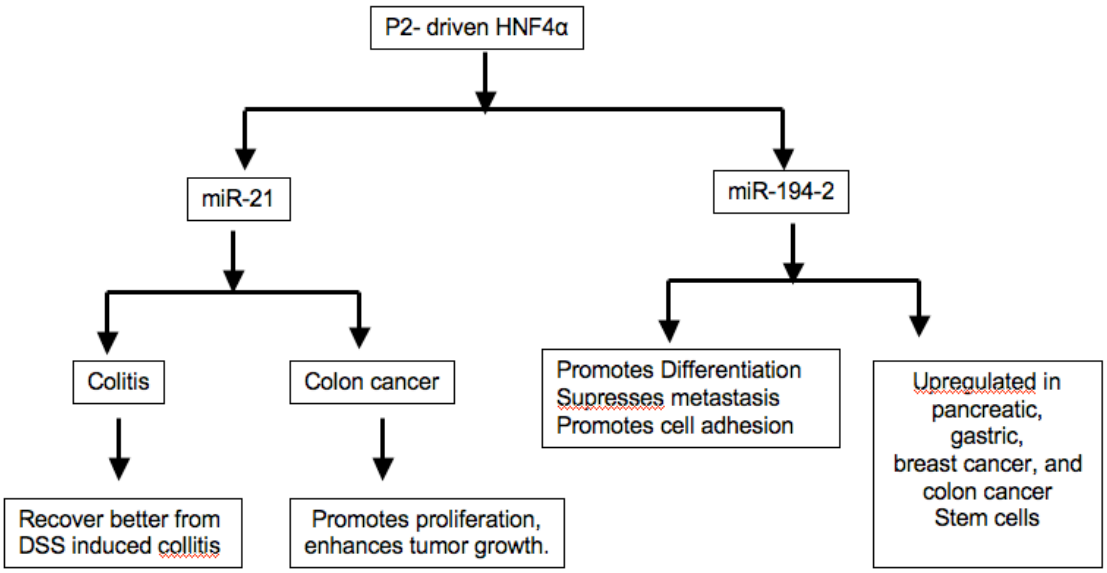

α7-HMZ
control
2.5% dss /5days
2.5% DSS/5days/
4 days recovery
miR 194-2
miR 21

Fig 5.2: Dual role of P2-driven HNF4α



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