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Understanding the Potential of microRNAs as Novel Therapeutic Targets in Cancer

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Understanding the Potential of MicroRNAs as Novel Therapeutic Targets in Cancer

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DISSERTATION

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Dedication

This work is dedicated to my wife, Katie, and our "dog-hter", Lucy, for their love and support during the toughest of times.

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review at *Hepatology*. The co-authors for this manuscript are Asha Balakrishnan, Noelle Huskey, Mona Jodari, Raymond Ng, Guisheng Song, Jesse Riordan, Brittany Anderton, Siu-Tim Cheng, Holger Willenbring, Adam Dupuy, Xin Chen, David Brown and Andrei Goga.

Abstract

Understanding the Potential of microRNAs as Novel Therapeutic Targets in Cancer

Cancer is a complex and heterogenous disease triggered by a myriad of genetic and lifestyle factors. Decades of research have furthered our understanding of key oncogenes, tumor suppressor genes and signaling pathways, leading to the development of numerous effective cancer therapies. However, the dysregulation of multiple signaling pathways through aberrant gene expression makes existing treatment ineffective in many cancers, including hepatocellular carcinoma (HCC) and triple negative breast cancers (TNBCs). There is thus an urgent need to explore and develop novel therapeutics for the treatment of these widespread and fatal cancers. It is well established that microRNAs (miRNAs) are key players in both HCC and TNBC development, and exhibit aberrant processing and expression profiles in these cancers. Because a single miRNA could potentially affect several clinically relevant targets, artificially altering the expression level of a miRNA may effectively shut down multiple oncogenic pathways, offering effective therapeutic perspectives. In this thesis, we explored the potential of targeting an oncogenic miRNA in HCC. We find that miR-494 is upregulated in multiple mouse models and aggressive human HCC. We demonstrate that it accelerates G1/S cell cycle transition through regulation of Mutated in Colorectal Cancer (MCC) gene expression and show that its inhibition reduces transformation in human liver tumor cell lines and inhibits tumor growth *in vivo*. Separately, we explored the possibility of exploiting endogenous miRNA

function to predict p53 expression, and consequently, therapeutic response in breast cancer. We find two SNPs in the p53 3' UTR, 485G>A and 826G>A, that are associated with mutant p53, and occur at higher frequencies in breast cancer patients than a diverse population. Further, we find that these SNPs lower p53 expression, possibly through creation of novel miRNA binding sites. Our findings indicate that miR-494 may have clinical value as a therapeutic target in HCC, and miRNA regulation of mutant p53 could be a predictor of therapeutic response in breast cancer.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Hepatocellular Carcinoma and Current Treatment Options

Liver cancer compromises diverse and histologically distinct primary hepatic neoplasms. These include hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (cholangiocarcinoma), hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and epitheliod haemangioendothelioma (Anthony, 2002). Among these, HCC is the most common and represents 83% of all cases (Farazi and DePinho, 2006). Major risk factors for HCC include chronic hepatitis B or C viral (HBV or HCV) infection, alcoholic liver disease and nonalcoholic fatty liver disease (El-Serag, 2011), accounting for the formation and progression of liver cirrhosis in about 80-90% of HCC patients.

It is estimated that HCC is the fifth most prevalent malignancy worldwide but the third most common cause of cancer related death (Bosch et al., 2004). The poor prognosis associated with HCC is largely due to late disease presentation and resistance to existing anticancer agents (Hertl and Cosimi, 2005). Systemic chemotherapy and radiotherapy, two conventional cancer therapies, have low response rates and no demonstrated survival benefits in HCC (Llovet and Bruix, 2008). While a breakthrough in systemic therapy was recently achieved when the multikinase inhibitor Sorafenib was approved for clinical use, phase III trials showed only a modest increase in patient median survival of about two to three months (Llovet et al., 2008). Surgical resection and/or transplantation provide the best options for long-term survival, but extensive disease and poor liver function limit

these treatments to only ~40% of HCC patients (Zhang et al., 2010). Clearly, there is an urgent need to develop novel treatments for advanced or recurrent HCC.

Despite the general lack of activity of conventional therapeutics in HCC, hepatocytes have been shown to readily take up antisense oligonucleotides (Morrissey et al., 2005; Soutschek et al., 2004; Zimmermann et al., 2006). In these studies, chemically modified, small interfering RNAs (siRNAs), when delivered systemically to mice and non-human primates in a liposomal formulation, showed efficient knockdown of target messenger RNA (mRNA) in the liver. These findings raise the hope that small nucleic acid based molecules that target endogenously expressed genes are a clinically viable therapeutic approach against HCC.

1.2 MicroRNA Biogenesis and Function

MicroRNAs (miRNAs) are a family of endogenous, single stranded, small RNAs typically ~20-23 nucleotides long that have emerged as key post-transcriptional regulators of gene expression (Bartel, 2004; He and Hannon, 2004). The founding members of the miRNA family, *lin-4* and *let-7*, were first discovered in the nematode worm *Caenorhabditis elegans*, through a screen for genes that exert temporal control over larval development (Ambros, 1989; Chalfie et al., 1981; Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). Soon after, *let-7* was found evolutionarily conserved throughout metazoans, with homologs readily detected in molluscs, zebrafish, flies, mice and humans through Basic Local Alignment Search Tool (BLAST) analysis (Pasquinelli et al., 2000). In humans, there are currently ~2000 known miRNAs predicted to regulate

60% of all protein coding genes (Friedman et al., 2009; Kozomara and Griffiths-Jones, 2011; Lewis et al., 2005). miRNAs are highly conserved, and their highly regulated expression allows them to perform essential functions in regulating diverse cellular processes (He and Hannon, 2004; Krol et al., 2010).

miRNA biogenesis occurs in four main steps **(Figure 1)** – transcription, nuclear processing, nuclear export and cytoplasmic processing (Kim, 2005). Transcription of miRNAs is mediated by RNA polymerase II in the nucleus and regulated in a similar fashion to that of protein-coding genes, allowing for tissue or temporal specific control of miRNA expression. Nascent miRNA transcripts, termed primary-miRNAs (pri-miRNAs), are typically several kilobases long with an ~120 bp hairpin structure containing the actual mature miRNA on one strand of the hairpin (Cai et al., 2004; Lee et al., 2004). After transcription, eukaryotic pri-miRNA transcripts are terminally modified by addition of a 7-methyl guanylate cap at the 5' end and a 3' poly(A) tail (Cai et al., 2004).

Following transcription, nuclear processing of pri-miRNAs occurs through recognition of target sequences flanking each hairpin structure by the RNA binding protein protein DiGeorge Syndrome Critical Region 8 (DGCR8). DGCR8 associates with the RNase III endonuclease Drosha to form a "Microprocessor complex" that cleaves the recognized hairpins from pri-miRNAs to generate precursor-miRNAs (pre-miRNAs) (Gregory et al., 2006). RNase III cleaved pre-miRNAs have a characteristic two-nucleotide overhang at their 3' end, enabling their recognition by the nuclear-cytoplasmic shuttle, Exportin-5 (Lund and Dahlberg, 2006). Pre-miRNA stem loops are subsequently shuttled from the

nucleus into the cytoplasm by Exportin-5 in a Ran-GTP dependent manner for further processing (Winter et al., 2009).

In the cytoplasm, pre-miRNAs are cleaved by another RNase III endonuclease, Dicer, into ~22 nucleotide long double stranded RNA duplexes that contain both the mature miRNA strand and its complementary, miRNA*, strand (Hutvagner et al., 2001). Recognition of pre-miRNAs by Dicer occurs through the characteristic two-nucleotide overhang at the 3' end previously generated by Drosha (Lund and Dahlberg, 2006). Dicer is able to bind to pre-miRNAs with the assistance of two RNA binding proteins, Human Immunodeficiency Virus Trans-Activating Response RNA Binding Protein (TRBP) and Protein Activator of Protein Kinase R Kinase (PACT) (Haase et al., 2005; Kok et al., 2007). The ~22 nucleotide miRNA duplexes do not persist for long in the cytoplasm. Studies on siRNA duplexes indicate that the relative thermodynamic stability of the two ends of the duplex determines which end persists (Hutvagner et al., 2001; Schwarz et al., 2003). The ribonucleoprotein complex (RNP), composed of Dicer, TRBP and Argonaute-2 (Ago2) aids in proper strand loading of the mature miRNA into the RNA-induced silencing complex (RISC) and it is thought that the complementary miRNA* strand is rapidly degraded upon its exclusion from the RISC (Hammond et al., 2001; Mourelatos et al., 2002). However, more recent deep sequencing data has revealed that although miRNA* molecules are generally less abundant than their mature miRNA counterparts, they can still accumulate in cells at physiological levels with biologically relevant consequences (Ruby et al., 2007).

In addition to Dicer and Ago2, the RISC also comprises of TRBP, PACT, fragile X mental retardation protein (FXRP), Tudor staphylococcal nuclease-domain-containing protein (Tudor-SN), the helicase MOV10 and the RNA recognition motif containing protein TNRC6B (Rana, 2007). Together, these protein factors making up the RISC allow regulation of mRNA translation by mature miRNAs. miRNAs generally bind through imperfect base pairing to the 3' untranslated region (3' UTR) of target mRNAs (Lewis et al., 2005), although a number of studies have reported target sites in the 5'UTR or coding regions (Kloosterman et al., 2004; Lytle et al., 2007). Binding specificity and efficiency is primarily determined by the degree of complementation between a 5-8 nucleotide sequence at the 5' end of the mature miRNA (position 2-8) and the target mRNA's 3' UTR (Lewis et al., 2005). This "seed sequence' on the miRNA, together with other factors such as supplemental and compensatory pairing at the 3' end of the miRNA, species conservation of 3' UTR target site and miRNA:mRNA binding stability make up the basis of miRNA target prediction softwares (Bartel, 2009; Grimson et al., 2007).

The degree of complementarity between the miRNA seed sequence and mRNA 3'UTR determines whether mRNA degradation or translational repression occurs upon miRNA:mRNA binding (Berezikov et al., 2005; Grishok et al., 2001; Lim et al., 2005). RISC mediated cleavage of target mRNAs via the RNase III catalytic domain of Ago2 results from high complementation between miRNA seed sequences and mRNA 3' UTRs (Orban and Izaurralde, 2005), while partial complementarity results in deadenylation of the mRNA poly(A) tail (Behm-Ansmant et al., 2006; Parker and Sheth, 2007). This reduces the stability of mRNA transcripts and is followed by translational repression in p-

bodies (Eulalio et al., 2007). However, this process is still poorly understood and requires further investigation.

miRNAs have been predicted to target almost 60% of all coding genes in the human genome and are responsible for regulating diverse cellular processes, including differentiation, development, cell proliferation and apoptosis (Lewis et al., 2005). In embryonic stem cells (ESCs), the Oct4/Sox2 regulated miR-302-367 miRNA cluster regulates somatic reprogramming and maintains ESC pluripotency through regulation of Cyclin D1 expression (Card et al., 2008). Also, miR-1 and miR-133 modulate skeletal muscle differentiation and proliferation through targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle gene expression, while miR-21 promotes cell proliferation through its regulation of programmed cell death 4 (PDCD4) and PTEN in multiple cell types (Chen et al., 2006; Qi et al., 2009). Understandably, the extensive involvement of miRNAs in various cellular events leads their dysregulation to be associated with multiple human diseases, including cancer.

Increasing evidence indicates that aberrant miRNA expression plays an essential role in the pathogenesis of HCC, either by controlling gene expression and subsequent signaling pathways, or by interacting with oncogenes or tumor suppressors (Calin and Croce, 2006; Huang and He, 2011). Several profiling studies have identified miRNA deregulation in human HCC. miR-18, miR-19, miR-21, miR-221 and miR-222, among others, have been reported to be highly upregulated in multiple studies (Jiang et al., 2008; Ladeiro et al., 2008; Murakami et al., 2006; Volinia et al., 2006). Because their targets are tumor

suppressor genes in HCC, these miRNAs are considered to function as oncogenes. Conversely, miR-101, miR-125a, miR-199 and miR-200 have reduced expression in HCC and are considered tumor suppressive miRNAs (Gramantieri et al., 2007; Murakami et al., 2006; Su et al., 2009). miRNA replacement or inhibition therapies have thus been suggested as potential novel HCC treatment options (Braconi and Patel, 2012). As proof of principle, adeno-associated virus (AAV) vector based overexpression of a single tumor suppressive miRNA, miR-26a, in the liver was recently shown to be successful at inhibiting primary tumor growth in mice (Kota et al., 2009). However, safety considerations exist over the clinical application of AAV-vector based gene therapy, and the use of antimiR oligonucleotides instead, presents a more feasible approach against HCC (Donsante et al., 2007). To this end, inhibition of aberrantly overexpressed miRNAs in the liver using such miRNA antagonists has demonstrated efficacy in tumor xenograft studies, metabolic disease and antiviral therapy (Elmen et al., 2008; Lanford et al., 2010; Park et al., 2011). However, whether miRNA antagonists will have therapeutic utility against primary liver tumor formation and which HCC associated miRNAs should be targeted to block tumor growth remains largely unexplored.

1.3 Dlk1-Dio3 miRNA megacluster and Hepatocellular Carcinoma

Many miRNA genes are found clustered together in the genome and are transcribed as a single transcript (Saini et al., 2007). Increasing evidence indicates that miRNAs within a cluster could act in concert to regulate multiple members of single or cooperating signaling pathways, amplifying the effects of miRNAs, and playing roles in cellular processes and human disease (Olive et al., 2010). The biological relevance of these

clusters is strongly mediated by the fact that many of them have a high degree of conservation between species (Altuvia et al., 2005). For example, the highly conserved miR-23a~27a~24-2 cluster is upregulated in multiple disease conditions including heart failure and ulcerative colitis (van Rooij et al., 2006; Wu et al., 2008). In cancer, one of the best-characterized oncogenic miRNA clusters is the miR-17~92 miRNA cluster. This MYC regulated, polycistronic transcript is derived from the miR-17~92 gene and yields six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (Tanzer and Stadler, 2004). Together, these miRNAs act to repress p21, BIM, PTEN, Connective Growth Tissue Factor (CTGF) and Thrombospondin 1 (TSP1) expression, depending upon cellular context (Ho et al., 2011; Olive et al., 2009; van Almen et al., 2011; Wang et al., 2010b). The pleiotropy demonstrated by these miRNAs results in a co-ordinated effort to suppress apoptosis but enhance proliferation and angiogenesis, leading to cancer progression in multiple cell types. Conversely, miRNAs encoded by the miR-15a~16-1 cluster, first reported to be downregulated in chronic lymphocytic leukemia (CLL), target several oncogenes, including JUN, Wilms Tumor 1 (WT1), ETS1 and BCL2 to suppress tumorigenesis in a concerted fashion (Calin et al., 2008; Cimmino et al., 2005; Gao et al., 2011; Gatt et al., 2010).

Although not as well studied, more recent evidence is emerging that miRNAs from the Dlk1-Dio3 miRNA megacluster could be playing essential roles in the development of HCC. The mouse Dlk1-Dio3 domain is located on chromosome 12qF1 and shares syntenic homology to the human 14q32 locus. Spanning ~800kb, it contains three protein-coding genes on the paternal strand – Delta Like Kinase 1 (DLK1),

Retrotransposon Like Gene 1 (RTL1) and Deiodinase, Iodothyronine Type III (DIO3) and two long non-coding RNAs on the maternal strand - GTL2 and RIAN (**Figure 2**). DLK1 is a member of the Notch signaling pathway and is involved in cell differentiation of several tissues (da Rocha et al., 2008). Upregulation of DLK1 has been observed in HCC, leading to its identification as an oncogene in HCC (Huang et al., 2007). RTL1 is essential for normal development of placental and embryonic tissues and DIO3 encodes a deiodinase protecting developing tissues from excessive amounts of thyroid hormone (da Rocha et al., 2008). Analysis of the Dlk1-Dio3 region reveals imprinting regulation involving heritable epigenetic marks that distinguish the paternal and maternal chromosome (Edwards and Ferguson-Smith, 2007). These include DNA methylation and post-translational modifications to the tails of core histones (Regha et al., 2007). A major element involved in regional imprinting regulation is the intergenic differentially methylated region (IG-DMR), located 13kb upstream of the Gtl2 promoter. This element, methylated on the maternal strand, has been shown in deletion studies to be essential for proper expression of paternal protein-coding genes and maternal non-coding RNAs (Lin et al., 2003). Mice showing paternal or maternal uniparental disomy for chromosome 12 (PatD12/MatD12), improperly express genes from the Dlk1-Dio3 locus, and die either in late gestation (PatD12) or the perinatal period (MatD12) (Georgiades et al., 2000; Tevendale et al., 2006). The Dlk1-Dio3 region also hosts 53 miRNAs, seven of which are located at the anti-RTL1 region and 46 within the miRNA containing gene (MIRG) region. In addition, numerous tandemly repeated C/D small nucleolar RNAs (snoRNAs) are located ~25kb downstream of RTL1 (Cavaille et al., 2002). The C/D box family of snoRNAs are typically involved in 2'-O-methylation of other RNA species such as

rRNAs and/or spliceosomal RNAs in the nucleolus (Bachellerie et al., 2002). While the C/D snoRNAs are poorly conserved, Dlk1-Dio3 miRNAs show high conservation among verterbrates and together make up one of the largest cluster of miRNAs in the verterbrate genome (Cavaille et al., 2001).

Dlk1-Dio3 miRNAs play an important role in development, and are expressed in a range of embryonic and extraembryonic cell types with postnatal expression being found predominantly in the brain (Seitz et al., 2004). Recent studies have indicated that improper imprinting of the Dlk1-Dio3 locus leads to suppression of Dlk1-Dio3 miRNA expression and failure to generate fully functional induced pluripotent stem cells (iPSCs) (Stadtfeld et al., 2010) during iPSC reprogramming. Further, activation of the imprinted Dlk1-Dio3 region correlates with pluripotency levels of mouse ESCs and miR-134, within this cluster, has been shown to modulate differentiation of these cells through suppression of Nanong expression (Liu et al., 2010b; Tay et al., 2008a; Tay et al., 2008b). A number of these miRNAs have also been shown to be deregulated and play roles in cancer development, emphasizing the increasing importance of miRNAs from within this locus. In breast cancer, p16 downregulates CDK1 through modulation of miR-410 expression, consequently inhibiting the Rb/E2F pathway, while miR-494 is downregulated in cholangiocarcinoma and exerts cell cycle control through control of CDK6 expression (Chien et al., 2011; Olaru et al., 2011; Yamanaka et al., 2012). miR-380 overexpression is observed in primary neuroblastoma tumors, suppresses p53 expession and predicts a poor outcome (Swarbrick et al., 2010) while miR-381 has been found to play a major role in human glioma progression through suppression of the

tumor suppressor LRRC4, which is associated with increased MEK/ERK and AKT signaling (Tang et al., 2011). In the liver, the findings of several studies have raised the possibility that Dlk1-Dio3 miRNAs might play an important role in HCC development. Two independent studies showed unbiased integration of either AAV-vector or *Sleeping Beauty* transposon elements into the Dlk1-Dio3 locus resulted in HCC development in mice (Donsante et al., 2007; Dupuy et al., 2009). However, these studies did not address if miRNAs from this locus were upregulated in the resultant liver tumors. More recently, upregulation of Dlk1-Dio3 miRNAs was detected in a TRE-c-MET-driven transgenic HCC mouse model, and also in a subset of HCC patients with high HCC stem cell marker expression and poor survival (Luk et al., 2011). Together, these studies provide strong evidence that miRNAs which play oncogenic roles in HCC exist within the Dlk1-Dio3 locus. However, these remain to be identified and characterized.

1.4 MYC and RAS Activation in HCC

Although the exact pathogenesis of HCC is poorly understood, key tumor suppressor and oncogene mutations have been identified in HCC (Farazi and DePinho, 2006). The two most commonly mutated genes in HCC are TP53 and beta-catenin (van Malenstein et al., 2011). Mutation of the tumor suppressor TP53 has been observed in greater than 50% of HCC patients (Minouchi et al., 2002). Mechanistically, it is thought that p53 mutation contributes to both HCC initiation and progression. In a transgenic mouse model, functional inactivation of p53 was documented in HCC (Yu et al., 1999). Separately, studies with HCC-prone mouse models demonstrated increased tumor development when mice with a mutant p53 allele were exposed to mutagenic Aflatoxin-B1 (Ghebranious and

Sell, 1998). Oncogenic beta-catenin is the second most frequently mutated gene, found in 20-40% of HCC (Miyoshi et al., 1998). Beta-catenin activates Wnt signaling, which plays a role in differentiation, proliferation, epithelial-mesenchymal transition and stem cell renewal (Huang and He, 2008). Beta-catenin mutations lead to its stabilization in the cytoplasm or accumulation in the nucleus, usually resulting in hyperactivated proliferation (Ishizaki et al., 2004). AXIN1, normally responsible for degrading beta-catenin, is mutated in ~10% of patients, leading to blockage of beta-catenin phosphorylation and subsequent stabilization (Satoh et al., 2000). Other less frequently found mutations include RB1 (0-14%), p16 (6-30%), IGF2R (18%), PTEN (3%) and KRAS2 (2%) (van Malenstein et al., 2011; Zucman-Rossi, 2010). Early attempts to provide molecular classifications of HCC tumors focused on these oncogene and tumor suppressor mutations, but were often inadequate owing to the complexities of HCC tumor development (Boyault et al., 2007; Llovet, 2007). However, a better understanding of the molecular changes in HCC has since emerged, allowing more refined tumor classification and a deeper understanding of the multistep process in HCC development. These other molecular changes associated with HCC include amplification or deletion at sites of genomic instability, misregulated biological signaling pathways, and transcriptome analysis of both mRNAs and miRNAs.

MYC and RAS are two canonical oncogenes not frequently mutated in HCC, but are either found at sites of genomic instability (MYC) or participate in frequently misregulated signaling pathways (RAS). MYC is a transcription factor containing a basic Helix-Loop-Helix Leucine zipper (bHLH/LZ) domain (Dang, 1999). Through its bHLH

domain, MYC binds to DNA at a consensus E-box site, while the LZ domain allows dimerization with its interaction partner MAX, another bHLH transcription factor (Landschulz et al., 1988; Murre et al., 1989). MAX is a ubiquitous protein, thus the activity of MYC/MAX heterodimers relies on the sophisticated control of MYC protein expression (Cascon and Robledo, 2012). In humans, the MYC gene is located at chromosome 8q24, a region frequently amplified in HCC, leading to the detection of MYC overexpression in up to 79% of alcohol and viral related HCC (Chan et al., 2004; Gan et al., 1993). Mechanistically, MYC is predicted to bind to approximately 15-20% of the genome (Meyer and Penn, 2008) and as both a transcriptional activator and repressor, directly and indirectly regulates genes that control cell cycle, protein synthesis, cytoskeleton and cell motility, cell metabolism and apoptosis (Abe et al., 2013; Hartl et al., 2006; Miller et al., 2012; Singh and Dalton, 2009; van Riggelen et al., 2010). In HCC, MYC activates the HBx gene, which transforms hepatocytes through multiple mechanisms (Terradillos et al., 1997). Another MYC activated gene during HCC development is HIF-1a, which co-operates with MYC to enhance expression of vascular endothelial growth factor-A (VEGFA), a critical angiogenesis gene (Huang, 2008). More recently, Myc has also been shown to regulate transcription of many miRNAs, including the oncogenic miR-17~92 cluster and miR-9, and the tumor suppressive miRNAs let-7, miR-195 and miR-34a (Frenzel et al., 2010).

The tumor oncoproteins H-RAS, K-RAS and N-RAS are founding members of a large superfamily of GTPase proteins (Young et al., 2009). The RAS superfamily consists of more than 150 proteins, which play a major role in cellular signal transduction through

multiple pathways, including the RAF/MAPK and PI3K pathways, resulting in cell growth, differentiation and survival (Gollob et al., 2006; Jiang and Liu, 2009; Rajalingam et al., 2007). Although not frequently mutated in HCC, RAS oncoproteins are often found overexpressed, leading to hyper-activated RAS signaling pathways. In the progression from cirrhosis to liver cancer, H-RAS is nearly universally over-expressed in tumor tissues and the active GTP-bound form is especially high in those tumors associated with the worst prognosis (Calvisi et al., 2006; Calvisi et al., 2007). In addition to H-RAS over-expression, RAS signaling pathways can also be activated through upstream activation of receptor tyrosine kinases, such as MET, EGFR or TGF-α, direct activating mutations of NRAS (6%) or KRAS (6%), or down-stream mutation of BRAF (3%) or PIK3CA (6%) (Thorgeirsson and Grisham, 2002). RAS is also intricately involved in miRNA pathways and is both regulated by (let-7), and a regulator of, miRNAs (miR-21, miR-221, miR-222) (Frezzetti et al., 2011; Johnson et al., 2005).

RAS and MYC are well known to cooperate to induce tumorigenesis. RAS signaling may affect MYC by two independent mechanisms. First, the RAS activated PI3K pathway inhibits glyocgen synthase-3, which is responsible for phosphorylating MYC at Thr58 and leading to its degradation. This increased RAS activity allows MYC to evade ubiquitin-mediated proteolysis (Downward, 2003). Second, RAS activates RAF-1, which in turn activates the ERK/MAPK signaling cascade, leading to phosphorylation and stabilization of MYC at Ser62 (Bachireddy et al., 2005). Co-expression of MYC and RAS in fibroblasts increases the number and size of soft agar colonies (Land et al., 1986; Lee et al., 1985; Yancopoulos et al., 1985), while animal studies in the prostate and breast

have shown that MYC and RAS co-expression gives rise to more rapid tumor formation (Podsypanina et al., 2008; Sinn et al., 1987; Thompson et al., 1989). In HCC, over-expression of both MYC and activated H-RAS in primary human liver tumors has been associated with aggressive forms of liver cancer with an especially poor prognosis (Calvisi et al., 2006; Tiniakos et al., 1989). While it is clear that MYC and RAS play important roles in HCC, little is known about the effect these canonical oncogenes exert individually and coordinately on miRNA expression in HCC.

1.6 Breast Cancer and p53

Similar to HCC, breast cancer is a heterogeneous disease. It is the second most common cancer, after lung cancer, with greater than 1,300,000 cases and 450,000 deaths worldwide. Clinically, breast cancer is categorized into three large molecular subgroups based upon the expression of three different hormone receptors in tumors. The presence or absence of each receptor guides the therapeutic approaches applied toward each subgroup. Briefly, the estrogen receptor (ER) positive group is the most numerous and diverse, and tumors of this subtype are responsive to endocrine therapy that antagonize the ER, such as Tamoxifen (van 't Veer et al., 2002). The Human Epidermal Growth Factor Receptor 2 (HER2) amplified group is effectively targeted by either monoclonal antibodies that disrupt HER2 function or tyrosine kinase inhibitors (TKIs) that antagonize downstream signaling pathways of HER2 (Slamon et al., 1987). Despite the success of treating ER and HER2 positive tumors, triple-negative breast cancers (TNBCs) which lack expression of ER, HER2 and progesterone receptor (PR) are a subgroup for which only conventional chemotherapy is available. These tumors have an increased incidence

in patients with germline BRCA1 mutations (Foulkes et al., 2003). Interestingly, TP53 mutations are frequently observed in TNBCs and are thought to be among the key driving factors of this aggressive subgroup. TP53 expression has also been demonstrated to predict worse overall and event-free survival in TNBC patients and is a prognostic marker for chemotherapeutic response (Biganzoli et al., 2011).

The tumor suppressor gene TP53 encodes a 53kDa DNA binding protein containing an N-terminus activation domain 1 (AD1) which activates several pro-apoptotic genes, AD2 and proline rich domain important for apoptotic activity, central DNA binding domain (DBD), a nuclear localization signal domain, homo-oligomerization domain and C-terminus domain which regulates DNA binding to the central DBD (Harms and Chen, 2005; Larsen et al., 2010; Piskacek et al., 2007; Venot et al., 1998). It is activated in response to several malignancy associated stress signals such as telomere erosion, hypoxia, microtubule inhibition, DNA damage, ribonucleotide depletion and loss of survival signals. Activated p53 binds to DNA and in turn, activates a host of other genes, including those involved in apoptosis (BAX, FAS, NOXA, PUMA), cell cycle arrest (p21), DNA repair (GADD45), angiogenesis (TSP1) and invasion (MMP2). How a cell responds to p53 activation is dependent upon cell type, cell environment and other oncogenic alterations sustained by the cell, although the overarching goal of p53 activation is to inhibit cell growth, either through cell cycle arrest or induction of apoptosis, thereby preventing tumor development (Vousden and Lu, 2002).

Wildtype p53 function in cancer can be lost by a variety of mechanisms, including mutations within the p53 gene as well as lesions that affect the post-transcriptional and post-translational regulation of the p53 protein (Vousden and Lu, 2002). p53 mutations most frequently occur (95%) within the DBD domain and are among the most common genetic change identified in human neoplasia, seen in about 50% of all cancers (Sigal and Rotter, 2000). However, this occurrence is much less frequent in breast cancers, as demonstrated by a comprehensive meta-analysis that revealed only approximately 20% of breast cancers express mutant p53 (Pharoah et al., 1999). It has been demonstrated that proteins such as ATM, ATR and CHK2 which function upstream of p53 to regulate its stability and function are instead frequently mutated in breast cancer, partially accounting for the loss of p53 function (Bell et al., 1999; Dork et al., 2001). However, it has also been demonstrated that the steady state level of p53 mRNA is lower in many breast cancers than in normal breast epithelium, suggesting that post transcriptional mechanisms may play a role in regulating p53 expression (Raman et al., 2000). These mechanisms include regulation by the RNA binding protein HuR, miRNAs such as miR-125b and miR-380 and mutations within the p53 3' UTR (Gefen et al., 2010; Mazan-Mamczarz et al., 2003; Swarbrick et al., 2010).

The 1.2kb p53 3' UTR directs gene expression by controlling mRNA stability, nuclear transport, intracellular translocalization and translational efficiency (Gebauer and Hentze, 2004; Kuersten and Goodwin, 2003). Conventionally, this operates in cis by RNA binding regulatory proteins and miRNAs. The RNA binding protein HuR binds to a target site in the p53 3' UTR in response to ultraviolet exposure, potently enhancing its

translation. miR-125b, miR-380 and miR-504 have also been shown to downregulate p53 protein expression through 3' UTR target sites (Lin et al., 2012).

Efficient regulation of protein expression may be lost when mutations are introduced into the 3' UTR, resulting in a disease state. A single nucleotide polymorphism (SNP) in the K-RAS 3' UTR inhibits regulation by let-7 and confers decreased survival in patients with oral cancers and increased risk to non-small cell lung cancer, while a SNP in the SLITRK1 3' UTR enhances the binding of miR-189, decreasing SLITRK1 protein levels and resulting in Tourette's syndrome (Abelson et al., 2005; Chin et al., 2008; Christensen et al., 2009). Further, SNPs in the 3' UTR of GDF8 (miR-1 and miR-206) and AGTR1 (miR-155) result in novel miRNA target sites created and contribute to the development of muscular hypertrophy and cardiovascular disease respectively (Clop et al., 2006; Martin et al., 2007). Whether such SNPs occur within the p53 3' UTR, and if they regulate p53 mRNA expression, is a crucial question to address, in order to further understand the regulatory mechanisms that govern p53 expression in breast cancer. Understanding how p53 is regulated in breast cancer could also allow its use as a predictor of chemotherapeutic response in TNBC.

1.7 Figures

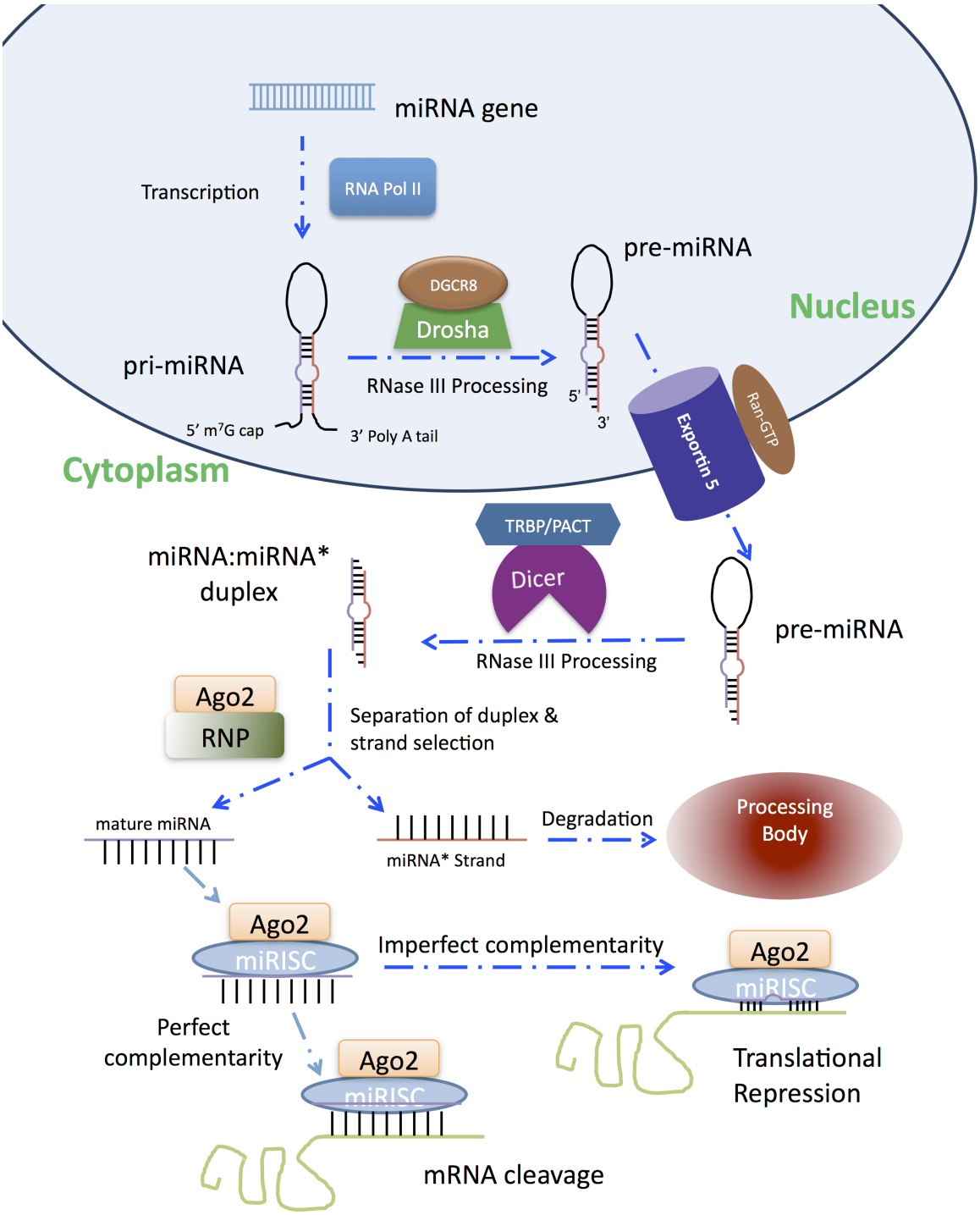


Figure 1. miRNA biogenesis

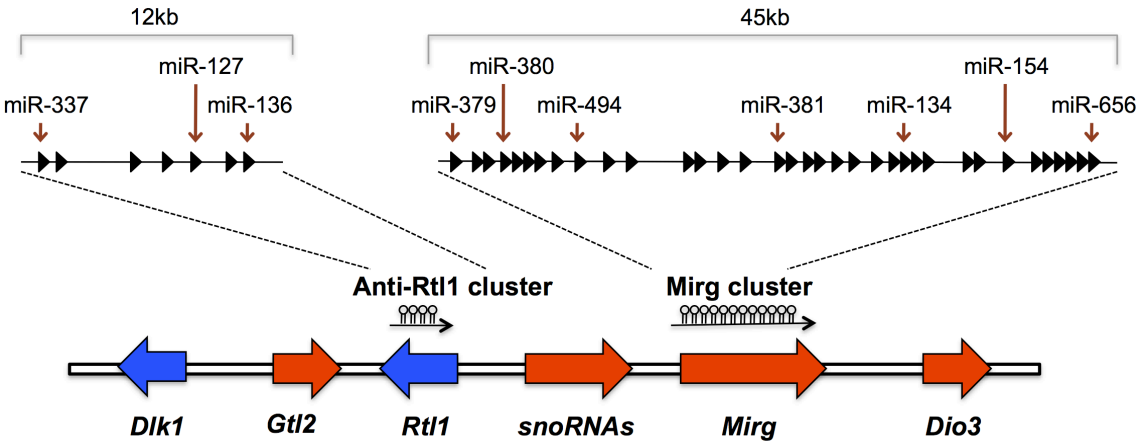


Figure 2. Genomic organization of the mouse Dlk1-Dio3 locus

Chapter 2: The Dlk1-Dio3 miRNA megacluster is upregulated in multiple HCC models

2.1 Introduction

HCC is currently an intractable cancer with few effective treatment options, and there is an urgent need to develop novel therapeutics for this disease. miRNAs can play numerous roles in normal cellular and tissue function, and their aberrant expression is associated with multiple human diseases, including HCC. Interestingly, recent primate and mice studies demonstrate that hepatocytes readily take up small nucleic acid based molecules, resulting in significant therapeutic benefit against metabolic disease and viral infection. Therefore, the use of small RNA based molecules to control aberrant miRNA expression in HCC could be an effective approach. However, whether miRNA antagonists will have therapeutic utility against primary liver tumor formation and which HCC associated miRNAs should be targeted to block tumor growth remains largely unexplored.

In this chapter, we utilized multiple transgenic mouse models to identify highly upregulated miRNAs in HCC. These models conditionally and selectively express MYC, RAS or MYC+RAS in the liver, leading to tumor formation, or utilize *Sleeping Beauty* Transposon insertional mutagenesis to generate HCC in an unbiased fashion. We reasoned that miRNAs upregulated in multiple liver tumor models could be playing important roles in HCC development and their inhibition, accordingly, could prevent or block tumor growth. We demonstrate that miRNAs from the Dlk1-Dio3 miRNA

megacluster are upregulated in distinct, unbiased tumor models and human HCC samples. From this cluster, we identify several miRNAs that accelerate colony formation of liver tumor cells in soft agar. Additionally, we find that Dlk1-Dio3 miRNA expression is MYC dependent. Together, our data indicate that several oncogenic miRNAs exist within this highly upregulated miRNA megacluster in HCC.

2.2 Results

Mouse liver tumors are distinct at the molecular level and are comparable to human liver cancers

To gain a better understanding of the miRNA changes that occur in HCC development, we sought to model HCC in the mouse such that the frequently activated MYC and RAS oncogenic pathways can be activated either alone or together. Liver tumors driven by the doxycycline-regulated expression of MYC (LAP-tTA/Tet-o-MYC or LT2/MYC) have been previously reported (Shachaf et al., 2004). In this study we developed a new *HRAS*^{V12}-driven model of liver cancer (LAP-tTA/Tet-o-RASV12 or LT2/RAS) and crossed these two strains to generate mice with liver tumors driven by co-expression of both *MYC* and *HRAS*^{V12} oncogenes (LAP-tTA/Tet-o-MYC/Tet-o-RAS or LT2/MYC/RAS).

Adult mice of each genotype were taken off doxycycline at 8 weeks to induce oncogene expression and tumor development. Survival of tumor-bearing mice differed depending on the specific oncogene expressed (**Figure 3 A**). Triple-transgenic (LT2/MYC/RAS) mice developed tumors and died within 7 weeks after both oncogenes were induced. In

contrast, RAS/LT2 and MYC/LT2 mice survived for 12-15 weeks and 30-35 weeks after doxycycline removal, respectively ($p < 0.0001$) (**Figure 3 A**). This indicates that expression of both potent oncogenes together in the liver (**Figure 3 B**) hastened tumor development and resulted in decreased survival. Our analyses of livers from the three tumor models further showed that *MYC* and *RAS* oncogenes induced morphologically and histologically distinct liver tumors (**Figure 3 C and E**). Likewise, combined expression of MYC and RAS together gave rise to tumors distinct from either oncogene alone (**Figure 3 C and E**). Our characterization of the mouse liver tumors from the three genotypes and control LT2 mice confirm that while histologically distinct, each of the transgenic models represents *bona-fide* liver tumors.

Transgenic Liver Tumor Models Share miRNA Expression Profiles with Aggressive Human HCCs

While it is clear that MYC and RAS play important roles in HCC, little is known about the effect these canonical oncogenes exert individually and coordinately on miRNA expression in HCC. RNA was prepared from the tumors of four independent mice from each genotype (LT2/MYC, LT2/RAS, LT2/MYC/RAS), as well as normal LT2 liver tissue for global miRNA expression profiling (DiscovArray, Assuragen). *In silico* analyses showed that the global miRNA expression profile from each genotype was distinct (**Figure 4**), suggesting that miRNA expression profiles can distinguish between tumors driven by specific oncogenes.

We have previously determined that mRNA expression signatures from these mouse tumor models cluster with those from human HCCs, and that these models have histologic features that resemble human HCC (Asha Balakrishnan, manuscript in preparation). To further establish the validity of our models in replicating human HCC, we tested if miRNA deregulation in our models was similar to that in human HCCs. We first compared the miRNA expression profiles of our mice with publicly available data from miRNA profiling performed in 97 human HCC samples (Luk et al., 2011). Hierarchal clustering of miRNAs common to both datasets showed that mouse tumor samples clustered with a subset of human HCC samples (n=18, Fisher's Exact Test $p<0.0001$, **Figure 5**) that have previously been associated with high alpha-fetoprotein (AFP) and HCC stem cell marker expression, as well as decreased survival (Luk et al., 2011).

We next performed a literature search for HCC-associated miRNAs identified through human HCC profiling studies and determined the expression of these miRNAs in our tumor models (expression cutoff >2 or <2 fold, $p<0.05$)(Connolly et al., 2008; Datta et al., 2008; Fornari et al., 2009; Meng et al., 2007; Su et al., 2009; Wang et al., 2010a; Wang et al., 2008; Wong et al., 2010; Xiong et al., 2010; Xu et al., 2009). We observed previously reported RAS dependent miRNAs, miR-21, -221 and -222, specifically upregulated in our RAS driven tumors and the MYC repressed miRNA, miR-195, specifically downregulated in MYC and MYC+RAS driven tumors(Chang et al., 2008; Frezzetti et al., 2011; Tsunoda et al., 2011). However, downregulation of tumor suppressive miRNAs miR-1, -29, -101, and upregulation of the oncomiR-17-92 cluster

were observed in all three tumor genotypes (**Table 1**). Together, these data indicate that although miRNA expression profiles in our tumor models are oncogene specific, they are comparable to human HCC, and that tumors from these transgenic models resemble human HCC.

A miRNA megacluster on mouse chromosome 12qF1 is upregulated in multiple HCC models.

To identify potentially oncogenic miRNAs from those aberrantly expressed in our tumor models, we hypothesized that the subset of miRNAs commonly upregulated in all three tumor genotypes are important in HCC development driven by MYC and/or RAS. Thus, we generated a venn diagaram of miRNAs that were at least 2 fold up- or down-regulated in our liver tumors ($-1 > \log_2 > 1$, $p<0.05$, **Table 2 and Table 3**) and found fifty-seven miRNAs commonly upregulated in all three genotypes, compared to normal LT2 mice (**Figure 6 A**). Since previous studies have shown that many miRNA genes are located in genomic clusters which can play important roles in mammalian development, human disease and cancer (Lagos-Quintana et al., 2003; Stadtfeld et al., 2010), we sought to determine if any of the 57 commonly upregulated miRNAs fall into clusters separated by 1Kb or less within the genome. We found that 36 of the 57 miRNAs were located in previously identified clusters (**Table 2**). These included the oncogenic miR-17-92 cluster and its homologs, previously shown to be regulated by MYC, and elevated in lymphoma and colon cancer (He et al., 2005; Mendell, 2008).

Surprisingly, 32 of the 57 commonly upregulated miRNAs were clustered in the same

genomic locus on mouse chromosome 12qF1 (**Figure 6 B**). This imprinted region, known as the Dlk1-Dio3 domain, is conserved among placental mammals and collectively comprises approximately 5% of all known miRNA genes (Cavaille et al., 2002). miRNAs from the Dlk1-Dio3 region are split into two separate clusters, which we termed the Anti-Rtl1 and Mirg clusters, respectively **(Figure 2**). We next asked if this miRNA megacluster is also upregulated in other distinct models of liver cancer. A transgenic *Sleeping Beauty* transposon based mutagenesis system results in diverse tumor types, including liver cancer which occurs with an ~35% frequency (Dupuy et al., 2009). We performed pyrosequencing on these liver tumors and identified two preferential genomic integration sites, one of which was within the Anti-Rtl1 cluster and represented 73% (8/11 mice) of the integrations. Further analysis revealed that five of these eight transposon integrations were in the same transcriptional orientation as the miRNAs. Sequencing of small RNAs from HCC samples revealed that miRNAs from the Dlk1-Dio3 megacluster were significantly upregulated in this group of tumors, suggesting that these mutations may promote tumorigenesis via upregulation of Dlk1-Dio3 megacluster miRNAs (**Figure 7**). Together, these results suggest that miRNAs within the Dlk1-Dio3 megacluster could be playing an oncogenic role in the development of HCC in mice.

miR-494 enhances transformation of liver tumor cells and is upregulated in human HCC.

Studies of oncogenic miRNA clusters have found that only one or a few miRNAs may account for the principal oncogenic potential of these clusters, such as miR-19, within the miR-17-92 cluster (Mu et al., 2009; Olive et al., 2009). Thus, we sought to identify

specific oncogenic miRNAs from within the Dlk1-Dio3 megacluster. To do so, we first tested the ability of Dlk1-Dio3 miRNAs to accelerate growth of liver tumor cells in soft agar. We used a mouse liver tumor cell line (LT2MR) that had reduced or absent expression of many Dlk1-Dio3 miRNAs (**Figure 10 B**), making it a suitable system to assess the effects of overexpression of miRNAs within the megacluster. To allow for efficient retroviral expression of miRNAs from this cluster, we split the Dlk1-Dio3 megacluster into 13 subclusters of miRNAs, each containing between 1 and 6 miRNAs. Each subcluster was amplified from human genomic DNA and cloned into retroviral expression vectors (**Figure 8 A**). Individual subclusters were stably expressed in LT2MR cells and their expression was verified by quantitative real time PCR (qRT-PCR) of a representative miRNA within each subcluster (**Figure 8 B**). Soft agar assays were performed and colonies counted after 2 weeks. We found four subclusters of miRNAs that significantly ($p<0.05$) increased the colony number of LT2MR cells grown in soft agar. Of these, the SM-4 and SM-5 subclusters, containing miR-494 and miR-495 respectively, were the most potent at increasing soft agar growth **(Figure 8 C**).

Our profiling studies showed miR-494 and miR-495 to be highly upregulated in all three tumor genotypes (39 and 60.8 fold, respectively in MYC, 2.36 and 10.7 fold, respectively in RAS and 57.7 and 132.5 fold, respectively in MYC+RAS, $p<0.05$), and this was further verified by qRT-PCR (**Figure 9 A**) performed on primary tumor samples. To investigate if these miRNAs might also be upregulated in primary tumors from human HCC, we performed qRT-PCR on 47 human HCC samples and their matched normal

tissues. 34% (n=16) and 27% (n=13) of samples had >1.5 fold miR-494 and miR-495 overexpression respectively, compared to normal liver tissue (**Figure 9 B**).

Dlk1-Dio3 miRNA expression is MYC dependent.

We next wondered how Dlk1-Dio3 miRNAs were regulated in our tumor models. Previous studies have indicated that the transcription factor MEF2A directly regulates expression of Dlk1-Dio3 miRNAs during skeletal muscle regeneration, and we asked if this was also the case in the liver (Snyder et al., 2013). To test this, we cloned and overexpressed MEF-2A stably in LT2MR cell lines (**Figure 10 A**). RNA was collected from these cells and qRT-PCR was performed to compare the expression of Dlk1-Dio3 miRNAs between control and MEF-2A overexpressing cell lines. We did not observe an increase in representative Dlk1-Dio3 miRNAs in MEF-2A overexpressing cell lines, indicating that MEF-2A is unlikely to be a direct regulator of Dlk1-Dio3 miRNA expression in the liver (**Figure 10 B**).

It has been suggested that proper imprinting at the IG-DMR is required for repression of non-coding RNAs within the Dlk1-Dio3 locus (Seitz et al., 2004). To test if the increase in Dlk1-Dio3 miRNAs in our tumor models was mediated by decreased methylation at the IG-DMR, we performed bisulfite sequencing on DNA extracted from the liver tumors and normal tissue of LT2 mice. We first identified a CpG island within the IG-DMR that contained 29 CpG sites using Methprimer (**Figure 11 A**). Bisulfite conversion was performed on LT2, LT2/MYC, LT2/RAS and LT2/MYC/RAS DNA. Methylation specific primers were used to sequence five clones from each genotype. Our data

indicated that there was no difference in methylation status at the IG-DMR between control and tumor mice, suggesting that loss of imprinting does not play a role in Dlk1-Dio3 miRNA upregulation in our models (**Figure 11 B**).

Several studies suggest that MYC could regulate Dlk1-Dio3 miRNA expression. In MMTV-c-MYC and TET-OFF-MYC lymphoma murine models, the expression of miR-494 was found to be MYC dependent (Sander et al., 2008; Zhu et al., 2011). We wondered if this was also the case in the liver. To test this, we first utilized cells isolated from LT2/MYC tumors that were doxycycline responsive. Addition of doxycycline to these cells in culture resulted in loss of MYC expression (**Figure 12 A**). RNA was isolated from these cells and qRT-PCR was performed to compare the expression of representative Dlk1-Dio3 miRNAs when MYC levels changed. We detected a decrease in representative Dlk1-Dio3 miRNAs miR-487b, miR-299-5p, miR-127, miR-412 and miR-494, as well as the previously identified MYC dependent miRNA miR-17-5p (**Figure 12 B**). However, expression levels of miR-382 and a non MYC-responsive miRNA, miR-21, remain unchanged. We next utilized a human HCC cell line, HepG2, which had high MYC expression. MYC expression was reduced by siRNA transfection and RNA was collected to determine miRNA expression (**Figure 12 C)**. Again, we detected decreased expression in miR-494, as well as the positive control miR-18a. miR-122 was used as a negative control and remain unchanged (**Figure 12 D**). Finally, we examined the expression of let-7g, miR-18a and miR-494 when MYC levels were altered *in vivo*. In this model, MYC expression was conditionally induced in LT2/MYC mice and either acutely terminated after five weeks, or left on (Hu et al., 2011). Consistent with our

observations in cell lines, we found that MYC expression *in vivo* increased expression of miR-18a and miR-494, but not let-7g, when compared to normal LT2 mice. However, acute loss of MYC expression resulted in a downregulation of miR-18a and miR-494 expression (**Figure 12 E**). Together, these results suggest that Dlk1-Dio3 miRNA expression is MYC dependent.

2.3 Discussion

There exists an urgent need to develop novel therapeutics against HCC. The affinity of hepatocytes for small nucleic acids suggests miRNA based therapy might be a promising approach. However, a better understanding of miRNA deregulation and function in HCC is first required. In this chapter, *de novo* tumor formation provided us with the ability to characterize oncogene specific miRNA expression changes. We report, for the first time, the miRNA expression changes associated with liver tumor formation driven by MYC, RAS and MYC+RAS expression changes *in vivo.* We find that the Dlk1-Dio3 miRNA megacluster is upregulated in multiple liver tumor models and identify miRNAs from within this cluster that accelerate cell growth in soft agar. Finally, we show that the expression of miR-494, together with several other Dlk1-Dio3 miRNAs is MYC dependent.

The common upregulation of a large megacluster of miRNAs from the Dlk1-Dio3 locus in multiple tumor models was initially surprising to us. However, recent studies have highlighted the increasing importance of this locus in HCC. Two separate studies found

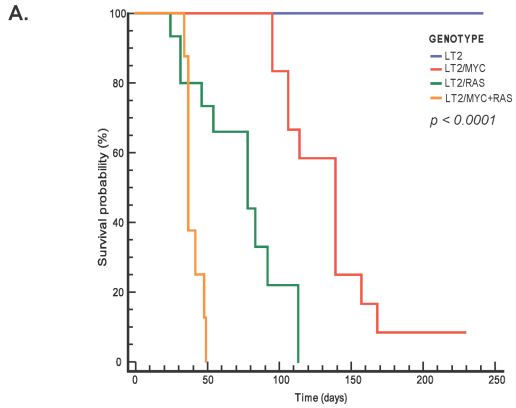
that insertions of active promoters at the Dlk1-Dio3 locus led to overexpression of miRNA transcripts from this locus and HCC development in mice (Donsante et al., 2007; Dupuy et al., 2009). Elevated expression of this miRNA cluster was also detected in a MET-driven model of liver cancer and a subset of human HCCs that correlated with a more aggressive, stem-like tumor subtype and worse prognosis (Luk et al., 2011), although whether any of the overexpressed miRNAs was important for liver tumor formation was not established. The convergence of these data suggests that Dlk1-Dio3 miRNAs could play an important role in HCC development. In this chapter, we dissected this cluster in a systematic manner, and identified several miRNAs that could be playing oncogenic roles in HCC development: miR-494, miR-495, miR-539, miR-889, miR-544, miR-655, miR-382, miR-134, miR-668, miR-485 and miR-453. Of these, miR-494 and miR-495 separately caused the greatest increase in soft agar colony formation. Further work remains to be done to dissect the contributions of miR-539, miR-889, miR-544 and miR-655 within the SM-7 subcluster and miR-382, miR-134, miR-668, miR-485 and miR-453 within the SM-8 cluster. Although several of these miRNAs have been found differentially expressed in other diseases, including cancer, their functional significance in HCC is not well understood and require further study (Benetatos et al., 2012). Since miR-494 and miR-495 were individually able to accelerate soft agar colony formation in a significant manner, we tested their expression in human HCC samples and were able to detect an upregulation of these two miRNAs in a subset of HCC. Work on miR-494 is performed and discussed in Chapter 3. miR-495 has been demonstrated to be upregulated by the transcription factor E12/E47 in breast cancer stem cells, promoting oncogenesis and hypoxia resistance via downregulation of E-cadherin and REDD1 (Hwang-Verslues

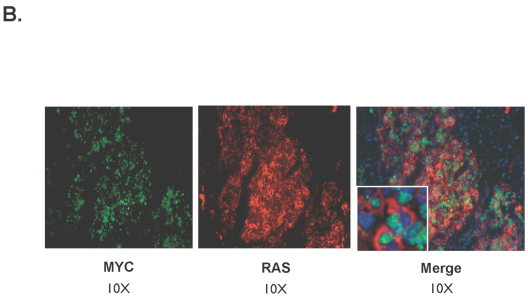
et al., 2011). It is also predicted to target the tumor suppressor PTEN (TargetScan). Conversely, it has been identified as a tumor suppressor miRNA downregulated in leukemia, and targeting PBX3 and MEIS1. It would thus be interesting to determine the role of miR-495 in the pathogenesis of HCC.

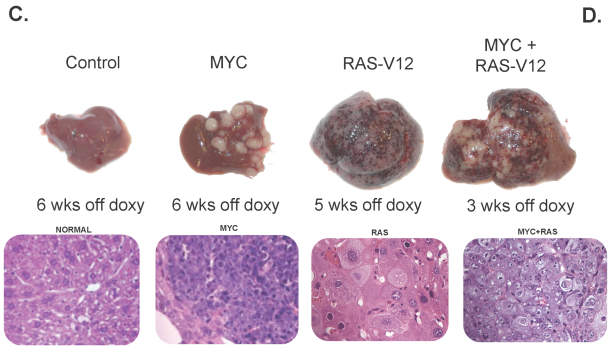
Regulation of Dlk1-Dio3 miRNA expression appears to involve a complex interplay of growth factors, transcription factors and DNA methylation. In this chapter, we demonstrate that expression of miR-487b, miR-299-5p, miR-127, miR-412 and miR-494 from within the Dlk1-Dio3 megacluster is MYC dependent, while miR-382 expression appears MYC independent. Although the transcriptional control of Dlk1-Dio3 miRNAs is poorly understood, it is thought several transcripts arise from within this locus. miR-433 and miR-127 were demonstrated to arise from independent, overlapping transcripts (Song and Wang, 2008a). This is consistent with our data, which suggests miR-382 could be transcriptionally regulated independent of miR-487b, miR-299-5p, miR-127, miR-412 and miR-494. Further, several other factors appear to regulate miRNA expression from this locus. SHP and ERRgamma induces miR-433 and miR-127 expression while knockdown of the hepatocyte growth factor receptor, c-met, downregulates miR-127 and miR-431, but not miR-433, expression (Luk et al., 2011; Song and Wang, 2008b). Although we find that several of these miRNAs are MYC dependent and MEF2A independent, it seems possible that other transcription factors could be also be regulating Dlk1-Dio3 miRNA expression. Previous studies also suggest that loss of imprinting could result in aberrant overexpression of miRNAs from this locus. Specifically, miR-127 is highly expressed in T24 bladder cancer cells after treatment with chromatin modifying

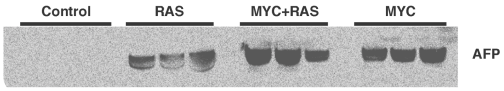
drugs that cause DNA demethylation (Saito et al., 2006). Although we were unable to show that decreased methylation at the IG-DMR is an explanation for increased Dlk1-Dio3 miRNA expression in tumors, we do not rule out the possibility that CpG islands closer to the promoters of these miRNA transcripts could be regulating their expression. Clearly, further work is required to identify these promoters, CpG islands and other factors that regulate Dlk1-Dio3 miRNAs in order to fully understand their regulation in HCC.

2.4 Figures and Tables









E.

Features	MYC	RAS	MYC+RAS
Tumor Morphology and Histology	• Pseudo-encapsulated rounded nodules. • Highly proliferating, poorly differentiated cells. • Irregular ovoid to round nuclei. • Increased nuclear to cytoplasmic ratios. • Prominent, frequently multiple, macronucleoli.	• Highly vascular. • Grow as nests and cords of poorly cohesive, disorganized cells infiltrating throughout the liver parenchyma. • Abundant vacuolated cytoplasm. • Enlarged round to oval nuclei. • Prominent nucleoli and occasional intranuclear cytoplasmic inclusions. • Frequent areas of vascular channels and cystic degeneration.	• Relatively heterogeneous lesions. • Frequently grow as lobulated rounded nodules composed of disorganized sheets of cells. • Nuclear enlargement with prominent, frequently multiple, macronucleoli. • Variable nuclear to cytoplasmic ratios. • Occasional groups of cells show moderate amounts of eosinophilic cytoplasm.
AFP expression	High	High	High
Human counterpart	Poorly differentiated HCC or HB	HCC	Aggressive variant of HCC or fetal variants of human HB

Figure 3. Doxycycline-regulated expression of MYC and/or RAS oncogenes give rise to distinct liver tumors. A. Kaplan-Meier survival curve showing the MYC+RAS mice develop tumors and die earlier than mice with either oncogene alone or controls. **B.** Immunoflorescence shows co-expression of MYC and RAS in triple transgenic mouse liver tumor tissue. **C.** Mouse liver tumors and corresponding H&E sections show that livers from mice from different genotypes are distinct from each other. **D.** Liver tumor marker, AFP expression in liver tumors from the three genotypes compared to normal LT2 livers show that these tumors are *bona fide* liver tumors. **E.** Table summarizing characteristic features of the tumors from the MYC, RAS and MYC+RAS genotypes.

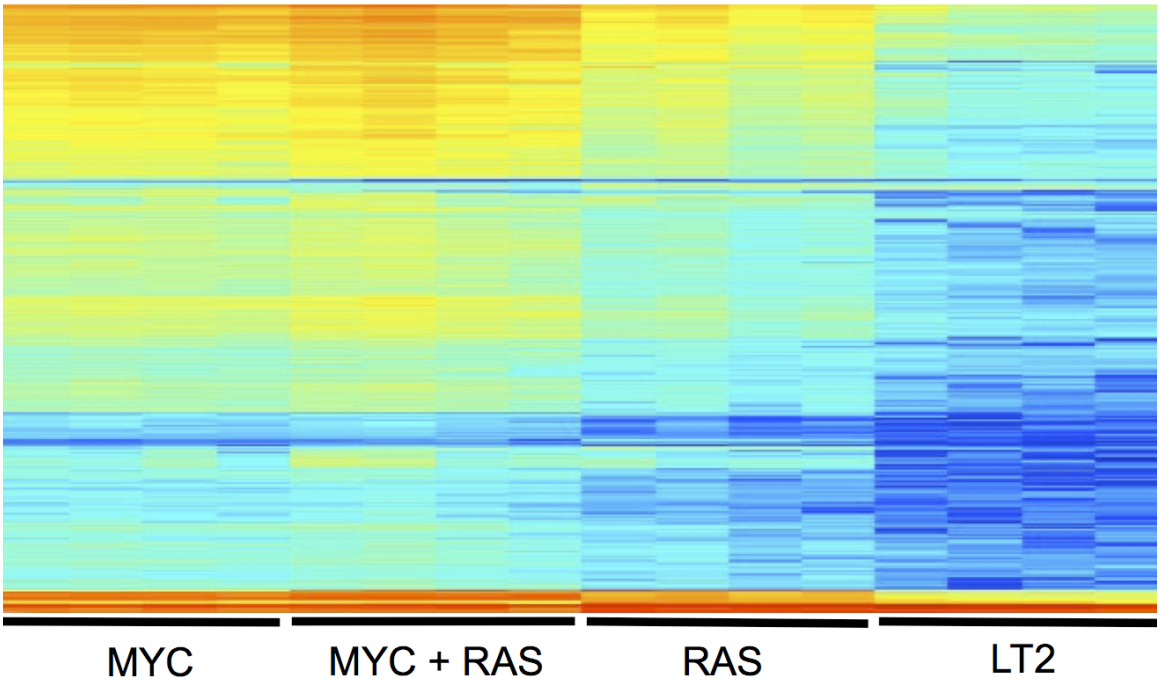


Figure 4. miRNA expression profile of LT2 mouse models. Heatmap of miRNA gene expression profiles shows that the four independent samples from each genotype cluster together

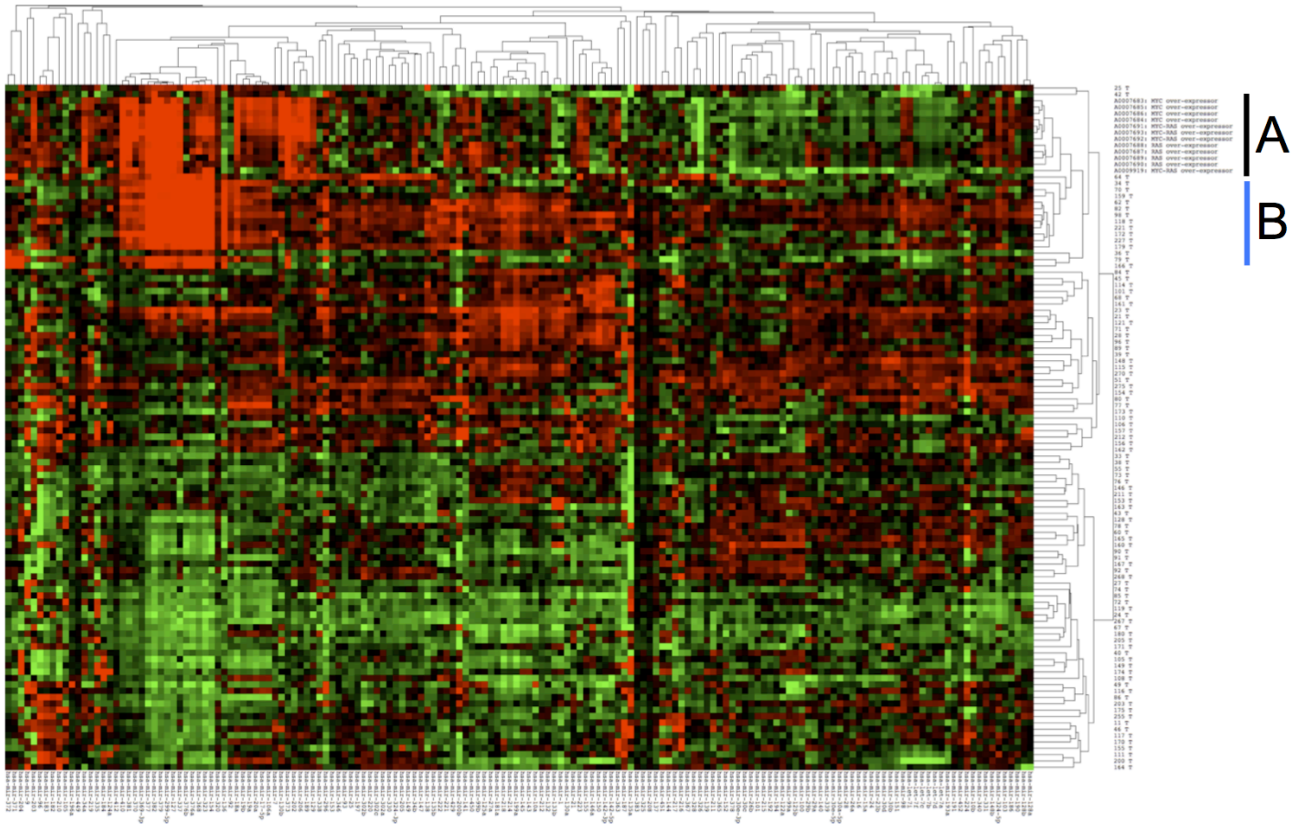
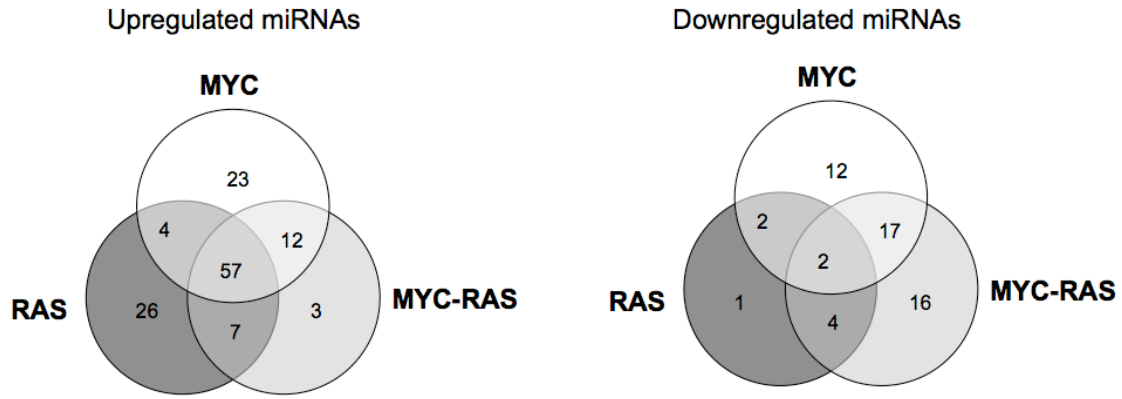


Figure 5. Comparison of miRNA expression between LT2 mouse models and human HCC samples. Heatmap of hierarchical clustering of human and mouse miRNA expression profiles shows mouse signatures (black line, A) cluster close to a subset of human HCCs (blue line, B).

A.



B.

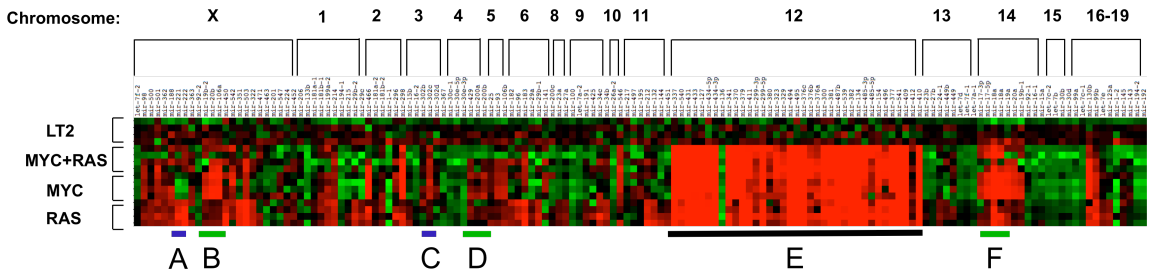


Figure 6. A miRNA megacluster on mouse chromosome 12qF1 is upregulated in LT2 tumor models. A. MiRNAs differentially expressed in tumor tissues were grouped according to unique or combined expression in each tumor genotype. MiRNAs significantly changed by at least 2-fold compared to normal liver ($p<0.05$) were used to generate the venn diagram. **B**. Hierarchical clustering of all miRNAs located within 1kb of each other in the mouse genome reveals an upregulated megacluster on Chr12qF1 in mouse tumors (E, underlined in black). miR-17-92 oncomiR cluster and its homologs are indicated with green lines (B, D and F). RAS regulated miR-221-222 cluster shows RAS specific expression (A), while MYC regulated miR-302b-d cluster shows MYC specific expression (C).

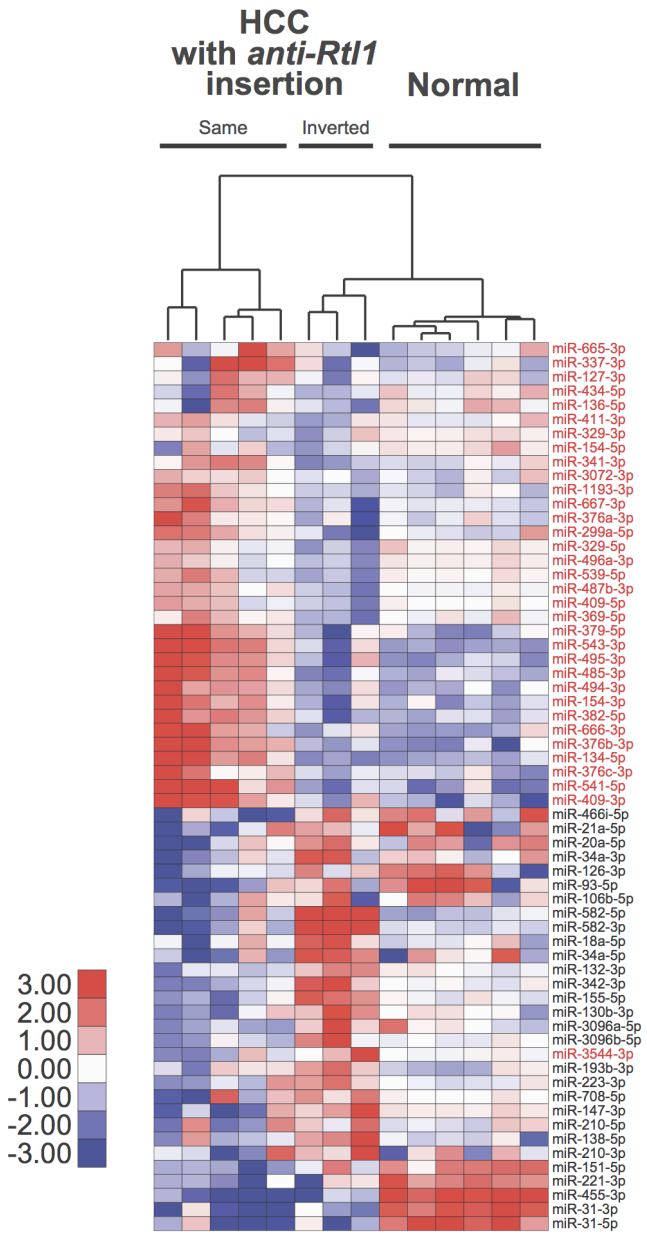
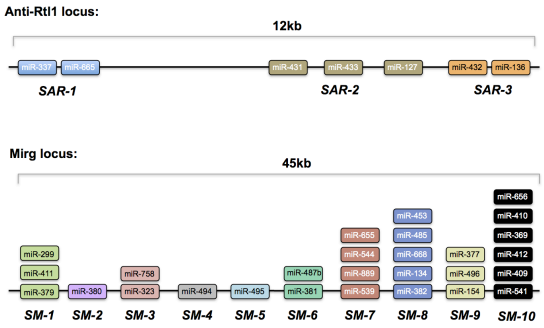


Figure 7. Dlk1-Dio3 miRNAs are upregulated in a Sleeping Beauty Transposon model of HCC. Sleeping beauty transposon integration in the same transcriptional orientation as the anti-Rtl1 cluster causes HCCs in mice, accompanied by an upregulation of Dlk1-Dio3 miRNAs (indicated in red). This is compared to HCCs with inverted SB insertions in the anti-Rtl1 cluster and normal livers that have no Dlk1-Dio3 miRNA upregulation.

A



B

Cell Line	Relative Parental Cell Expression	Relative Expression in Engineered Cells
LT2 SM-1 (miR-299-5p)	1	37.89073705
LT2 SM-2 (miR-380)	1	4.752939733
LT2 SM-3 (miR-323)	1	1352.760116
LT2 SM-4 (miR-494)	1	2.290649217
LT2 SM-5 (miR-495)	1	1099.456788
LT2 SM-6 (miR-487b)	1	342.7339699
LT2 SM-7 (miR-544)	1	2.26212382
LT2 SM-8 (miR-134)	1	270.3482461
LT2 SM-9 (miR-154)	1	69.72118392
LT2 SM-10 (miR-412)	1	2.622350016
LT2 SAR-1 (miR-337)	1	242.0213826
LT2 SAR-2 (miR-127)	1	2900.98867
LT2 SAR-3 (miR-136)	1	1.189834657

C

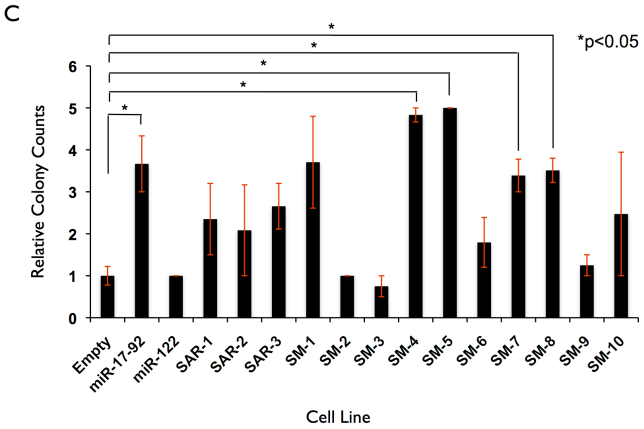


Figure 8. Multiple miRNAs within the Dlk1-Dio3 miRNA accelerate cell growth in soft agar. A. Schematic of miRNA subcluster cloning from the Dlk1-Dio3 region. **B.** Verification of miRNA subscluster expression in LT2MR stable cell lines. qRT-PCR was performed to detect expression of representative miRNAs. **C.** Quantification of soft agar colonies formed by each LT2MR stable cell line. miR-17-92 and miR-122 were used as positive and negative controls respectively. Values are average of 3 independent experiments. SM-4, 5, 7 and 8 subclusters significantly increased transformation when compared to LT2MR cells stably expressing a control pMSCV vector (*p<0.05).

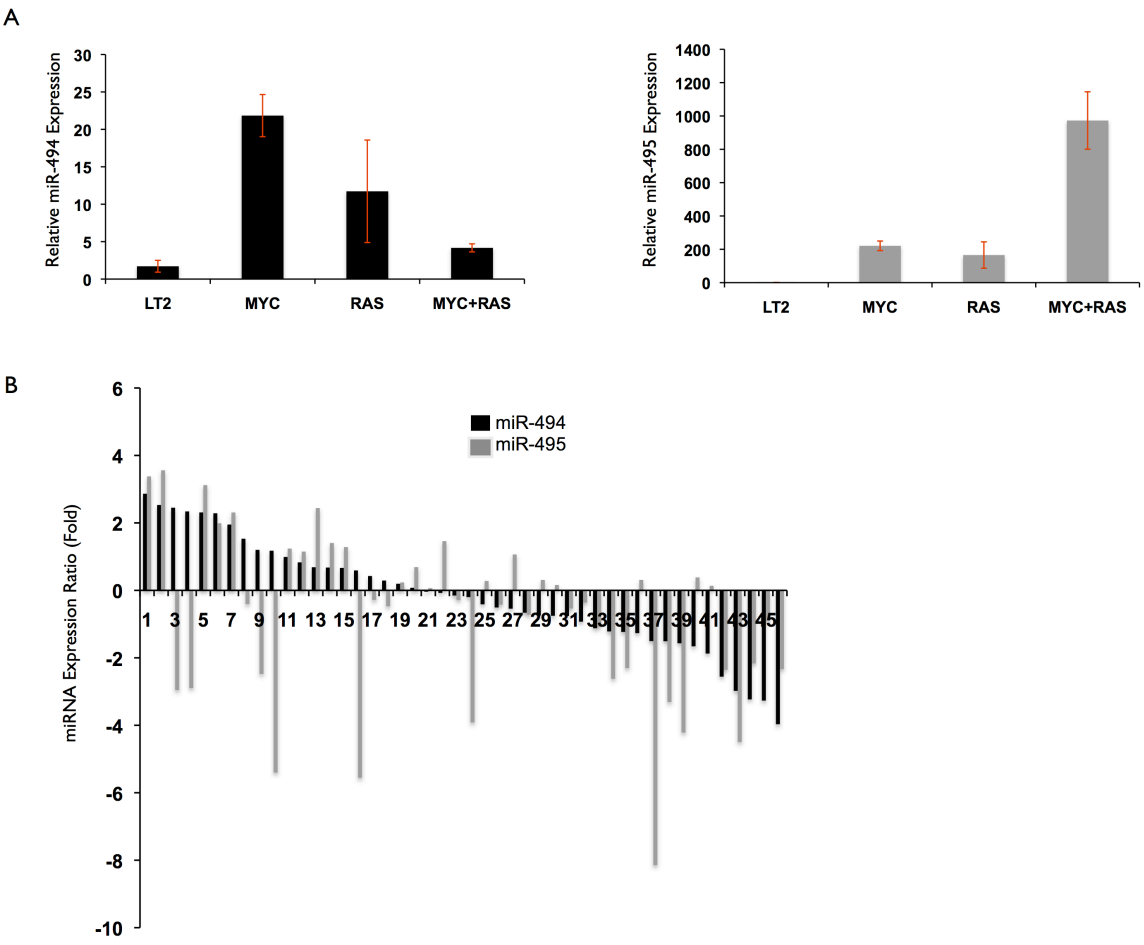
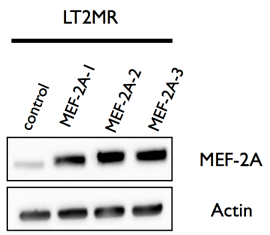


Figure 9. miR-494 and miR-495 are highly expressed in mouse tumor models and human HCC samples A. Verification of miR-494 and 495 expression in mouse liver tumors. qRT-PCR was performed to detect expression of respective miRNAs. **B.** Relative expression of miR-494 and miR-495 from 47 HCC tumor and matched normal samples were analyzed by qRT-PCR. miRNA abundance was normalized to RNU48. Differences in expression are shown as fold change over matched normal tissue.

A



B

	LT2MR	LT2MR-MEF-2A
miR-154	Unexpressed	Unexpressed
miR-323	Unexpressed	Unexpressed
miR-487b	1	0.59
miR-299-5p	Unexpressed	Unexpressed
miR-127	1	0.11
miR-134	1	1.27
miR-136	Unexpressed	Unexpressed
miR-544	Unexpressed	Unexpressed
miR-382	1	0.67
miR-337	Unexpressed	Unexpressed
miR-412	1	2.59
miR-494	1	0.23
miR-495	1	0.73
miR-122	1	9.06
miR-16	1	0.67

Figure 10. Dlk1-Dio3 miRNA expression is not MEF2A dependent in LT2MR cells. **A.** Three separate MEF2A overexpressing LT2MR clones were generated. MEF2A cDNA was cloned from human cDNA and expressed in pMSCV-puro lentivectors. Stable cell lines expressing either MSCV-puro empty vector or MSCV-puro-MEF2A were generated in LT2MR cells. **B.** qRT-PCR was performed to detect Dlk1-Dio3 miRNA expression. Values in LT2-MEF2A column are average of three separate clones shown in **Figure 10 A.** miR-122 and miR-16 were used as negative controls as they are not known to be MEF2A responsive. sno202 was used as a housekeeping control. Unexpressed indicates that miRNA expression was undetected at 40 cycles.

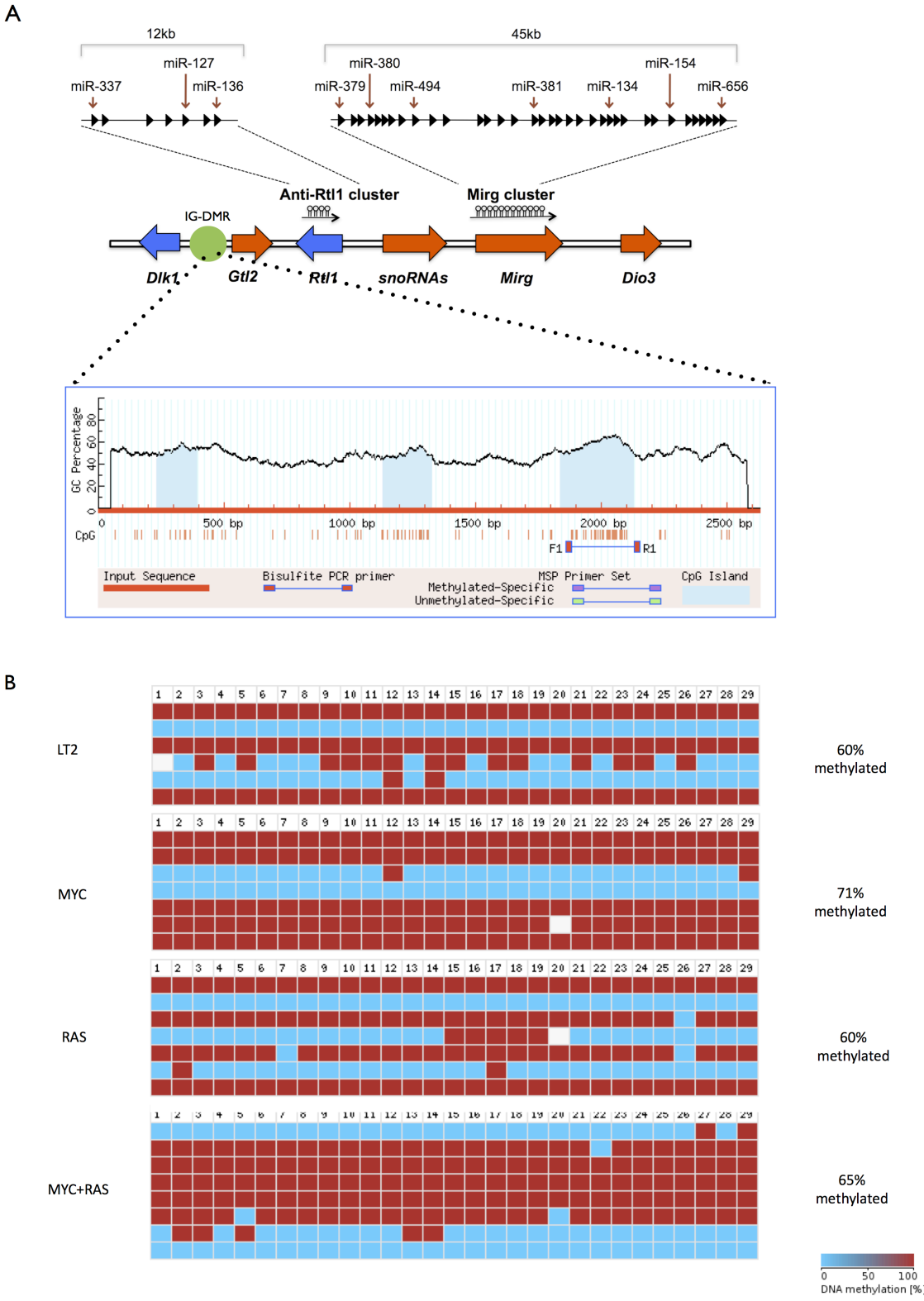


Figure 11. Methylation at the IG-DMR region is not changed in LT2 mouse models.

A. Methprimer was used to identify CpG islands within the 2.5kb long IG-DMR (in

green). Three CpG islands were identified (in light blue). **B.** The third CpG island was selected for methylation analysis because it contained the highest concentration of CpG sites (29). Bisulfite sequencing of five independent clones indicated that there was no difference in methylation between LT2 normal mice and tumor mice.

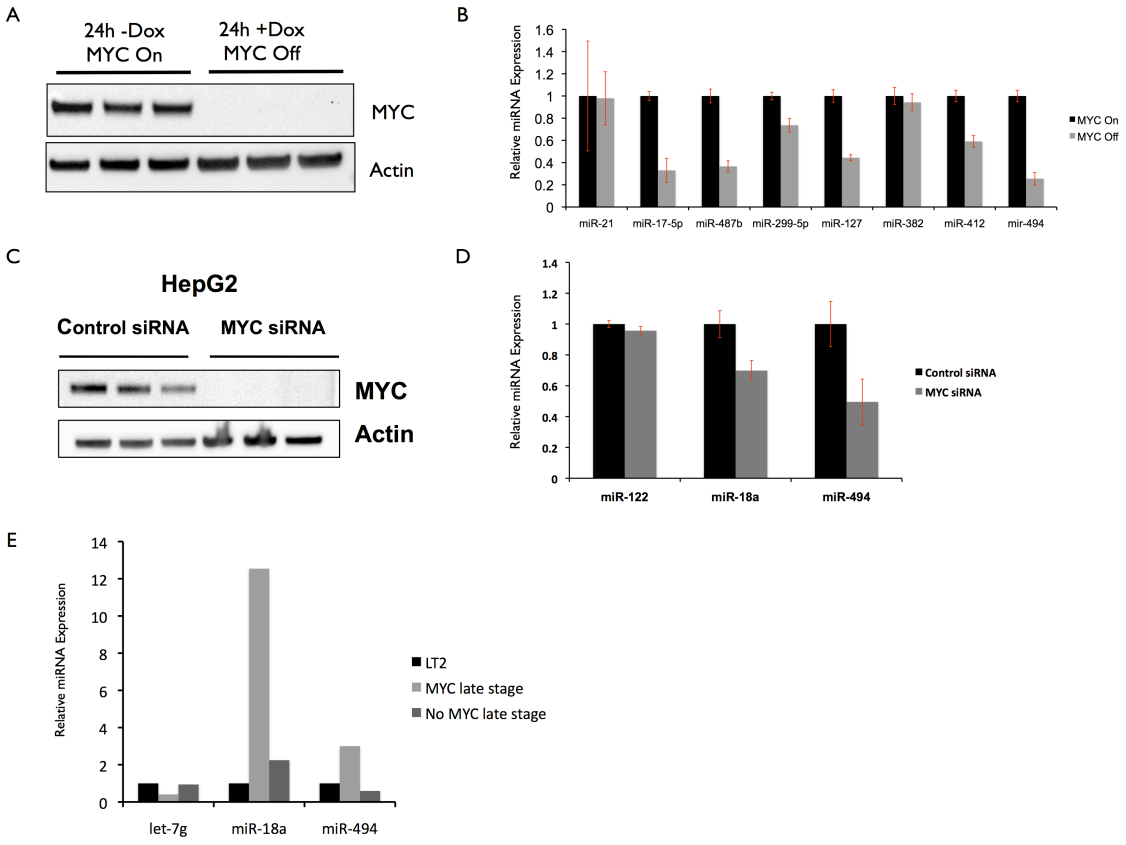


Figure 12. Dlk1-Dio3 miRNA expression is MYC dependent. A. 3332 cells previously isolated from LT2/MYC tumors are responsive to doxycycline. Addition of doxycycline to cell culture media results in loss of MYC expression at 24 hours. **B.** qRT-PCR data indicates that representative Dlk1-Dio3 miRNAs are downregulated when MYC expression is lost. miR-21 and miR-17-5p were used as negative and positive controls respectively Values are averaged between three independent experiments. **C.** HepG2 cells express a high level of MYC. **D**. qRT-PCR data indicates that miR-18a and miR-494 expression is downregulated when HepG2 cells are transfected with MYC siRNA. miR-122 was used as a negative control. Values are averaged between three separate transfections. **E.** An *in vivo* regression model indicates that MYC tumors (MYC late stage) have high miR-18a and miR-494 expression compared to normal liver (LT2).

However, acute removal of MYC (No MYC late stage) results in downregulation of miR-18a and miR-494 expression. Let-7g was used as a negative control.

microRNA	Human HCC	Mouse Liver Models			References
		MYC	RAS	MYC+RAS	
miR-1	↓	↓	↓	↓	Datta et al. 2008
miR-17-92	↑	↑	↑	↑	Connolly et al. 2008
miR-21	↑	-	↑	-	Meng et al. 2007
miR-29	↓	↓	↓	↓	Xiong et al. 2010,
miR-101	↓	↓	↓	↓	Su et al. 2009
miR-122	↓	↓	↓	↓	Fornari et al. 2009
miR-181b	↑	-	↑	-	Wang et al, 2010
miR-195	↓	↓	-	↓	Xu et al. 2009
miR-221	↑	↓	↑	-	Fornari et al. 2008
miR-222	↑	↓	↑	↑	Wong et al. 2010

Table 1. Expression patterns of tumor-associated miRNAs are similar in human HCC and mouse liver tumor models.

miRNA	RAS-Norm	RAS-Norm p-value	miRNA	Myc-Norm	MYC-Norm p-value	miRNA	MycRas-Norm	MYCRAS-Norm p-value
miR-125a	1.197678216	0.000858517	**miR-106a**	2.614389857	2.20359E-06	**miR-106a**	1.405569273	0.012787913
miR-127	5.786061658	6.83462E-05	miR-106b	1.682212205	0.002455358	miR-127	6.075464934	0.000304125
miR-128	2.55001028	0.039716376	miR-127	4.814456285	0.00024149	miR-128	4.633618735	0.006989907
miR-130b	2.622439002	0.013039244	miR-128	4.376990553	0.003054302	**miR-129-5p**	4.429199989	0.016684775
miR-132	1.855848822	0.00146702	**miR-129-5p**	4.570637896	0.005798465	miR-130b	3.584470765	0.012149198
miR-134	4.138355114	0.000329446	miR-130b	6.111131841	0.000194917	miR-134	5.514297956	0.000394673
miR-144	1.678416329	0.042652006	miR-134	4.281857741	0.000195768	miR-146b	5.027624249	9.65477E-05
miR-146b	5.73447743	1.87024E-06	miR-146b	1.725715653	0.001038269	miR-154	3.580361792	0.008396065
miR-154	4.138133923	0.003978347	miR-154	2.685552435	0.010264433	miR-154*	5.36325942	0.000610319
miR-154*	3.329593274	9.53372E-05	miR-154*	3.871653199	0.000708141	**miR-17-5p**	1.106552082	0.02309923
miR-155	2.494824473	0.008141322	miR-17-3p	2.368955514	0.002890331	miR-183	1.289608807	0.017189818
miR-181b	1.007705278	0.00971591	**miR-17-5p**	2.418776003	6.35077E-05	miR-186	1.304195753	0.018311901
miR-182	1.516389438	0.000754449	miR-186	2.021988909	0.005879677	miR-18a	3.975469544	0.002037405
miR-183	2.399518052	0.000504605	miR-18a	5.699199283	1.45777E-05	**miR-18a***	2.273411437	0.030164192
miR-186	2.147291356	0.001017327	**miR-18a***	3.281603521	0.001822295	miR-18b	2.836833112	0.002493315
miR-18a	2.73599427	0.002630504	miR-18b	4.609599217	7.46931E-06	**miR-20b**	1.125510015	0.022701337
miR-18b	1.992597285	0.001980246	miR-19a	2.993253423	0.01519511	miR-290	5.192667771	0.001643784
miR-193b	3.737534343	2.44744E-05	miR-19b	2.36901559	0.00863101	miR-292-5p	4.442862409	0.001104587
miR-199a	1.490813707	0.016900668	miR-200b	1.237388108	0.011638093	miR-298	4.189379654	0.001603987
miR-210	2.318239305	0.003110702	miR-20a	2.387467774	9.75037E-05	miR-299-3p	2.100203892	0.001110698
miR-212	2.456278841	0.007655115	**miR-20b**	2.738768515	0.000219164	miR-299-5p	4.839839929	0.001110764
miR-214	1.601558572	0.001012003	miR-214	1.023166716	0.007780312	miR-300	4.806016482	0.004103692
miR-221	2.054644955	0.000322165	miR-223	1.460238731	0.001205136	**miR-302c***	3.430893843	0.004178854
miR-222	2.913424356	0.001435567	miR-290	5.209713259	0.000311196	**miR-323**	3.43106417	0.020179201
miR-223	1.817166674	0.000529294	miR-292-5p	4.479125167	0.000203645	miR-329	4.610796797	0.002183761
miR-290	2.679252893	0.006810932	miR-297	6.065525948	0.00039947	miR-337	4.840551773	0.000691058
miR-292-5p	2.744434626	0.004042008	miR-298	5.311602185	0.000188193	miR-341	3.369104669	0.002333911
miR-296	1.976305663	0.017109346	miR-299-3p	1.469666022	0.028099349	miR-34a	3.21345154	0.002208555
miR-298	2.971642488	0.011828086	miR-299-5p	3.396460765	0.011795497	**miR-363***	2.135609435	0.00249151
miR-299-5p	4.910484695	7.65909E-05	miR-300	3.69874254	0.004014523	miR-368	2.164348863	0.023710332
miR-29b	1.871907998	0.004299565	miR-301	3.177526702	0.001990264	miR-369-3p	3.852610553	0.00417986
miR-300	3.202263585	0.003918281	**miR-302c***	3.650841947	0.001451459	miR-369-5p	4.576160979	0.002007668
miR-31	1.255901924	0.000101537	**miR-323**	1.817010682	0.038017312	miR-370	4.23137027	0.000715654
miR-322	1.903571319	1.95682E-05	miR-324-5p	1.560412559	0.007156794	miR-373*	5.616363603	0.000229892
miR-329	3.54111941	8.44961E-05	miR-329	3.711175408	0.00038779	**miR-376a**	2.739750192	0.002866253
miR-331	1.453778676	0.000727254	miR-331	1.138972	0.005060238	miR-376b	4.568651899	0.004665187
miR-337	4.130309848	0.000171638	miR-337	4.445047823	0.00037428	miR-376c	5.487253318	0.000199797
miR-341	1.949629122	0.027874238	miR-341	3.158483314	0.002213266	miR-377	6.007757255	0.001280201
miR-34a	4.731822239	9.58618E-06	miR-342	1.647470365	0.000112449	miR-379	4.737477302	0.001680086
miR-34b	2.278015147	0.005899053	miR-345	1.010471049	0.045902428	miR-380-3p	4.157754152	0.00306738
miR-350	2.039184755	0.000477777	miR-34a	3.094989773	0.000471334	miR-380-5p	1.662699586	0.012516826
miR-351	2.501180246	6.39869E-06	**miR-363***	2.353559808	0.00028068	miR-381	4.181942442	0.001715543
miR-362	1.197152384	0.021201496	miR-369-3p	2.306187559	0.042128084	miR-382	5.618045132	0.000506858
miR-368	2.063974837	0.020075232	miR-369-5p	3.565768027	0.000449028	miR-409	5.637647671	0.002177747
miR-369-3p	3.07170275	0.015716058	miR-370	3.783513222	0.000313791	miR-409-3p	6.530892136	0.002385755
miR-369-5p	3.994698005	0.00028411	miR-373*	5.322650112	2.52396E-05	miR-410	6.473113802	0.000356262
miR-370	1.879854121	0.00787486	miR-374	1.509950223	0.001232148	miR-411	4.371690698	0.002966608
miR-373*	3.252549489	0.000551813	**miR-376a**	2.08209766	0.004669071	miR-421-3p	1.867334917	0.00431779
miR-376b	3.789985363	0.00271862	miR-376b	3.667528127	0.006110153	miR-431	6.011866045	0.000670183
miR-376c	4.825838089	1.4185E-05	miR-376c	4.613727377	0.000111862	miR-432	1.786262462	0.034975142
miR-377	5.003609979	0.000151555	miR-377	3.902345417	0.033454356	miR-433	5.14882667	0.000762482
miR-379	4.15420147	0.000208357	miR-379	3.544007916	0.000585743	miR-433-5p	2.288642928	0.04950412
miR-380-3p	2.703689778	0.003832981	miR-380-3p	3.562262087	0.000877376	miR-434-3p	4.650856637	0.000386922
miR-381	2.606012872	0.011106748	miR-381	2.801486937	0.018709772	miR-434-5p	5.315247973	0.005299544
miR-382	4.844442307	1.25357E-05	miR-382	3.936444692	0.000362391	**miR-483**	4.741791993	0.000551152
miR-383	2.106274462	0.019351186	miR-409	3.739140816	0.001350343	miR-483*	5.452388824	0.000567899
miR-409	3.32681004	0.000314295	miR-409-3p	4.655657152	0.001163167	miR-485-3p	4.909846773	0.014488881
miR-409-3p	4.449536462	0.000457221	miR-410	5.232774891	0.00021103	miR-485-5p	2.659942881	0.017553044
miR-410	5.058226778	0.000101931	miR-411	3.108644553	0.005371115	miR-487b	5.481625462	0.001185514
miR-411	3.835738093	0.001222548	miR-421-3p	2.952047054	7.80211E-05	miR-492	2.680958162	0.024237735
miR-421-3p	1.262766636	0.004919313	miR-431	5.16307	0.00021484	miR-493-5p	3.412356484	0.007597309
miR-424	3.262636647	2.23849E-05	miR-433	3.545146921	0.000421759	miR-494	5.077926016	0.000518352
miR-431	4.961064926	0.000279597	miR-433-5p	1.640249151	0.015101792	miR-495	6.551495059	0.000822359
miR-433	3.769683661	0.000235839	miR-434-3p	3.669970598	0.000453699	miR-496	3.312176749	0.006435747
miR-433-5p	1.953300924	0.01268743	miR-434-5p	3.817065386	0.0028627	miR-501	1.356041965	0.038547403
miR-434-3p	4.575082749	4.07924E-05	miR-466	7.212339537	2.13937E-05	miR-503	2.043116562	0.003642548
miR-434-5p	3.903040435	0.004107215	miR-467	6.053304996	0.000361068	**miR-509**	1.831988546	0.024073909
miR-450	2.557242887	0.001247715	**miR-483**	5.263817633	0.00039535	miR-539	5.772890726	0.001861711
miR-466	1.970426974	0.032238274	miR-483*	5.777417802	8.67647E-05	miR-540	4.911244753	0.000622188
miR-483*	3.760494208	0.001863512	miR-485-3p	2.992024268	0.043395032	miR-541	6.055654797	0.000255272
miR-484	1.61094881	0.000852538	miR-487b	4.205360213	0.000276087	miR-592	1.6351806	0.031951255
miR-485-3p	4.294326854	0.007886436	miR-491	1.125163144	0.025216611	**miR-654**	3.461728042	0.015382599
miR-485-5p	2.023829172	0.000381541	miR-492	2.31006377	0.012850415	miR-99b	1.001597879	0.034153505
miR-487b	3.357279022	0.000595517	miR-494	4.834021842	6.60298E-05	miR141	1.492967023	0.038487671
miR-492	1.33349722	0.045458549	miR-495	5.4860273	0.000163082	miR203	6.611063725	0.000802488
miR-493-5p	2.279370487	0.022568708	miR-496	1.877967179	0.020511658	miR206	4.358189621	0.000912054
miR-494	2.88095312	0.000641872	miR-501	2.068508279	0.030119643	miR207	4.971693848	0.000185952
miR-495	4.860067632	0.000111752	miR-503	2.119886032	0.000417828	miR219	3.744821424	0.001154982
miR-496	3.49699467	0.000403937	**miR-509**	1.230091085	0.001599418	miR23	3.876599418	0.006453741
miR-501	2.407193992	0.018809399	miR-539	4.493509718	0.000664297			
miR-503	3.437840619	4.0559E-05	miR-540	3.606037951	0.001075572			
miR-539	3.154979285	0.004588304	miR-541	4.766046267	0.000208043			
miR-540	3.389126239	0.000812262	miR-542-5p	1.242970304	0.042746483			
miR-541	5.058517646	9.72819E-06	miR-546	1.623299529	0.043393922			
miR-542-3p	2.148329045	0.000199608	**miR-654**	2.465047	0.018862393			
miR-592	2.007741716	0.009045424	miR-7	1.465196915	0.000867018			
miR-96	2.371286005	0.00478311	miR-93	1.456597443	0.004036194			
miR-99b	2.457702637	5.6702E-05	miR-98	1.287938371	0.000922903			
miR141	2.447623026	0.001568701	miR-99b	1.842693063	0.000305857			
miR203	4.984789649	0.000729669	miR141	1.507038828	0.011029159			
miR206	2.106885551	0.024332162	miR203	6.246291278	0.000194513			
miR207	3.282558075	0.000439628	miR206	4.310055985	0.000264647			
miR219	1.60459017	0.008934791	miR207	4.449321045	0.000248781			
miR23	3.05122727	0.003541336	miR219	3.350014327	0.000494015			
			miR220	2.465290108	0.004073084			
			miR429	1.828146723	0.00957366			

Upregulated in all three genotypes
Upregulated in MYC and RAS
Upregulated in RAS and MYCRAS
Upregulated in MYC and MYCRAS

Table 2. Upregulated miRNAs with differential expression ($log_2 > 1$ and $p<0.05$) sorted by tumor specific expression in MYC, RAS or MYC+RAS mice. miRNAs from this table were used to generate the venn diagram seen in Figure 6 A.

miRNA	RAS-Norm	RAS-Norm p-value	miRNA	MYC-Norm	MYC-Norm p-value	miRNA	MYCRAS-Norm	MYCRAS-Norm p-value
miR-1	-1.771201741	0.064676381	let-7a	-1.008585432	0.000176778	**let-7b**	-1.02136736	1.41012E-05
miR-139	-1.283456726	9.50873E-05	**let-7b**	-1.094586641	0.000446483	**let-7d***	-1.093503846	0.003995063
miR-142	-1.850319656	0.047810107	let-7c	-1.01447099	0.000117781	let-7g	-1.080279284	5.79807E-05
miR-203	-1.536689231	7.16879E-05	**let-7d***	-1.823564923	0.014334516	**miR-100**	-1.175146001	0.000970488
miR-375	-1.592565166	0.045711464	miR-1	-1.724874854	0.040397109	**miR-101b**	-1.79546686	0.005642515
miR-378	-1.257101979	0.016191914	**miR-100**	-1.473555849	0.003837352	miR-103	-1.152520966	0.0002792
miR-422a	-2.243046134	2.49184E-06	**miR-101b**	-1.53018606	0.001405045	miR-107	-1.222994633	0.000220756
miR-422b	-2.132670255	1.38446E-05	**miR-122a**	-1.224268723	0.001557913	miR-10a	-1.149826032	0.009653222
miR-455	-2.225065972	0.0005251	**miR-125b**	-1.509261736	0.00092248	**miR-122a**	-1.034619202	0.006043804
			miR-130a	-1.133575053	0.00345496	**miR-125b**	-1.312171684	0.000621322
			miR-139	-2.031235444	0.000469256	miR-126	-1.194013224	0.001817237
			miR-142	-1.288385084	0.028578806	**miR-130a**	-1.253488284	0.002347366
			miR-148a	-2.323421889	0.039420948	miR-139	-2.051706412	5.89362E-05
			miR-192	-1.634783243	0.000217181	**miR-148a**	-1.410258955	0.000533852
			miR-193a	-1.055925472	0.049876242	miR-15a	-1.664953244	0.003686532
			miR-194	-1.685639661	0.000664324	miR-16	-1.256665995	5.18134E-05
			miR-215	-1.210252299	0.029122721	miR-181	-1.171781624	0.00581864
			miR-218	-1.805428715	0.017845539	miR-185	-1.018368153	0.002628359
			miR-22	-2.355985899	0.000959458	miR-187	-1.955644397	0.033263827
			miR-221	-1.51480346	0.004783616	**miR-192**	-1.286723957	0.001329715
			miR-222	-1.084136209	0.011641008	**miR-194**	-1.445294736	0.00061786
			miR-26a	-1.118272573	0.000581623	miR-200a	-1.252843628	0.032972638
			miR-29a	-1.385136978	0.006534768	miR-203	-1.122398147	0.044989531
			miR-29c	-1.759002992	0.007223067	**miR-22**	-2.132785782	0.003790026
			miR-30a-3p	-1.254547385	0.001515924	**miR-26a**	-1.265000325	4.68143E-05
			miR-30d	-1.042143704	0.000176089	miR-27b	-1.34327445	0.000794852
			miR-30e-3p	-1.05473302	0.009717337	**miR-29a**	-1.338456872	0.008603489
			miR-328	-2.182857225	0.030544095	**miR-29c**	-1.319041783	0.038146768
			miR-455	-1.679446763	0.005127725	miR-30a-5p	-1.193133483	0.000517031
			miR-486	-1.755647989	0.020888982	miR-30b	-1.347150959	0.00125622
			miR-574	-1.107102253	0.003570584	miR-30c	-1.008225907	0.000480221
			miR-652	-1.102931183	0.002780329	**miR-30d**	-1.234827942	7.21761E-05
			miR-99a	-1.442994048	0.002378516	**miR-328**	-2.668526896	0.000221358
						miR-339	-1.180478276	0.009773421
						miR-378	-2.818165916	0.008396824
						miR-422a	-1.356238058	0.000171173
						miR-422b	-1.57283057	7.68073E-05
						miR-455	-1.711847255	0.017081176
						miR-486	-1.422186094	0.011780482

Downregulated in all three genotypes
Downregulated in MYC and RAS
Downregulated in RAS and MYCRAS
Downregulated in MYC and MYCRAS

Table 3. Downregulated miRNAs with differential expression ($log_2 < -1$ and $p<0.05$) sorted by tumor specific expression in MYC, RAS or MYC+RAS mice. miRNAs from this table were used to generate the venn diagram seen in Figure 6 A.

Chapter 3: miR-494 accelerates G1/S cell cycle transition and its inhibition blocks tumor growth *in vivo*

3.1 Introduction

In the previous chapter, we identified, using a systematic approach, several miRNAs that could be playing oncogenic roles in HCC development: miR-494, miR-495, miR-539, miR-889, miR-544, miR-655, miR-382, miR-134, miR-668, miR-485 and miR-453. Since our primary goal was to identify and characterize oncogenic miRNAs that could potentially be novel therapeutic targets in HCC, we selected miR-494 for further study based on the following criteria: First, miR-494 and miR-495, as individual miRNAs, caused the greatest increase in soft agar colony growth. Second, while the expression of miR-494 and miR-495 was high in both mouse liver tumors and human HCC, miR-494 overexpression was observed in a greater number of human HCC samples than miR-495 (34% compared to 27%).

In this chapter, we characterized the role of miR-494 in HCC using both mouse and human liver tumor models. We find that miR-494 accelerates the G1/S cell cycle transition and causes increased proliferation through suppression of the Mutated in Colorectal Cancer (MCC) target gene. We show that its inhibition reduces transformation of human HCC cells. Further, we demonstrate that *in vivo* delivery of a miR-494 antagonist to MYC driven liver tumors slows tumor growth through cell cycle inhibition.

Our results present the possibility of targeting miR-494 in HCC using miRNA antagonists.

3.2 Results

miR-494 Overexpression Increases Cellular Proliferation and S-Phase Entry

Since miR-494 was amongst the most potent at increasing soft agar growth, we sought to determine if it could directly effect tumor cell proliferation. To address this, we engineered LT2MR cell lines that stably overexpress miR-494, using a pMSCV retroviral vector. Although, we had previously detected a moderate level of endogenous miR-494 expression in LT2MR cells (**Figure 10 B**, ct value ~21), we found that miR-494 expression was further increased in pMSCV-494 infected cells, relative to control-infected cells (**Figure 13 A**). Over a period of four days, we observed that miR-494 overexpression increased the rate of LT2MR proliferation, when compared to control cells (**Figure 14**). Conversely, stable expression of a miR-494 antagonist (pmiRZip-494) resulted in diminished endogenous miR-494 expression (**Figure 13 B**) and proliferation of LT2MR cells, compared to pmixRZIP-control (**Figure 14,** p=0.01).

Given the increase in cellular proliferation caused by miR-494 overexpression, we asked if this was mediated through alterations in cell cycle control. To test this, we first performed cell cycle analysis using propidium iodide (PI) staining on miR-494 mimic and inhibitor transfected cells. Transfection of a miR-494 mimic resulted in a decreased percentage of cells in the G1-phase and increased S-phase cells (**Figure 15 A**,

***p=0.001, *p=0.01). Transfection of these cells with a miR-494 hairpin inhibitor (Dharmacon) resulted in an accumulation of cells in G1 and a decrease of cells in S phase (**Figure 15 B**, **p=0.003). These results suggest that miR-494 overexpression results in increased G1/S transition in LT2MR cells, while its downregulation is associated with a delay in G1/S transition. To evaluate the frequency of cell transition through S-phase, we treated cells with bromodeoxyuridine (BrdU) and measured its incorporation using flow cytometric analysis. We observed that miR-494 mimic transfection caused an increase in BrdU labeled cells, while miR-494 inhibitor transfection resulted in decreased BrdU labeled cells, compared to respective controls (**Figure 15 C and D**).

We wondered if the increase in S-phase entry caused by miR-494 was associated with changes in the expression of cell cycle inhibitor proteins. We tested for expression of two key regulators of the G1/S transition, p21 and p27, by western blotting. We detected a decrease in expression of both cell cycle inhibitors in miR-494 overexpressing cells (**Figure 16 A**). In contrast, knockdown of miR-494 resulted in increased expression of these cell cycle inhibitors (**Figure 16 B**). Thus, miR-494 overexpression is associated with increased S-phase entry in LT2MR cells, as well as decreased p21 and p27 abundance, known inhibitors of cell proliferation.

We attempted to verify these findings in a separate cell line, Hepa1-6 mouse hepatoma cells, which have high miR-494 expression. miR-494 inhibition decreased proliferation in Hepa1-6 cells, associated with a delay in G1/S transition and an increase in p21 and p27 **(Figure 17 A-E)**. Together, these results demonstrate that miR-494 overexpression

increases G1/S transition in LT2MR and Hepa1-6 cells, coinciding with decreased p21 and p27 abundance, known inhibitors of cell proliferation.

The Tumor Suppressor MCC is a Direct Target of miR-494

We hypothesized that the ability of miR-494 to accelerate cell cycle progression was occurring through its regulation of one or more gene targets associated with growth inhibitory properties. We first performed an *in silico* search, using TargetScan (Grimson et al., 2007), to identify candidate miR-494 targets with predicted binding sites conserved across multiple species. These putative targets were then evaluated using DAVID pathway analysis to identify genes established to block cell cycle progression. Seven candidate genes with features of tumor suppressors were identified: Transforming Acidic Coiled-coil Containing Protein 2 (TACC2), Transforming Growth Factor Beta-2 (TGFB2), Retinoblastoma-1 (RB1), Retinoblastoma-1 Inducible Coiled-Coil-1 (RB1CC1), Cell Division Cycle-73 (CDC73), WEE1 and Mutated in Colorectal Cancer (MCC) (**Figure 18 A**). To evaluate if these genes were direct miR-494 targets, we cloned an approximately 300 bp fragment of their 3' UTRs that contained the predicted miR-494 binding sites into luciferase reporter constructs. Co-transfection of candidate reporters was performed in LT2MR cells with either a control or miR-494 mimic, and luciferase expression was normalized to a beta-galactosidase plasmid. These reporter assays revealed that miR-494 had negligible effect on the RB1, RB1CC1, CDC73 and WEE1 UTRs, and caused an increase in TACC2 and TGFB2 UTR reporter expression (**Figure 18 B**). Since our earlier hypothesis was guided by the fact that miR-494 should have a repressive effect on candidate UTRs, we reasoned that none of these six gene candidates

were targets through which miR-494 was exerting its increased proliferative effects. However, we observed an approximately 70% ($p<0.05$) decrease in reporter expression when miR-494 was co-transfected with the MCC 3' UTR luciferase reporter (**Figure 18 D**). Consistent with these findings, co-transfection of this reporter with a miR-494 inhibitor resulted in a 40% ($p<0.05$) increase in reporter expression (**Figure 18 D**). These results indicated that miR-494 could be directly targeting MCC through its 3' UTR.

The 5203 bp mouse MCC 3' UTR contains one 7-mer binding site for miR-494 (seed region, 5049-5055nt) that is conserved in human, mouse and chimpanzee transcripts (**Figure 18 C**). To determine if miR-494 could directly target MCC through its 3' UTR, we performed site directed mutagenesis of four nucleotides within the putative seed-sequence binding site. We observed a loss of reporter repression when this mutant reporter was co-transfected with a miR-494 mimic. Expression of the MCC mutant 3' UTR reporter was also unresponsive to co-transfection of cells with a miR-494 inhibitor **(Figure 18 D**).

We next examined if endogenous MCC expression could be altered by miR-494 expression. To test this, we first compared the expression of MCC mRNA in LT2MR cells transfected with either miR-494 mimic or inhibitor. In mimic transfected cells, we detected an approximately 60% decrease in MCC mRNA abundance ($p<0.05$). Conversely, MCC mRNA was significantly increased ($p<0.05$) in inhibitor-transfected cells (**Figure 18 E**). We also analyzed the protein expression of MCC in mimic and inhibitor transfected cells by western blot. Consistent with the observations made with

MCC mRNA, we detected an 80% decrease in MCC protein expression with mimic transfected cells and a 60% increase in expression with antimiR transfected cells ($p<0.001$, **Figure 18 F**), compared to control transfected cells. Collectively, these results indicate that MCC is a direct target of miR-494 in mouse liver tumors cells.

MCC modulates G1/S transition in liver tumor cells

To our knowledge, the role of MCC to regulate cellular proliferation in liver cancer had not been previously examined. To assess the functional contribution of this miR-494 target to proliferation, we silenced MCC expression by RNA interference. Consistent with our observations of increased proliferation mediated by miR-494, reduction of MCC levels caused an increase in cell proliferation over four days in LT2MR cells (**Figure 19 A**). Cell cycle analysis by PI staining revealed an increased percentage of S-phase cells, consistent with increased G1/S transition (**Figure 19 B**) which was further confirmed by BrdU incorporation assays (**Figure 19 C**). Conversely, transient overexpression of MCC using a recombinant retrovirus resulted in decreased cell proliferation (**Figure 19 A**). This was associated with delayed G1/S transition in LT2MR cells, as observed by PI analysis and BrdU staining (**Figure 19 B and C**). These observations were similar to the effects seen when miR-494 expression was lowered in LT2MR cells.

We reasoned that the expression of regulators of G1/S cell cycle transition might be altered following MCC expression changes. To explore potential mechanisms through which MCC regulates proliferation, western blot analysis of p27 and p21 was performed. We found a decrease in p27 but not p21 protein expression when cells were transfected

with MCC siRNA (**Figure 19 D**). This suggests that the G1/S delay caused by MCC occurs through p27, but also raises the possibility that other targets of miR-494 might be involved in suppressing p21 expression, independently of MCC.

To further explore the extent through which miR-494 functions to regulate cell proliferation through modulating MCC expression, we attempted to rescue the delay in G1/S transition caused by miR-494 knockdown. To do this, we co-transfected pmiR-ZIP control or 494 with either control or MCC siRNA followed by assessment of BrdU incorporation. We found that the delay in S-phase entry caused by miR-494 knockdown was reversed by co-transfection of MCC siRNA (**Figure 19 E**). Together, these results indicate that miR-494 causes increased proliferation in LT2MR cells through suppression of MCC expression.

Although MCC overexpression has been shown to delay G1/S transition, its exact mechanism of cell cycle regulation is still poorly understood. MCC has previously been found to inhibit beta-catenin dependent transcription by preventing its translocation into the nucleus(Fukuyama et al., 2008). Since the p27 transcriptional repressor Id3 is a Wnt/beta-catenin target(Chassot et al., 2007; Zhang et al., 2012a), we hypothesized that this could be a possible mechanism through which MCC was modulating p27 levels (**Figure 20 A**). To test this, we first quantified p27 and Id3 mRNA levels in MCC siRNA transfected cells by qRT-PCR. While we observed a downregulation of p27 mRNA, there was no significant difference in Id3 mRNA levels between control and MCC siRNA transfected cells (**Figure 20 B**). Using both western blot and immunoflorescence staining

techniques, we tested if reduction in MCC levels resulted in nuclear translocation of beta-catenin. However, we were unable to detect an increase in nuclear beta-catenin when cells were transfected with MCC siRNA (**Figure 20 C and D**). These results suggest that the observed changes in p27 levels are not occurring through a beta-catenin dependent mechanism. Nevertheless we consistently observed a MCC-dependent upregulation of p27, which had not been previously reported, and was associated with decreased G1/S cell cycle transition.

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miR-494 Knockdown Decreases Transformation in Human HCC Cells

Since our studies thus far were performed in mouse liver tumor cells, we sought to determine if MCC was also a miR-494 target in human HCC cells and primary tumors, and more crucially, if miR-494 inhibition in human HCC cells could reduce transformation. We first examined MCC mRNA expression in ten of the highest miR-494 expressing human HCC samples, as determined in **Figure 9 B**. We observed a substantially reduced expression of MCC mRNA in 8 of 10 samples when compared to non-tumor adjacent tissue (**Figure 21 A**), suggesting that MCC is also a miR-494 target in human HCC.

We next asked if miR-494 expression could alter transformation in human HCC cell lines. We used two human HCC cell lines, Huh7 and Hep3B, with detectable MCC expression. We found that Huh7 and Hep3B cells had 18 and 12 fold greater miR-494 expression, respectively, when compared to normal human liver tissue (**Figure 21 B**). To test if MCC was a miR-494 target in these cancer cells, we engineered stable cell lines to

express pmiRZip-494, which achieved approximately 50% miR-494 inhibition (**Figure 21 C**). Western blot analysis revealed that miR-494 inhibition resulted in increased MCC and p27 expression, similar to our prior observations in mouse LT2MR cells (**Figure 21 C**). We also tested for the presence of PARP by western blot, in order to determine if miR-494 was modulating apoptosis. However, the absence of cleaved PARP led us to conclude that miR-494 was acting solely through cell cycle control (data not shown). To assess the effects of miR-494 inhibition in these cell lines, we performed soft agar colony formation assays and scored the number of colonies formed after two weeks. We observed a significant decrease in colony formation in cells with miR-494 inhibition (**Figure 21 D**). These results indicate that reduction of miR-494 expression increases MCC expression and impairs transformation in human HCC cells.

In Vivo Delivery of a Drug-like miR-494 Antagonist Attenuates Liver Tumor Formation

One of the most stringent tests of a therapeutic is the ability to block primary tumor formation. Since miR-494 inhibition resulted in diminished proliferation of human HCC cells, but did not cause cell death, we reasoned that inhibition of miR-494 might decrease primary tumor formation *in vivo*. To test this, we took advantage of our ability to form *de novo* liver tumors in LT2/MYC mice. Mice were taken off doxycyline to activate the MYC oncogene and initiate liver tumors. Five weeks after MYC activation, we treated LT2/MYC mice for three weeks with either antimiRs against miR-494 or a scrambled sequence (25mg/kg, Regulus Therapeutics). We calculated the extent of tumor burden by quantifying the total area, as previously described for this mouse (Kota et al., 2009). A

significant decrease in tumor burden was observed in the antimiR-494 treated group compared to mice injected with the control antimiR ($p<0.05$, **Figure 22 A and B**). We confirmed by qRT-PCR that miR-494 expression was decreased in antimiR-494 treated mice compared to the control treated group (**Figure 22 C**). We next asked if miR-494 inhibition was attenuating tumor formation *in vivo* through suppression of MCC expression, similar to what we had observed in mouse and human HCC cells. To test this, we compared the levels of MCC and p27 expression between control and antimiR-494 treated groups. We observed an increase in both MCC and p27 expression in the tumors of antimiR-494 treated mice, suggesting that antimiR-494 treatment is attenuating tumor formation *in vivo* by slowing down cell cycle progression (**Figure 22 D and E**). TUNEL staining also revealed that there was no difference in the amount of apoptosis in either treatment, suggesting that miR-494 acts to slow tumor progression solely through cell cycle control (**Figure 22 F**). Our results indicate that knockdown of miR-494 may be a novel and effective approach to slow HCC progression.

3.3 Discussion

The role of miR-494 in tumor development appears to be tissue dependent. It has been shown to increase proliferation in H460 lung cancer cells and functions as an oncogene in breast and transformed bronchial epithelial cells (Liu et al., 2010a; Liu et al., 2012; Romano et al., 2012). However, it inhibits proliferation and induces cell cycle arrest in lung cancer and cholangiocarcinoma (Ohdaira et al., 2012; Olaru et al., 2011). Our results demonstrate that miR-494 increases proliferation in HCC through an acceleration of G1/S

transition, but the pleiotropic nature of miRNAs suggests that it could have additional gene targets, which may play further roles in tumorigenesis. miR-494 has previously been shown to target PTEN (Liu et al., 2010a), and we investigated this in the context of our tumor models. While we were unable to detect PTEN downregulation when miR-494 was overexpressed in LT2MR cells (data not shown), we find that miR-494, instead targets the tumor suppressor MCC. miRNAs have been shown to act through the regulation of multiple gene targets (He and Hannon, 2004), and we explored that possibility by testing six other predicted targets of miR-494. Although we found none of those candidate reporters to be attenuated by miR-494 expression, we do not rule out the possibility of them being targets in other cell types, or that miR-494 could have other tumor suppressor targets in HCC.

MCC is found on human chromosome 5q21 and is closely linked to the Adenomatous polyposis coli (APC) gene (Kinzler et al., 1991). Loss of heterozygosity at this genomic region has been detected in colorectal, breast, lung and esophageal cancers (Boynton et al., 1992; Medeiros et al., 1994; Miki et al., 1991; Poursoltan et al., 2012; Thompson et al., 1993). Although MCC has previously been shown to slow proliferation and induce G1/S arrest in NIH3T3 and HCT-15 colorectal cancer cells (Matsumine et al., 1996; Pangon et al., 2010), we were uncertain of its biological effects in the liver. Little is also known about its mechanism of cell cycle regulation. We are the first to demonstrate that MCC affects p27 expression during the G1/S transition and could play a tumor suppressive role in HCC. To explore possible mechanisms by which MCC effects p27 expression, we attempted to verify the previous finding that MCC inhibits Wnt/beta

catenin dependent transcription (Fukuyama et al., 2008). However, our results indicate that this interaction does not extend to our model and suggest that other mechanisms for p27 regulation by MCC could be involved.

Most prior *in vivo* studies aimed at modulating miRNA expression have been performed using orthotopic transplant models (He et al., 2012; Li et al., 2011; Park et al., 2011; Zhang et al., 2012b). The data generated from these models is promising and will no doubt remain a fruitful area of research. However, we felt that treatment of liver tumors, as they form, with a miRNA antagonist would more closely replicate the clinical paradigms in which such therapies could eventually be deployed. To this end, we took advantage of our ability to form *de novo* liver tumors driven by MYC in mice. In this same model, AAV based overexpression of miR-26a, a tumor suppressive miRNA in HCC, was previously shown to diminish tumor growth (Kota et al., 2009). To our knowledge, we are the first to demonstrate that therapeutic delivery of a miRNA antagonist is successful at limiting the growth of existing *de novo* liver tumors. These findings represent a significant step toward the use of antimiR based therapy *in vivo*, and indicate that the delivery of antagonists against other highly upregulated miRNAs, such as miR-21 and 221, could be a promising therapeutic approach in HCC (Meng et al., 2007; Pineau et al., 2010). In addition, increased miR-494 expression has also been observed in an MMTV-c-MYC breast tumor model and retinoblastoma tumors, suggesting that miR-494 inhibition could also be a potential therapeutic approach in those cancer types (Zhao et al., 2009; Zhu et al., 2011). The findings of this chapter highlight the therapeutic potential of targeting miR-494 in HCC and suggest miR-494 inhibition

could also be a potential therapeutic approach in other cancer types in which it is overexpressed.

3.4 Figures and Tables

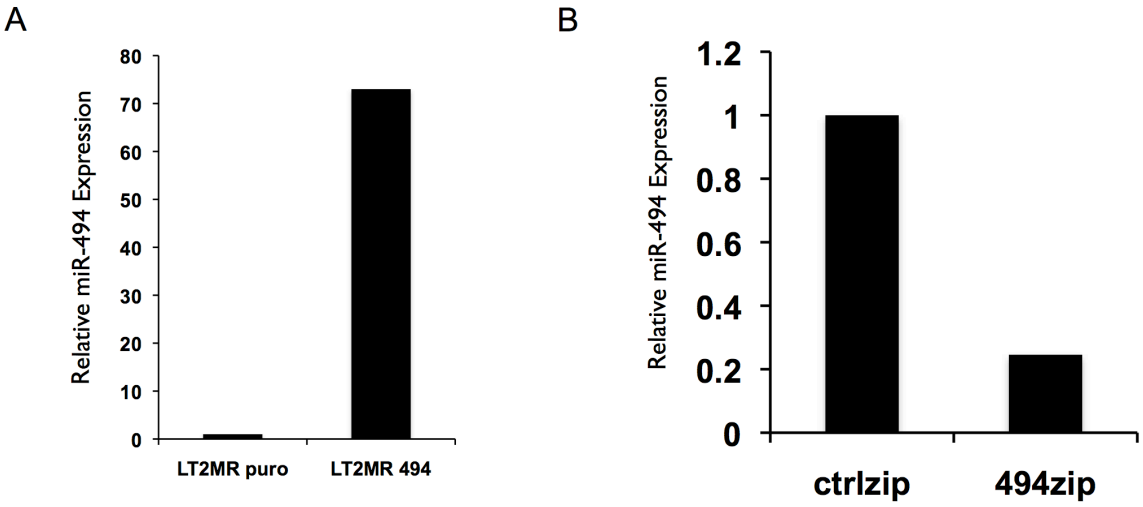


Figure 13. Quantification of miR-494 levels in engineered LT2MR cell lines by qRT-PCR. Sno202 was used as an internal control.

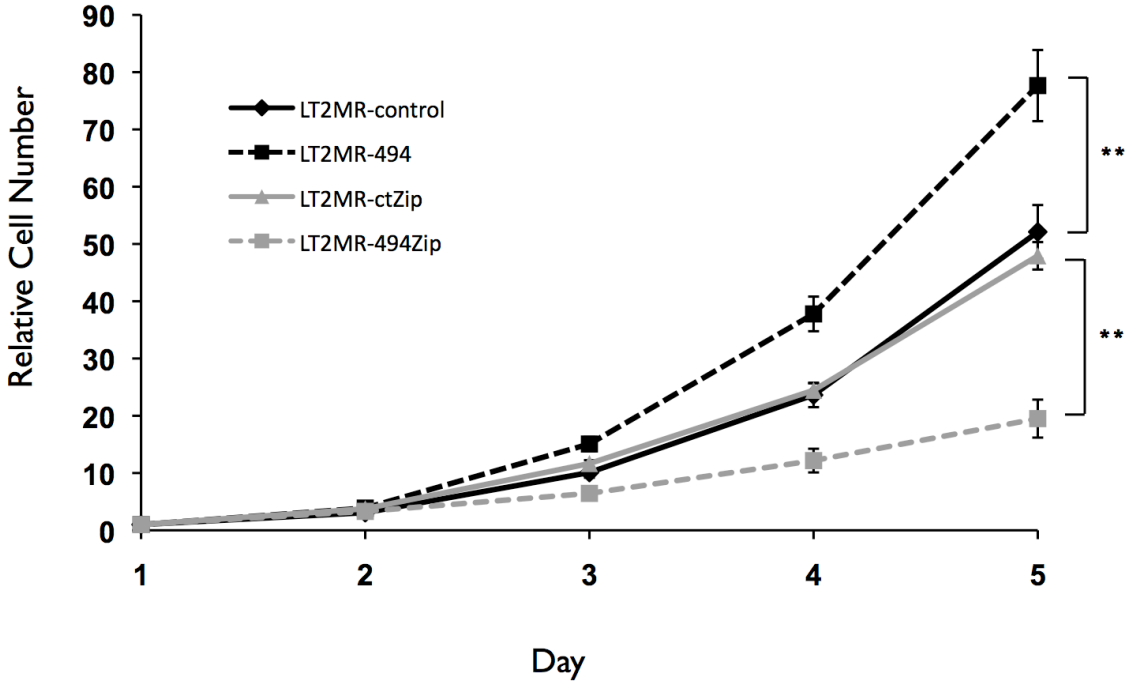


Figure 14. miR-494 affects proliferation of LT2MR cells. LT2MR cells undergo increased proliferation with enforced miR-494 expression and decreased proliferation with miR-494 inhibition. 50,000 cells were plated in triplicate and counted at days 1, 2, 3, 4 and 5 after transfection of pMSCV puro or pMSCV 494, pmiR-ZIP-control or pmiR-ZIP-494. Cell numbers were normalized to day 1. Experiment was repeated 3 times and values are from 1 representative experiment. **p=0.01.

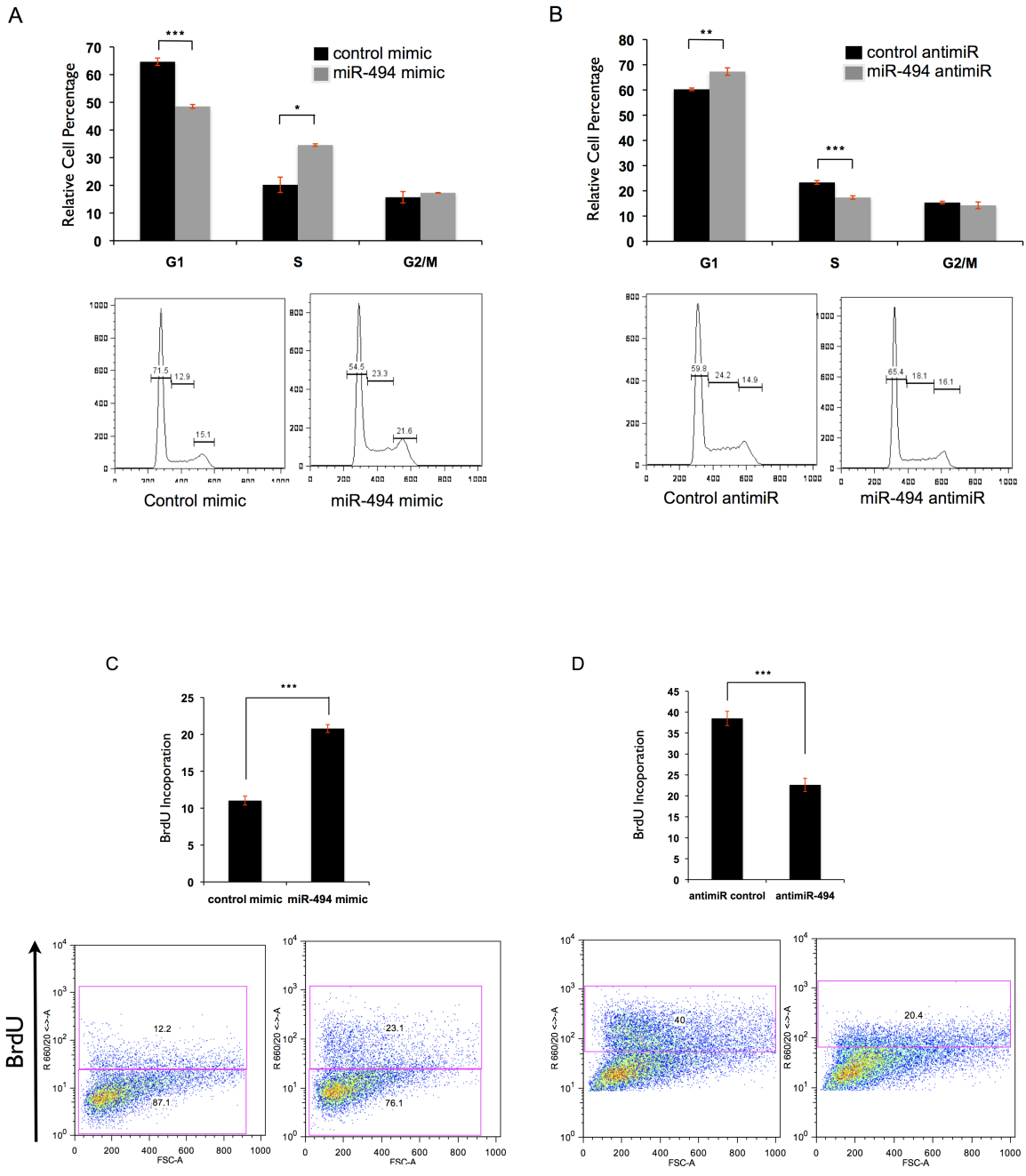


Figure 15. miR-494 affects G1/S transition in LT2MR cells. A. LT2MR cells exhibit accelerated G1/S transition with enforced miR-494 expression. **B.** LT2MR cells exhibit delayed G1/S transition with miR-494 inhibition. LT2MR cells transfected with miR-494 or control mimic and miR-494 or control inhibitor for 48 hours were stained with PI for FACS analysis. Cell cycle profiles were analyzed using FlowJo Analysis Software.

Experiment was done with three replicates per sample and values were averaged to generate graphs. *p=0.01, **p=0.003, ***p=0.001. Figures A and B are representative of three experiments**. C.** LT2MR cells exhibit increased BrdU incorporation with enforced miR-494 expression. **D.** LT2MR cells exhibit decreased BrdU incorporation with miR-494 inhibition. LT2MR cells were transfected with either miRNA mimics or miRNA inhibitors for 48h and exposed to BrdU for 40 min. Flow cytometry was used to measure BrdU incorporation. Experiment was repeated 3 times with 3 replicates per sample and values are from 1 representative experiment. ***p=0.008. Representative FACS plots are shown.

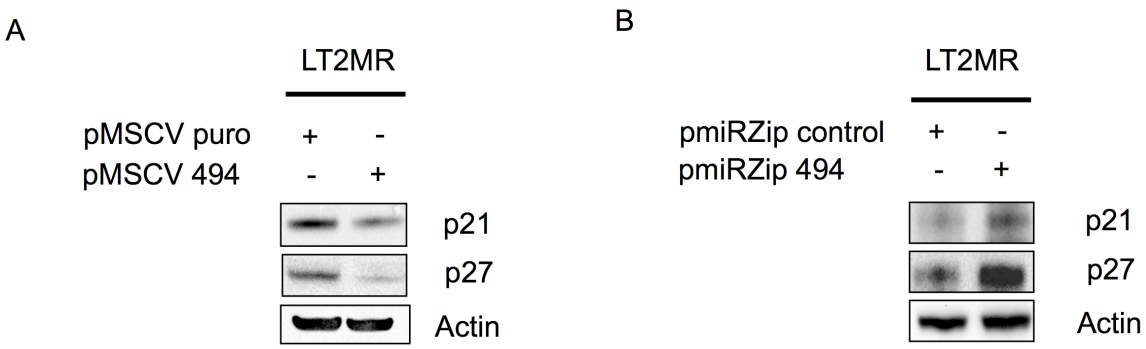


Figure 16. Expression of cell cycle inhibitors corresponds to proliferation. **A.** Stable LT2MR cells overexpressing miR-494 exhibit decreased p21 and p27 expression. **B.** Stable LT2MR cell lines with miR-494 inhibition exhibit increased p21 and p27 inhibition.

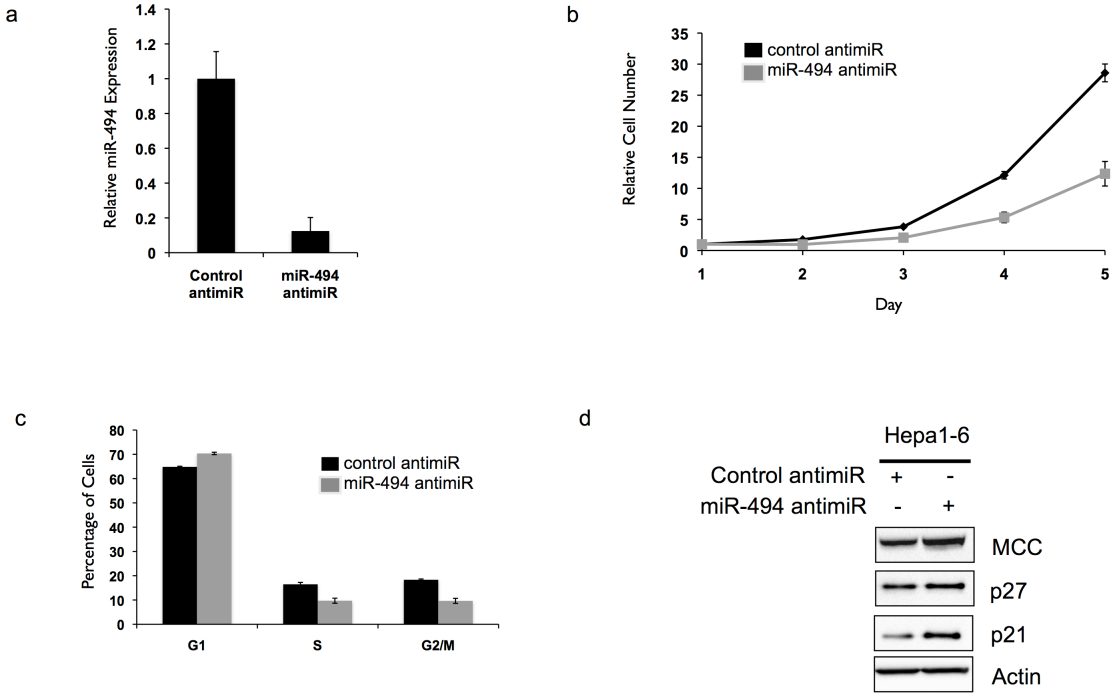


Figure 17. miR-494 knockdown in Hepa1-6 cells impairs proliferation through G1/S transition delay. **A.** qRT-PCR Quantification of miR-494 expression in Hepa1-6 cell lines transfected with either a control or miR-494 antimiR. **B.** Proliferation assay performed on Hepa1-6 cells transfected with either control or miR-494 antimiR. **C.** PI assay performed on Hepa1-6 cells transfected with either control or miR-494 antimiR. **D.** Western blot analysis of MCC, p27 and p21 expression of Hepa1-6 cells cells transfected with either control or miR-494 antimiR.

A

Use TargetScan and miRanda to identify putative miR-494 targets. Eliminate redundant targets (5000 genes)

↓

Input putative targets into DAVID and identify cell cycle related genes (249 genes)

↓

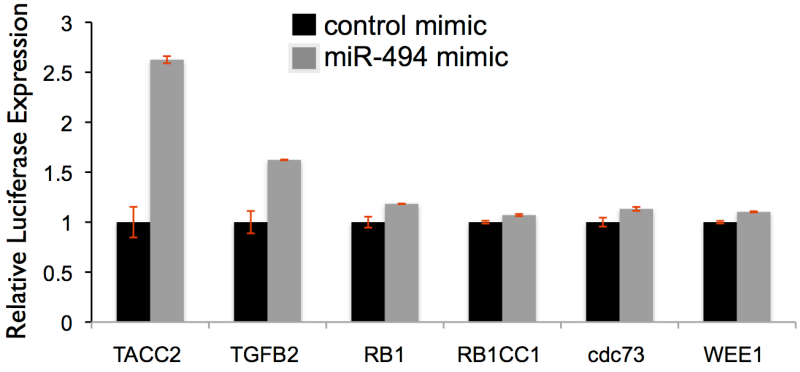
Identify all putative cell cycle targets with binding sites conserved between mouse and human (80 genes)

↓

Using GeneCards, identify negative regulators of cell cycle (7 genes)

1) TACC2
2) TGFB2
3) RB1
4) RB1CC1
5) CDC73
6) WEE1
7) MCC

B



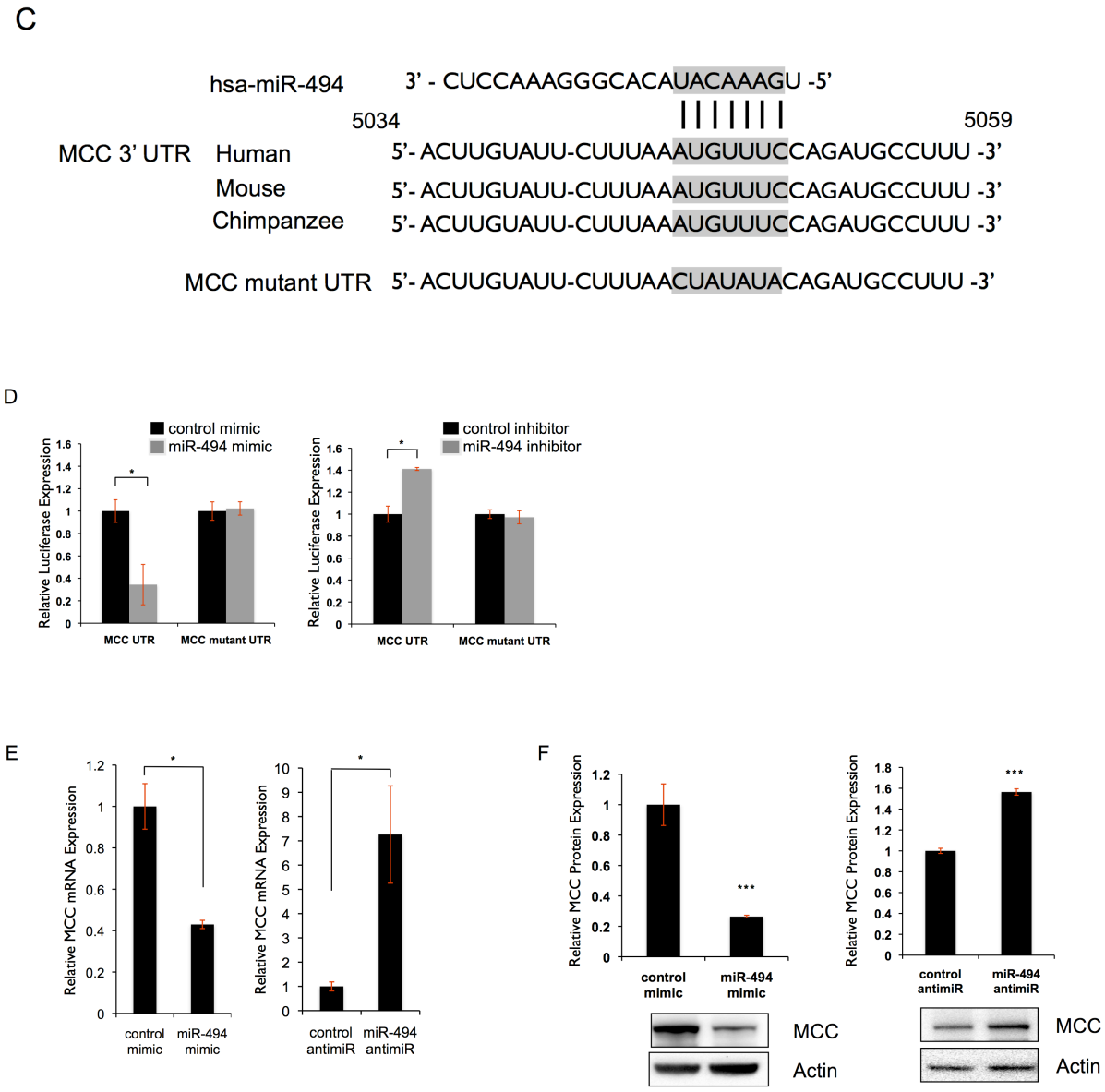


Figure 18. MCC is a direct target of miR-494. A. Schematic illustrating the strategy used to narrow down putative miR-494 targets. **B.** Luciferase reporter assay on TACC2, TGFB2, RB1, RB1CC1, CDC73 and WEE1 3' UTRs show that none of these UTRs are negatively regulated by miR-494. **C.** Putative binding site of miR-494 in MCC 3' UTR is conserved across multiple species. Alignment of human, mouse and chimpanzee MCC 3' UTRs shows a highly conserved region predicted to bind to miR-494. Predicted 7-mer

binding seed of miR-494 to MCC 3' UTR indicated with vertical lines. Putative binding site was mutated as indicated. **D.** MCC 3' UTR is a direct target of miR-494. A representative 300bp of the MCC 3' UTR containing the predicted miR-494 binding site was cloned into a pMIR-REPORT luciferase vector. Activity of the reporter was decreased when co-transfected into LT2MR cells with miR-494 mimic and increased when co-transfected with miR-494 antimiR, compared to control co-transfections. *$p<0.05$. In both cases, site directed mutagenesis of the predicted miR-494 binding site resulted in loss of reporter response. **E.** MCC mRNA expression is responsive to miR-494. LT2MR cells were transfected with miR-494 mimic or hairpin inhibitor and tested for MCC mRNA expression after 24h by qRT-PCR. Values normalized to sno202 and expressed as a relative of control transfected cells. *$p<0.05$**. F.** MCC protein expression is responsive to miR-494. LT2MR cells were transfected with miR-494 mimic or inhibitor and tested for MCC protein expression after 48h by western blot. Graph is an average of three independent transfections and values were quantified using a BioRad Chemidoc. ***$p<0.001$.

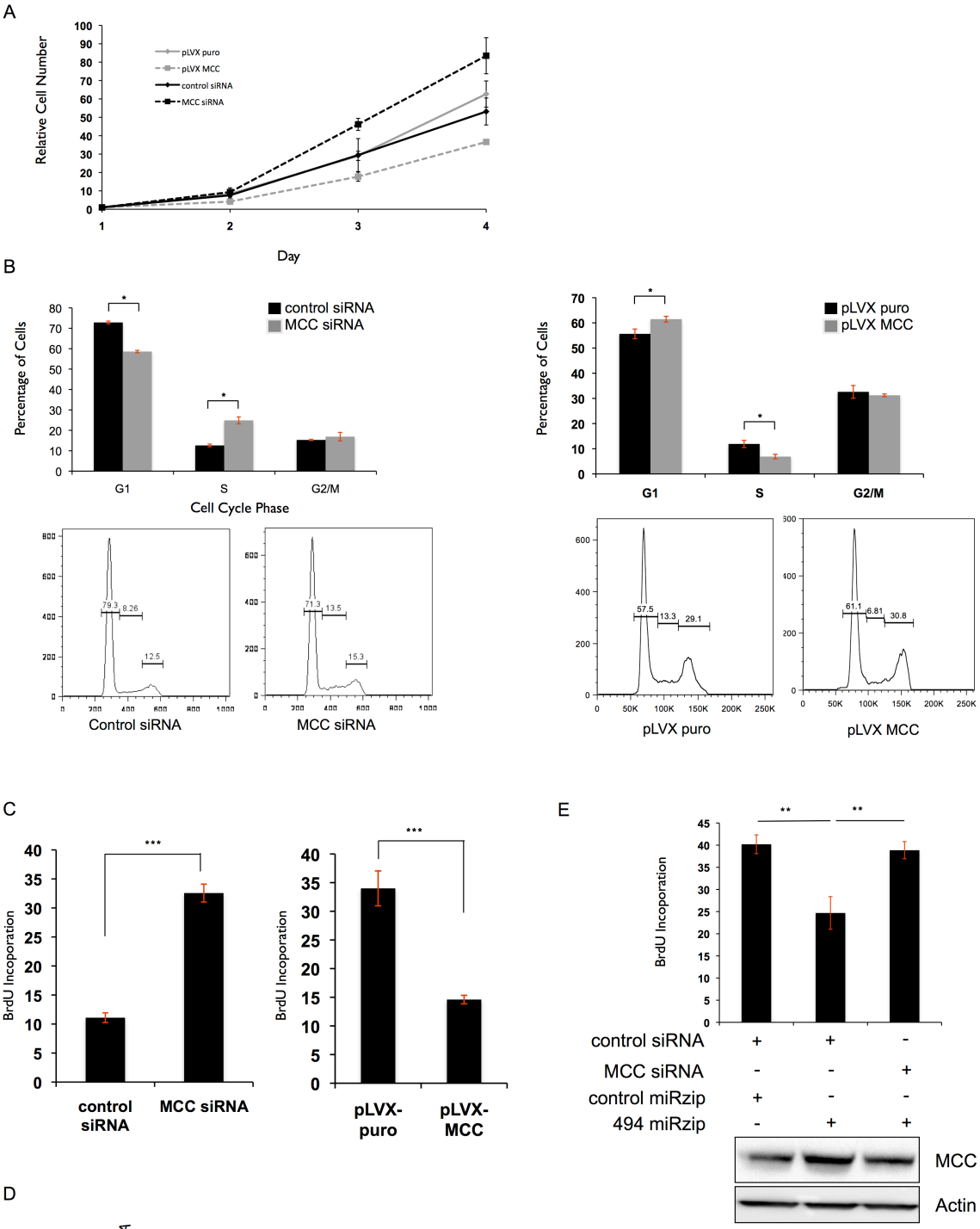

A
Relative Cell Number
pLVX puro
pLVX MCC
control siRNA
MCC siRNA
Day
B
Percentage of Cells
control siRNA
MCC siRNA
G1
S
G2/M
Cell Cycle Phase
79.3
8.26
12.5
71.3
13.5
15.3
Control siRNA
MCC siRNA
pLVX puro
pLVX MCC
57.5
13.3
29.1
61.1
6.81
30.8
C
BrdU Incoporation
control siRNA
MCC siRNA
pLVX-puro
pLVX-MCC
D
Control siRNA
MCC siRNA
pLVX puro
pLVX MCC
MCC
p27
p21
Actin
E
BrdU Incoporation
control siRNA
MCC siRNA
control miRzip
494 miRzip
MCC
Actin

Figure 19. miR-494 modulates G1/S transition through suppression of MCC expression. A. LT2MR cells undergo increased proliferation with MCC inhibition and decreased proliferation with enforced MCC expression. Experiments were repeated 3 times and values are from 1 representative experiment. **B.** LT2MR cells exhibit accelerated G1/S transition with MCC inhibition and G1/S delay with MCC overexpression. LT2MR cells transfected with control or MCC siRNA (top panel) and pLVX puro or pLVX MCC for 48 hours were stained with PI for FACS analysis. Cell cycle profiles were analyzed using FlowJo Analysis Software. Experiment was done with three replicates per sample and figure is representative of three experiments. **C.** Increased proliferation is due to accelerated G1/S transition in MCC siRNA transfected cells. LT2MR cells were transfected with either control or MCC siRNA (left panel) or pLVX puro or pLVX MCC (right panel) for 48h and exposed to BrdU for 40 min. Flow cytometry was used to measure BrdU incorporation. Experiment was repeated 3 times with 3 replicates per sample and values are from 1 representative experiment. ***$p<0.001$. **D.** Expression of cell cycle inhibitors corresponds to proliferation rate. LT2MR cells were transfected with either control or MCC siRNA (left panel) or pLVX puro or pLVX MCC (right panel) for 48h and tested for expression of p27 and p21 by western blot. **E.** Downregulation of MCC attenuates the effects of miR-494 inhibition on cell proliferaion. LT2MR cells were transfected with pmiRZip control or pmiRZip 494 and control or MCC siRNA as indicated and tested for MCC expression after 48h by western blot. Cell proliferation was assessed in these cells by measuring BrdU uptake over a period of 40 min. **$p<0.01$.

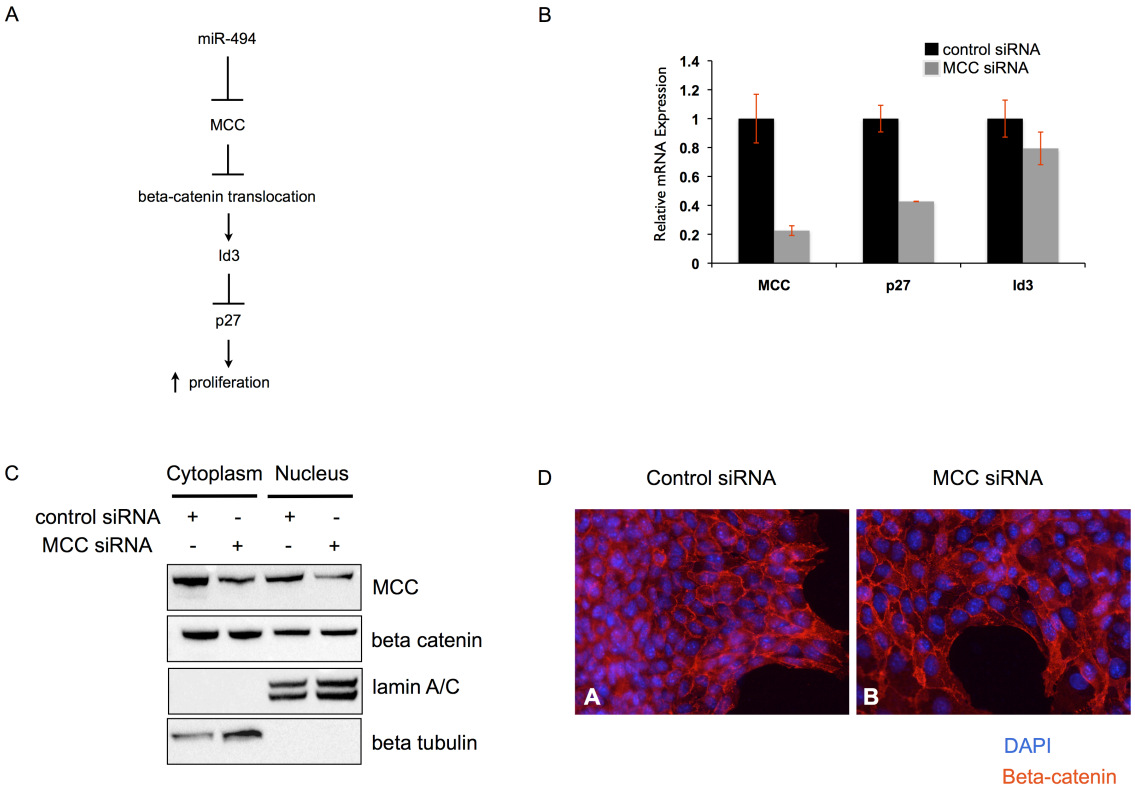


Figure 20. MCC does not modulate p27 levels through beta-catenin in LT2MR cells. **A**. Proposed mechanism of p27 regulation by MCC. **B.** qRT-PCR quantification of MCC, p27 and Id3 mRNA levels in control and MCC siRNA transfected cells. **C.** Analysis of beta catenin levels in cytoplasmic and nuclear fractions in control and MCC siRNA transfected cells. Lamin A/C and beta-tubulin were used as loading controls for nuclear and cytoplasmic fractions, respectively. **D**. Immunoflorescence staining of LT2MR cells transfected with either control or MCC siRNA to detect beta-catenin localization.

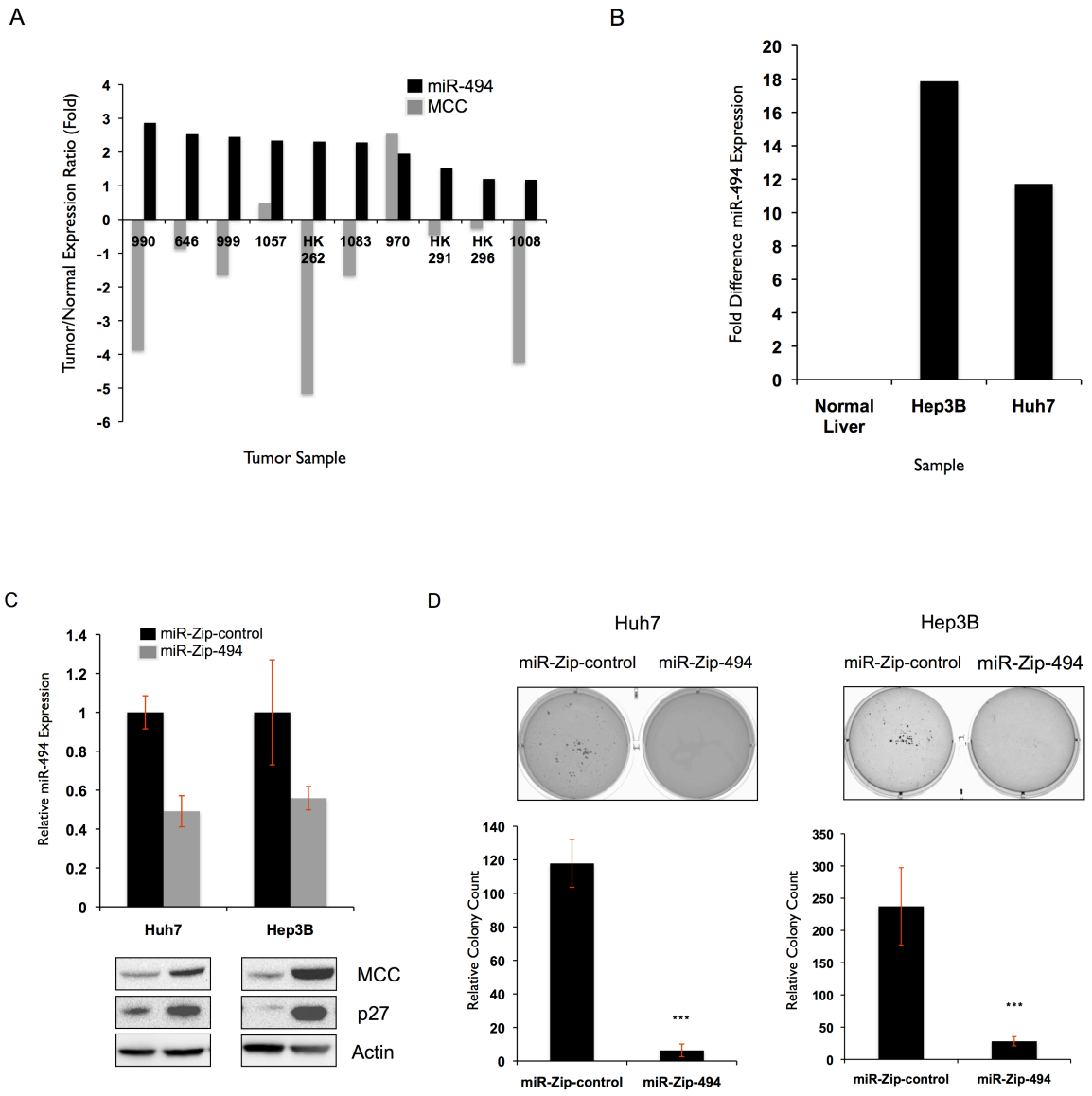
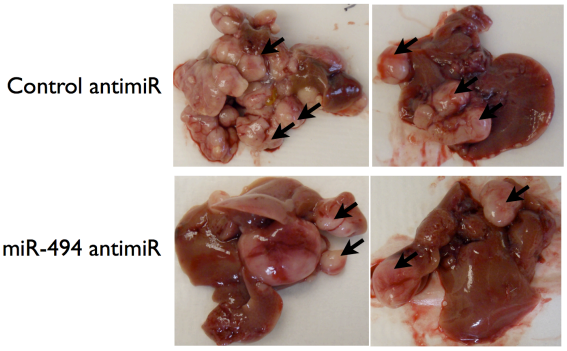


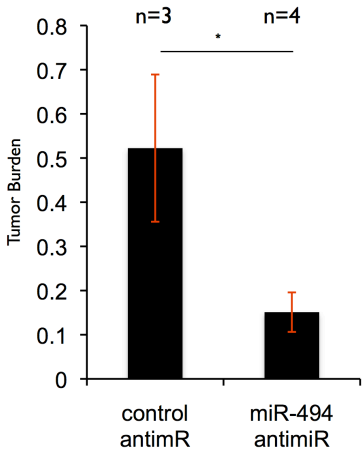
Figure 21. miR-494 targets MCC in human samples and its inhibition decreases transformation. A. MCC mRNA expression is reduced in human HCC samples with high miR-494 expression. Ten samples with highest miR-494 expression were selected from 47 human HCC samples and tested for MCC expression by qRT-PCR. miR-494 and MCC levels were normalized to RNU48 and beta-actin, respectively. Differences in expression are shown as fold change over matched non-tumor tissue. **B**. Quantification of miR-494 levels in Huh7 and Hep3B cells relative to normal liver tissue. **C.** miR-494

knockdown increases MCC expression in Huh7 and Hep3B cells. miR-494 was stably knocked down in Huh7 and Hep3B cell lines with p-miR-ZIP-494. miR-494 expression was tested by qRT-PCR and calculated relative to p-miR-ZIP-control cell lines. MCC and p27 expression was tested by western blot. **D**. miR-494 inhibition decreases colony formation in Huh7 and Hep3B cells. Stable p-miR-ZIP control or 494 Huh7 and Hep3B cells were plated in soft agar for 2 weeks. Experiment was repeated three times in triplicate. ***$p<0.001$.

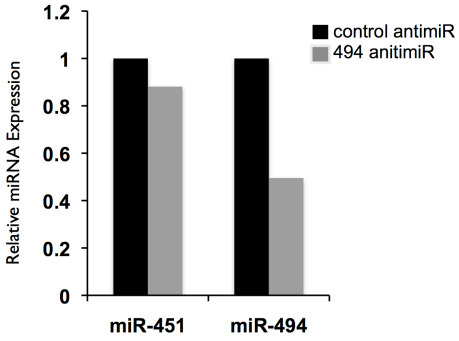
A


Control antimiR
miR-494 antimiR

B


n=3
n=4
*
Tumor Burden
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
control antimR
miR-494 antimiR

C


Relative miRNA Expression
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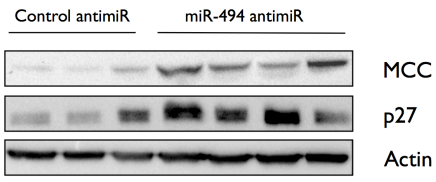
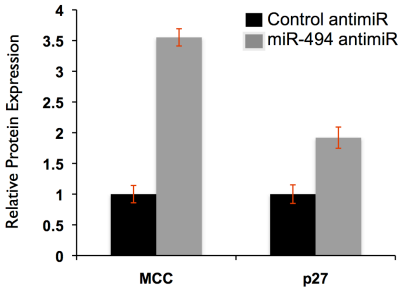
1
0.8
0.6
0.4
0.2
0
control antimiR
494 anitimiR
miR-451
miR-494

D


Control antimiR
miR-494 antimiR
MCC
p27
Actin

E


Relative Protein Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
Control antimiR
miR-494 antimiR
MCC
p27

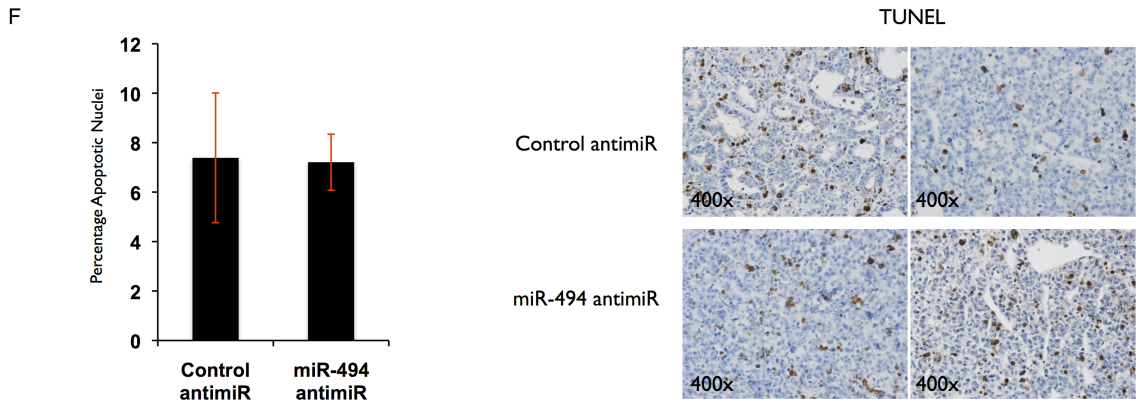


Figure 22. mir-494 inhibition slows tumor growth *in vivo* through cell cycle inhibition. A and B. miR-494 inhibition decreases liver tumor growth *in vivo*. **A.** Representative pictures of livers of LT2/MYC mice (5 weeks off dox) injected with control (n=3) or miR-494 (n=4) antimiRs over a period of 3 weeks (total 8 weeks off dox), twice a week. **B.** Relative tumor burden in mice as quantified by ImageJ. *$p<0.05$. **C.** Verification of miR-494 expression in tumors treated with either control or miR-494 antimiR. miR-451 expression was tested as a negative control. qRT-PCR was performed to detect expression of respective miRNAs. **D and E.** miR-494 inhibition increases MCC and p27 expression *in vivo*. **D**. Protein expression of MCC and p27 in liver tumors of control and miR-494 antimiR injected mice**. E.** AntimiR-494 treated tumors show increased MCC and p27 protein expression. Protein expression values were quantified using Biorad Chemidoc software. MCC and p27 expression was normalized to actin and the average expression of each protein was calculated in each treatment group to generate graph. F. Quantification of TUNEL staining performed on control and miR-494 antimiR treated animals. Representative pictures are shown

Chapter 4: SNPs in the p53 3' UTR modulate its expression

4.1 Introduction

In the preceding chapters, we identified and characterized an oncogenic miRNA in HCC and found that its inhibition could be a potential therapeutic approach in treating liver cancer. Despite our initial promising results with miR-494 antagonists *in vivo*, several questions still remain over the delivery, control, sustainability and tissue specificity of RNA based therapy in humans (Gramantieri et al., 2008; Zhang et al., 2010). Thus, alternative approaches involving miRNA-based therapy are still under exploration. One such approach is the identification of mutations in the 3' UTRs of oncogenes or tumor suppressor genes that could alter miRNA-binding sites, resulting in mis-expression of these key tumorigenic players.

The p53 tumor suppressor gene is one of the main molecular decision makers of stress response in human cells. Depending upon the cellular context and type of stress, p53 elicits apoptosis, DNA repair, cell cycle arrest or metabolic homeostasis maintenance by inducing appropriate sets of genes (Gasco et al., 2002; Menendez et al., 2009). It is frequently found mutated in a wide variety of tumor types, including breast cancer (26% of cases, COSMIC database). Of the three described molecular subtypes of breast cancer, p53 mutations are most commonly found in TNBCs (54%) and correlate with poor prognosis in this intractable subgroup. Mutant p53 can play multiple roles in breast cancer development (Gasco et al., 2002). However, its role in mediating sensitivity to spontaneous and UV-induced apoptosis portends its use as a predictive marker for

chemotherapy efficacy (Walerych et al., 2012). In particular, p53 mutant breast cancer cells have been demonstrated to be more resistant to anthracyclines than cells with wildtype p53 (Bertheau et al., 2007; Lowe et al., 1994). Clearly, elucidating the mechanisms that govern p53 expression is important to further understand the role p53 plays in breast cancer development and modulating chemotherapeutic response. Since miRNAs can fine-tune protein expression, we wondered if mutations existed in the p53 3' UTR that could modulate its expression by altering miRNA binding sites.

In this chapter, we sequenced the p53 3' UTR from 45 human breast cancer tumors and identify 2 SNPs present in these patients. We find these SNPs are associated with patients that have mutant p53 and result in lowered p53 expression. These findings suggest that SNPs in the p53 3' UTR could be a predictive marker of chemotherapeutic response in breast cancer patients.

4.2 Results

Two SNPs were identified in the p53 3' UTR

In order to identify mutations within the p53 3' UTR, we sequenced genomic DNA from the breast tumors of 45 patients using the strategy outlined in **Figure 23**. Briefly, the 1.2kb long UTR was split into two halves with a 150bp overlap, and sequenced using two primer pairs. Analysis of sequencing data revealed two mutations, the first present at position 485 (G>A) and the second at position 826 (G>A) in the p53 3' UTR. The 485G>A mutation was detected in 5% of patients (n=2) while the 826G>A mutation was detected in 11% of patients (n=4, **Table 4**). Interestingly, all six mutations were found in

patients with mutant p53, although a two-tailed Fisher's exact test showed no significance (p=0.3122, **Table 5**). Previous studies identifying 3' UTR mutations in other genes have demonstrated that these mutations occurred as SNPs of variable frequencies in the population. We wondered if this was also the case with the p53 3' UTR mutations in our samples. To test this, we performed a search on the NCBI Single Nucleotide Polymorphism Database (dbSNP) and found that both mutations were documented SNPs. The 485G>A mutation (dbSNP: rs4968187) occurs with a 4% frequency while the 826G>A mutation (dbSNP: 17884306) occurs with a 5% frequency among a mixed African, American, Asian and European population.

SNPs result in decreased p53 expression

To test if these two SNPs had functional significance on p53 expression, we performed luciferase reporter assays. Luciferase reporter vectors containing either wildtype p53 UTR, 485G>A p53 UTR or 826G>A p53 UTR were co-transfeced with Renilla luciferase vectors and readings were taken after 48 hours. Compared to wildtype p53 3' UTR, the 485G>A mutation significantly lowered reporter expression by about 15% while the 826G>A mutation significantly lowered expression of the reporter by about 20% (**Figure 24**). We attempted to determine if these SNPs disrupted any predicted miRNA binding sites in the p53 3' UTR. However, no there were no predicted binding sites for any miRNAs at these loci, according to TargetScan (**Figure 25**). We hypothesize that perhaps novel miRNA binding sites are being created by these SNPs and are an area for further study.

4.3 Discussion

p53 is a tumor suppressor gene frequently found mutated in TNBC and correlated with poor prognosis in this subgroup. Besides being a key oncogenic driver in TNBC, mutant p53 also plays a role in modulating chemotherapeutic sensitivity of this intractable subgroup. Thus, there is a need to understand the regulatory mechanisms that govern p53 expression in order to better predict chemotherapeutic response in TNBC and other breast cancers. In this chapter, we identify two SNPs present in the 3' UTR of p53 that alter p53 expression. We find that these SNPs occur at a frequency of 5% and 11% of our samples, compared to 4% and 5% in a database containing American, European, Asian and African populations. Further, we find their occurrence exclusively in patients with mutant p53.

The role of miRNA SNPs in disease is just being defined. For example, a SNP in mature miR-125a, upregulated in breast cancer, decreases its expression through alterations in pri-miRNA processing (Duan et al., 2007). Other studies have identified SNPs in miRNA binding sites that interefere with gene expression, resulting in increased disease incidence (Abelson et al., 2005; Chin et al., 2008). While we were able to show that SNPs in the p53 3' UTR altered reporter expression *in vitro*, we were unable to correlate their occurrence to the expression of p53 in patients due to limited patient data resources. It would be useful to determine if these SNPs are associated with lowered p53, and if they predict a worse chemotherapeutic outcome in patients. It would also be useful to sequence a larger sample of tumor DNA, in order to identify other 3' UTR SNPs that could possibly alter p53 expression. Mechanistically, lowered reporter expression

suggests that these SNPs are creating an illegitimate binding site for miRNAs in the p53 3' UTR. The identification of such miRNAs is important, in order to further understand the roles that miRNAs play in cancer development. The data in this chapter suggest that SNPs in the 3' UTR of mutant p53 could be a factor in determining chemotherapeutic response through alterations in miRNA binding, although further mechanistic and clinical work needs to be done to fully understand the implications of these findings.

4.4 Figures and Tables

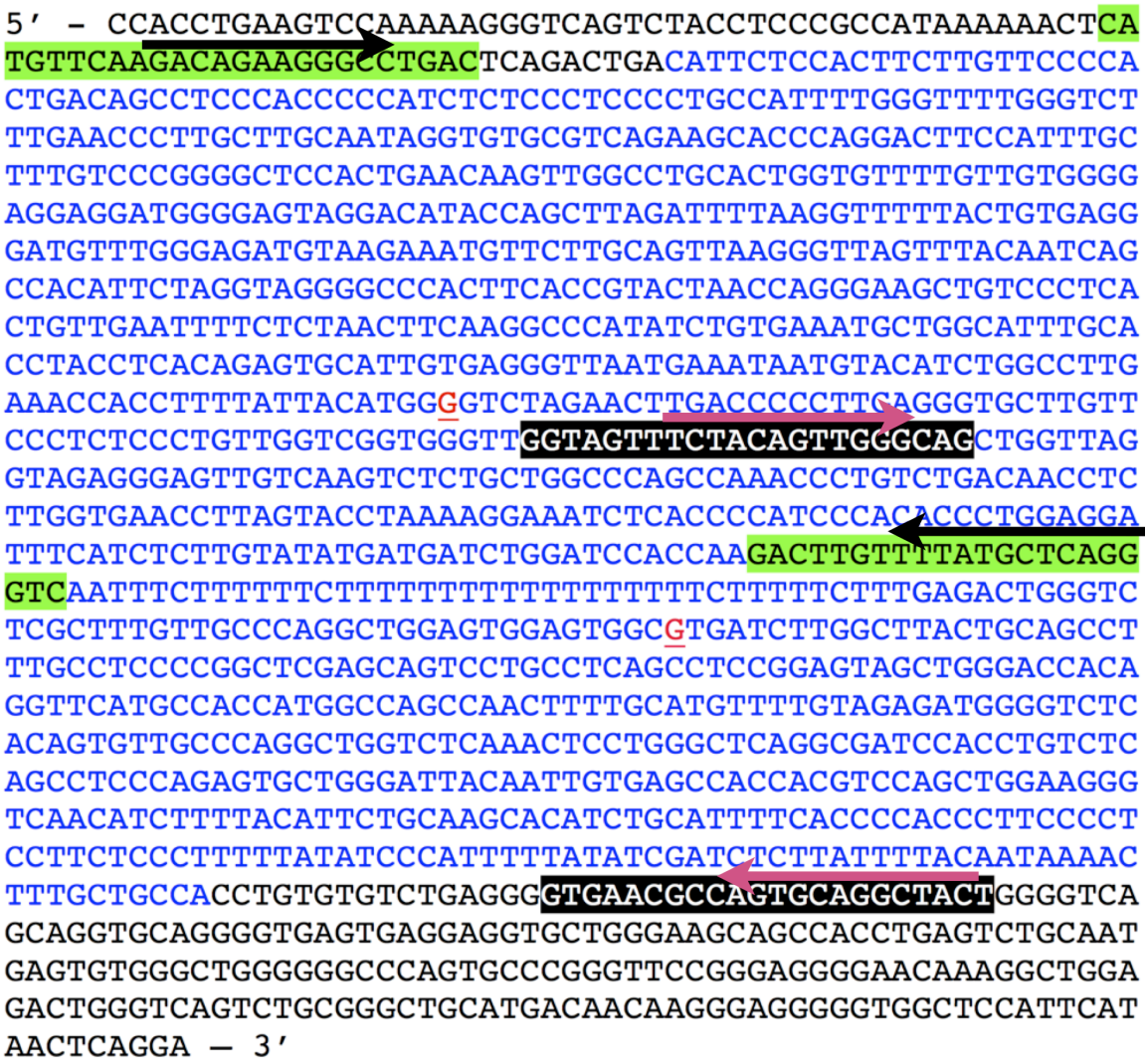


Figure 23. Schematic of the wildtype human TP53 3' UTR. TP53 3' UTR is indicated in blue lettering. The 1.2kb long 3' UTR was broken into 2 segments for sequencing. The first segment, 775bp long, was sequenced using the primers shaded in green. The second segment, 683bp long, was sequenced using the primers shaded in black. Two mutations common to several samples were identified shown in red lettering. The first is 485 G>A and the second is 826 G>A.

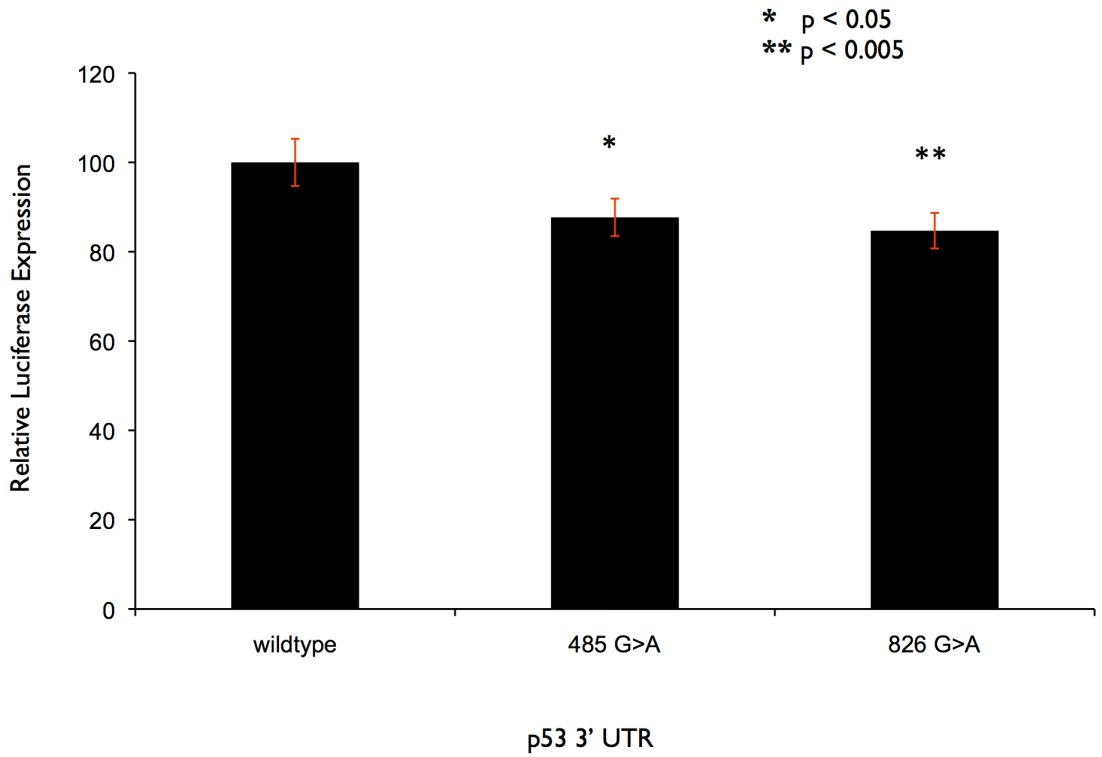


Figure 24. SNPs in 3' UTR alter p53 expression. UTRs with SNPs detected in patient samples were cloned into luciferase reporter vectors and tested for expression in 293T cells. Both SNPs caused about a 15-20% decrease in untreated 3T3 cells after 48 hours.

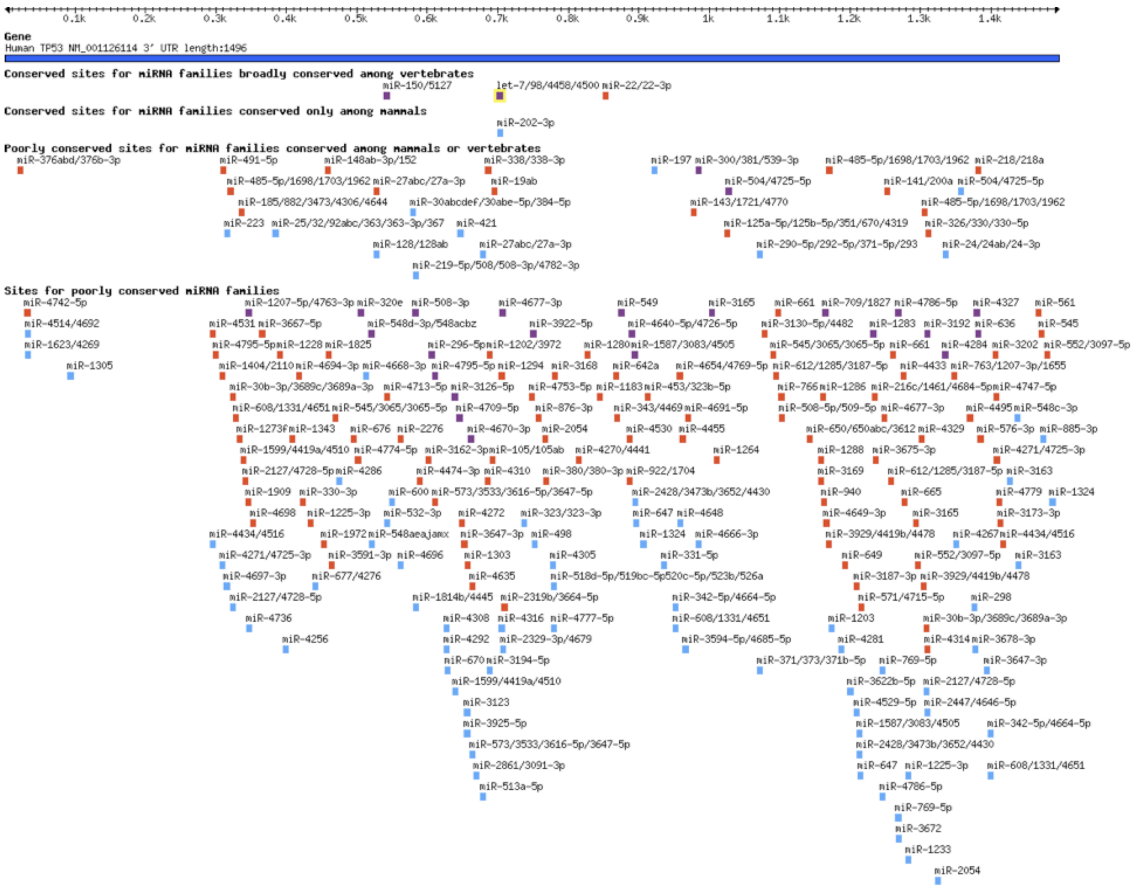


Figure 25. miRNAs predicted by TargetScan to bind to the TP53 3' UTR. No miRNAs are predicted to bind at nucleotide 485 or 826 in the human TP53 3' UTR.

Sample ID	ER Status	TP53 status	Mutations Detected
7	+	Mutant	-
70	+	Mutant	-
108	+	Mutant	-
155	+	Mutant	826 G>A
208	-	Mutant	-
54	-	Mutant	485 G>A
64	-	Mutant	-
98	-	Mutant	-
210	-	Mutant	-
225	-	Mutant	-
85	-	Wildtype	-
118	-	Wildtype	-
178	-	Wildtype	-
306	-	Wildtype	-
359	-	Wildtype	-
470	+	Wildtype	-
91	+	Wildtype	-
11	+	Wildtype	-
128	+	Wildtype	-
242	+	Wildtype	-
33	-	Mutant	-
47	-	Mutant	-
59	-	Mutant	-
60	-	Mutant	826 G>A
78	-	Mutant	-
82	-	Mutant	485 G>A
107	-	Mutant	-
154	+	Mutant	-
192	+	Mutant	-
237	+	Mutant	-
252	+	Mutant	-
255	+	Mutant	-
304	-	Mutant	-
324	-	Mutant	-
341	-	Mutant	826 G>A
376	-	Mutant	-
395	-	Mutant	-
400	-	Mutant	-
447	-	Mutant	826 G>A
453	-	Mutant	-
460	-	Mutant	-
472	+	Mutant	-
478	-	Mutant	-
483	-	Mutant	-
507	-	Mutant	-

Table 4. p53 3' UTR sequencing results

	SNP absent	SNP present	Total
p53 wildtype	10	0	10
p53 mutant	29	6	35
Total	39	6	45

Table 5. Summary of sequencing results

Conclusions and Future Directions

In this thesis, we explored the potential of miRNAs as novel therapeutic targets in HCC and breast cancer. Using multiple mouse models, we identified miR-494 as an upregulated miRNA in HCC. We find that it plays a role in modulating the cell cycle and most importantly, demonstrate that its inhibition can limit tumor growth *in vivo*. Our data also indicates that numerous other miRNAs in the Dlk1-Dio3 miRNA megacluster could be oncogenic in HCC, and these should be further explored. Although our results are encouraging, much work remains to be done before miR-494, or any other miRNA, can be targeted in the clinic. Because a miRNA can affect the expression of several downstream targets, modulating the expression of miR-494 could lead to undesirable off-target effects, and this needs to be further studied before clinical application. The main obstacle in RNA based therapy is the effective delivery of the effector molecule, which should preferably be controllable, sustained and tissue-specific. Since AAV vector mediated delivery of miR-26a to mice was demonstrated to suppress tumorigenesis in mice, gene therapy using virally delivered miRNAs has been proposed. However, several safety issues are raised with the clinical use of viral therapy, the main being host genome integration. Instead, several groups have found that chemical modifications to miRNA mimics and antagomiRs, as well as incorporation into lipid envelopes allow efficient delivery *in vivo*. However, these results are still under study and more work needs to be done to determine effective delivery methods *in vivo*.

Since altering miRNA expression *in vivo* is still under study, we wondered if there were other ways to take advantage of miRNA function in cancer therapy. In chapter three, we explored that possibility by sequencing the p53 3' UTR of 45 breast cancer patients, reasoning that mutations in the 3' UTR of p53 could modulate its expression, by creating or abrogating miRNA-binding sites. We find two SNPs in the p53 3' UTR that alter its expression, occur at a higher frequency than previously established and associated with mutant p53. These exciting findings suggest that breast cancer patients could benefit from a form of "personalized medicine", where sequencing of their genome could be carried out to inform chemotherapeutic response. However, more work is required before this can occur, including identifying the miRNAs that now target and suppress the SNP containing p53 3' UTRs. Further, it would be useful to sequence the 3' UTRs of other key oncogenes or tumor suppressor genes in breast cancer, such as ER, HER2, HER3, in order to determine if similar mechanisms governing their expression exist.

In conclusion, miRNAs provide tremendous potential as therapeutic targets in HCC. This is underlined by the findings described in this thesis. However, much work remains to be done in order to fully understand miRNA function *in vivo,* in order to fully exploit their therapeutic potential in the clinic.

Materials and Methods

Mouse Strains

LAP-tTA/TRE-MYC (LT2/MYC) double-transgenic mice have been described previously(Shachaf et al., 2004). TRE-RASV12 mice(Chin et al., 1999) were crossed with mice containing the tetracycline-transactivator under the control of liver specific promoter, liver enriched activator protein (LAP), to generate LAP-tTA/TRE-RAS (LT2/RAS) double-transgenic mice. Crossing LT2/MYC males with LT2/RAS female mice generated the triple-transgenic mice, LAP-tTA/TRE-MYC/TRE-RAS (LT2/MYC/RAS). Mice were maintained on doxycycline (200 mg/kg doxy food) to suppress oncogene expression until 8 weeks of age. Doxycycline was then removed to allow the induction of transgene expression and tumor formation. Mice were monitored weekly for tumor development by palpating the abdomen. The Committee for Animal Research at the University of California, San Francisco, approved all animal experiments.

Human Tissues

Human HCC and associated noncancerous liver tissues, collected during liver resections at University of Hong Kong, were frozen in liquid nitrogen within half an hour after removal. All HCC samples were found by two pathologists to contain more than 80% tumor cells. The Ethics Committee of the University of Hong Kong and Internal Review Board of UCSF approved use of human samples. Informed consent was obtained from all patients.

Sleeping Beauty Tumor Induction and miRNA analysis

Spontaneous HCCs were developed in mice using the *Sleeping Beauty* (Huang et al.) transposon insertional mutagenesis system as previously described(Dupuy et al., 2009). Ubiquitous mobilization of mutagenic transposons was achieved by crossing mice harboring T2/Onc3 transposon concatemers to mice expressing *SB* transposase from the *Rosa26* locus. Liver tumors resulting from transposon mutagenesis were collected and snap-frozen in liquid nitrogen for later DNA and RNA sequencing analyses. All tumors used in this study were collected from mice using procedures approved and monitored by the Institutional Animal Care and Use Committee at the National Cancer Institute-Frederick. Transposon integration sites in tumors were identified as previously described(Dupuy et al., 2009). Briefly, ligation-mediated PCR (LM-PCR) was conducted on genomic DNA extracted from *SB*-induced liver tumors. Amplified transposon/genome junctions were detected by pyrosequencing. Total RNA was collected from *SB*-induced HCC and normal liver samples using the miRNeasy kit (Qiagen). The flashPAGE Fractionator system (Life Technologies) was used to isolate RNAs shorter than 40nt. Library preparation and sequencing were performed using the SOLiD small RNA expression workflow (Life Technologies). For data normalization, the raw number of reads for each miRNA was converted to reads per 100,000 mapped reads. This was followed by $\log_2$ transformation of the normalized value + 1. Unsupervised clustering was performed on samples based on normalized expression of genes with variation in Euclidean distance among samples of at least 1.5 standard deviations using Cluster 3 software. Heat maps were generated using Java TreeView software.

Microarray Analysis

RNA from normal liver (LT2) and tumor tissue (LT2/MYC, LT2/RAS, LT2/MYC/RAS) was extracted, enriched for miRNAs and biotin-labeled using Ambion's miRVana, flashPAGE and miRVana miRNA labeling kits respectively. Samples (n=4) from each genotype were hybridized to a custom Affymetrix Genechip designed to miRNA probes derived from Sanger miRBase v9.2. This array contained a total of 14216 probes, of which 1233 matched to 301 unique mouse miRNAs. For each probe, an estimated background value was subtracted that was derived from the median signal of a set of GC-matched anti-genomic controls. Detection calls were based on a Wilcoxon rank-sum test of the miRNA probe signal compared to the distribution of signals from GC-content matched anti-genomic probes and normalized according to the variance stabilization method described (Huber et al., 2002). Post-normalized data scale is reported as generalized $\log_2$ data. For statistical hypothesis testing, one-way ANOVA was applied and probes are considered significantly differentially expressed based on a default p-value of 0.05 and $\log_2$ difference >1 or <-1. The microarray data has been deposited at the NCBI GEO repository under accession number GSSE44570.

Plasmids and Cloning

pMSCVhygro and pMSCVpuro (Clontech, Mountain View, CA) retroviral vectors were used to express H-RAS-V12 and miRNA subclusters respectively. pmiRzip control and miR-494 lentivectors were obtained from System Biosciences. pLVX-AcGFP-N1 (Clontech) lentivector was used to express MCC cDNA cloned from mouse genomic DNA with the following primers: MCC-F 5'-ATG AAT TCT GGA GTT GCG GTG AAA-3' and MCC-R 5'-TTA GAG TGA CGT TTC GTT GGT-3'. miRNA subclusters were cloned from human genomic DNA using the following primers: SAR-1-F 5'-GCC

ATA GGA GGC AGT AGG-3', SAR-1-R 5'-AGG CAG AAG ACA GAG GTC CA-3'; SAR-2-F 5'-GCT ACC AAG GAA TTC CA-3', SAR-2-R 5'- CCA CTC TCC CTA CTG CCT GAA -3'; SAR-3-F 5'-ACC ATG GCC ATA AGT TC-3', SAR-3-R 5'- GCT GGC TCG ATA CAA GAA GG -3'; SM-1-F 5'-CAA ATC CAG CCT CAG AAA GC-3', SM-1-R 5'-CCT GTT TCA CAT CCC TCG TT -3'; SM-2-F 5'-TTC ACT GAC CTC ACG GTA-3' SM-2-R 5'-ACT GAG TTC TTG GCT GGT GG-3'; SM-3-F 5'-TTG TGA GGC TTT CTG GAG GT-3', SM-3-R 5'-ACA TTG AGG GTT AGC GCA AT-3'; SM-4-F 5'-GCT TGA AGA GCT TT-3', SM-4-R 5'-TCC ATG GCT GGG GCC TGA GAG-3'; SM-5-F 5'-AAG ACT TCC TGG GAT CC-3', SM-5-R 5'-TCC CCA GCC TCC CTG GC-3'; SM-6-F 5'-GGA GTG ATG CCT GGA AAA GA-3', SM-6-R 5'-CAC CAC CAC CAC CAA TGA TA-3'; SM-7-F 5'-GCA TGA GCG AGT GTG TGA CT-3', SM-7-R 5'-TCA AAG AGG AGG GAA CCT CA-3'; SM-8-F 5'-CAG AAA CCC TGG GGT CTA CA-3', SM-8-R 5'-TCC CCA AGA ATG CTG AAA TC-3'; SM-9-F 5'-AAT ACA GGG CGT GTG CTT TC-3', SM-9-R 5'-GCG ACA TCT GTT CTC ATC CA-3'; SM-10-F 5'-AGG CTC TGA TGC AGG TCA GT-3', SM-10-R 5'-AGG CCT CTG GTC ATC TTC CT-3';

Cell Lines and Cell Cultures

Huh7 and Hep3B cell lines were obtained from UCSF Cell Culture Facility and propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. LT2M cells were isolated from mouse LT2/MYC tumors as previously described and propagated in RPMI 1640 media supplemented with 10% fetal bovine serum. LT2M cells were engineered to stably express RAS by retroviral infection

with pMSCV-HRAS V12 virus. Hygromycin and puromycin were used at 200 and 2 ug/ml respectively to select for stable transformants. To generate virus, 293FT cells were co-transfected with pMSCV retroviral vectors and pCL-ECO packaging plasmid or pmiR-Zip lentiviral vectors and ViraPower Lentiviral Packaging Mix (Invitrogen, Carlsbad, CA). Viral supernatant was harvested 48h after transfection and used to infect target cells. Soft agar assays were performed as previously described. Briefly, 10,000 cells were plated in 0.3% agar over a bottom layer of 1% agar. After 14 days, crystal violet was used to visualize colonies, and these were counted by visual inspection.

RNA Isolation and RT-PCR

Total RNA was isolated from tumor tissue and cell lines using Ambion mirVana isolation kit (Life Technologies, Carlsbad, CA) and DNase treated with Ambion Turbo DNA-*free* (Life Technologies) before use for downstream applications. For mRNA RT-PCR, cDNA conversion was carried out using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR for human MCC was carried out on a BioRad Chromo 4 Instrument using Life Technologies Taqman probe Hs00182059_m1. For SYBR green qRT-PCR detection of mouse MCC, the following primer set was used: MCC-F 5'- GGA GAC CTC TCC ATG AAA TCA G, MCC-R 5'-AGA ATA TCC TCC CAG CTC TC-3'.

Transfections

Transient transfection of plasmids was carried out using FuGENE HD Transfection Reagent (Promega, Madison, WI) according to manufacturer's instructions. MCC siRNA

ON-target plus (L-056552-00-0005, Dharmacon, Lafayette, CO) and control siRNA (D-001810-01-05, Dharmacon) were used at final concentrations of 50nm. miR-494 (C-310659-05-0005, Dharmacon) and control miRIDIAN mimic (CN-001000-01-05, Dharmacon) were used at final concentrations of 30nm. miR-494 (IH-310659-06-0005, Dharmacon) and control hairpin inhibitor (IN-001005-01-05, Dharmacon) were used at final concentrations of 100nm. All small RNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. For co-transfections of plasmids and mimics, Fugene HD was used.

Cell Proliferation Assay and FACS Analysis

For proliferation assay, cells were initially transfected with siRNA or expression vectors in a 100mm dish. 24 hours later, 20,000 cells were counted and plated in triplicate in 6 well dishes. Cell counts were carried out over a period of 4 days using a hemocytometer and inverted light microscope. For cell cycle analysis, 50,000 cells were plated in a 6 well plate and transfected. After 48 hours, cells were harvested, fixed and stained with propidium iodide as described previously(Horiuchi et al., 2012). BrdU staining was carried out using an APC BrdU flow kit (BD Biosciences, San Jose, CA). DNA content and BrdU uptake were measured using a BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo Software (Tree Star Inc, Ashland, OR).

Western Blotting

Western blots were performed as previously described(29). The following antibodies were used: anti-AFP Ab-2 (RB365A1, Thermo Scientific, Waltham, MA), anti-beta-actin

(AC-74, Sigma Aldrich, St. Louis, MO), anti-p21 (Clone SX118, BD Pharmingen), anti-p27Kip1 (Clone 57, BD Pharmingen), anti-MCC (Clone 1, BD Pharmingen).

Luciferase Reporter Assay

A 300bp fragment of the MCC 3'UTR containing the putative miR-494 binding site was cloned from mouse genomic DNA and inserted into a pMIR-REPORT luciferase reporter vector (Ambion) by restriction digest. The following primers were used: MCC-UTR-F 5'- AGA GAG AGC TCA GAT GCT CTA CCA TTC GTT CTC C-3' and MCC-UTR-R 5'-AGA GAA AGC TTA ACG TTT GTA AAA TAT TAT AAA TGT CT-3'. LT2MR cells (20,000 cells/well) were plated in 24-well plates. 200ng of pMIR-REPORT-MCC-3'UTR was co-transfected with either miRIDIAN mimics (30nm) or miRIDIAN inhibitors (50nm) and 50ng pMIR-REPORT Beta Galactosidase vector (Ambion) using Lipofectamine 2000 (Invitrogen). Luciferase and beta-galactosidase readings were taken 48h after transfection using the luciferase reporter assay and Beta-Glo Assay Systems (Promega). Firefly luciferase activity was normalized to beta galactosidase expression for each sample. Site directed mutagenesis of MCC 3'UTR at the putative miR-494 binding site was carried out using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) with the following primers: MCC-SDM-F 5'-CCT TGT AAT TTT TAG ACT CAC TGT AGC TTT AAC TAT ATA CAG ATG CCT TTG GTT TTT AGC TGC T-3', MCC-SDM-R 5'- AGC AGC TAA AAA CCA AAG GCA TCT GTA TAT AGT TAA AGC TAC AGT GAG TCT AAA AAT TAC AAG G-3'.

In vivo AntimiR Injections

Control and miR-494 antimiRs were provided by Dr. E. Marcusson (Regulus Therapeutics, San Diego, CA). Aged matched LT2/MYC mice were taken off doxycycline at 8 weeks of age to turn on MYC expression. After 5 weeks off doxycycline, mice received intraperitoneal injections with either control or miR-494 antimiR at a dose of 25mg/kg. Injections were carried out twice a week. Mice were sacrificed after three weeks of injections and tumor samples were collected for RNA and protein analysis. Liver tumors and adjacent non-tumor tissues were scanned and ImageJ software was used to quantify tumor burden, as previously described.

Statistical Analysis

Data are expressed as the mean ± standard error (S.E.M) from at least 3 separate experiments performed in triplicate, unless otherwise noted. Differences in groups were analyzed using a double-sided student's t test.

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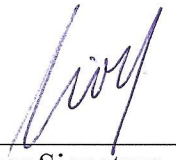

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