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Probing microRNA's function in cancer

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Abstract of the Dissertation

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To date, cancer research has focused on alterations of protein coding genes. However, recent evidence suggests that alterations of non-coding RNA, particularly micro-RNAs (miRNAs), also contribute to tumorigenesis. For example, an oncogenic polycistronic miRNA cluster, *mir-17-92,* cooperates with *c-myc* to accelerate B-cell lymphomas in mice. There's also evidence that miRNAs such as *mir-15*, *mi-16*, *and let-7* function as tumor suppressors. Together with Dr. Lin He, I have identified the mir-34 family as direct transcriptional target genes of the tumor suppressor p53 for mediating cell-cycle arrest. Using retroviral expression vectors, I showed that constitutive or conditional expression of miR-34a in murine liver tumor cells resulted in delayed tumor progression, suggesting delivery of miR-34a as a potential therapeutic tool. To study the loss-of function phenotype of miR-34a, I generated knockout animals harboring genetic ablation of miR-34a. I also performed a genome wide miRNA screen and identified candidate oncogenic miRNAs. My thesis work established miR-34a as an essential component of the p53 tumor suppressor network and emphasized the importance of miRNAs in human cancer.

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Abbreviations:

HCC	Hepatocellular Carcinoma
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
FACS	Fluorescence Activated Cell Sorting
GFP	Green Fluorescence protein
H&E	hematoxylin and eosin stain
IF	Immunofluorescence
IHC	Immunohistochemistry
IRES	Internal Ribosome Entry Site
LB	Luria Broth
LPC	Liver progenitor cells (hepatoblasts)
LTR	Long Terminal Repeat
MEF	Mouse Embryonic Fibroblasts
miRNA	micro-RNA
MLP	MSCV LTR Mir30 Puro-IRES-GFP Vector
MLS	MSCV LTR Mir30 SV40-GFP Vector
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA Interference
ROMA	Representational Oligonucleotide Microarray Analysis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PIG	MSCV LTR Puro-IRES-GFP Vector
pSM2	plasmid SHAG-MAGIC 2 vector
PVDF	Polyvinylidene fluoride
RISC	the RNA-induced silencing complex
shRNA	short hairpin RNA
SIN	Self-inactivating
siRNA	small-interfering RNA
TSG	Tumor suppressor gene
CHIP	Chromatin immunoprecipitation

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Chapter 1

Introduction

Cancer arises from genetic lesions that lead to uncontrolled proliferation, cell survival, loss of differentiation and invasive growth (Hanahan and Weinberg 2000). So far, cancer studies have focused on genetic alterations in protein coding genes. It was not until recently that non-coding RNAs, in particular miRNAs, have been showen to play important roles in cancer (Voorhoeve et al, 2006; Johnson et al, 2005; He et al, 2005; Calin, et al, 2005, 2004, 2002; Tagawa, et al, 2005; Kluiver et al, 2005; Eis, et al, 2005). Since then, a number of studies support the idea that miRNAs can be components of oncogene and tumor suppressor networks. Therefore, it's important to explore the potential of miRNAs as new diagnostic indicators and potential therapeutic targets.

1.1 miRNAs biogenesis

First identified as small, non-coding RNAs essential for the timing of larval development in worms, a large number of miRNAs have been and are still being discovered in nearly all metazoans. Most animal miRNAs share common biogenesis and effector machineries (Zamore and Haley, 2005; Bartel, 2004; Ambros, 2004; He and Hannon, 2004; Lee, et al, 2003). Mature miRNAs often range from 20–22 nucleotides in length as a result of two sequential processing reactions. Nascent miRNA transcripts (pri-miRNAs) are first transcribed from the genome, and then processed by two ribonuclease III enzymes, Drosha and Dicer, to generate mature miRNAs. Generally, Drosha cleavage of pri-miRNAs yields ~70nt precursors (pre-miRNA) with stem-loop

structures, and subsequent Dicer cleavage generates mature miRNA duplexes. After maturation, usually, one strand from the miRNA duplex is incorporated into the effector complex, the RNA-induced silencing complex (RISC). RISC recognizes specific target mRNAs through imperfect base-pairing, and down-regulates their expression by post-transcriptional gene silencing (Fig1.1).

1.2 The biological role of miRNAs

MicroRNAs recognize their target genes by binding to their complementary base-pairing sites on the target mRNA. A series of mutational analyses indicated that the most critical interactions between the microRNA and its targets occur within the 5' region of the microRNA (Doench and Sharp, 2004). Therefore, the eight nucleotides at the 5' end of a miRNA are designated as the "seed" sequence, whose complementarity to the target mRNA has been employed to search for candidate targets. In a recent study by Lewis et al., more than 5300 human genes were predicted as conserved miRNA targets, representing 30% of the human genome. However, imprecise base-pairing and complex recognition between the microRNA and its target mRNA have imposed a technical barrier to identifying true miRNA targets. Therefore, computational predictions by themselves are not sufficient, and they've been combined with independent experimental validations to remove noise.

Through functional studies and targets searches, miRNAs are involved in

many aspects of biological pathways. To date, more than 500 miRNAs have been identified from the mammalian genome (Griffiths-Jones, 2004). Many miRNAs play important roles during development. For example, *lin-4* and *let-7,* the founding members of miRNA family*,* regulate larval developmental timing in *C. elegans* (Lee, et al, 1993; Wightman, et al, 1993; Bagga, et al, 2005). In addition, miRNAs can also regulate signaling pathways, the best example of which is the regulation of *notch* signaling by *Drosophila mir-1* (Kwon, et al, 2005) and *C. elegans mir-61* (Yoo, et al, 2005). Finally, miRNAs can regulate proliferation and apoptosis, both of which are important cellular processes directly relevant to tumorigenesis. For example, a *Drosophila* miRNA, *bantam*, regulates expression of a pro-apoptotic BH3-only protein, *hid* (Brennecke, et al, 2003).

1.3 miRNAs and cancer

The first observations linking microRNAs to cancer was that some microRNA genes reside at genomic regions frequently mutated in cancer. For example, *mir-15* and *mir-16* were located at 13q14, a chromosomal locus that is deleted in more than 50% of B cell chronic lymphocytic leukemias (Calin, et al, 2002). Low expression of miR-15 and miR-16 was detected in more than 68% of this cancer type, and tumor-specific mutations in the miR15/16 precursor have also been detected (Calin, et al, 2005). These results indicated that miR15 and miR-16 could be tumor suppressor genes. Another candidate microRNA tumor suppressor is let-7, whose reduced expression is observed in lung cancer.

Decreased expression of let-7 is often associated with poorer survival in patients. In addition, enforced *let-7* expression in the lung cancer cell line A549 reduced colony formation *in vitro* (Takamizawa, et al, 2004).

Several other studies also suggested that microRNAs could be oncogenes. A non-coding RNA, BIC, was initially identified as a viral insertion site in an ALV induced lymphoma. It is now clear now that BIC is the precursor of a miRNA, mir155, whose increased expression has been observed in human cancers, including diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma (Kluiver, et al, 2005; Eis, et al, 2005). Similar amplification of a polycistronic microRNA cluster, *mir-17-92*, which is at chromosome 13q31, was observed in DLBCL, follicular lymphoma, mantle cell lymphoma and lung cancer. Increased expression of the *mir17-92* precursor has been observed in 65% of human lymphoma samples and a smaller percentage of other tumor types as well. More importantly, over-expression of mir17-19b, a truncated cluster of *mir17-92* cooperates with *c-myc* to accelerate B-cell lymphomagenesis by suppressing *c-myc*-induced apoptosis (He, et al, 2005). These findings provided some of the first functional evidence that miRNAs, or in a broader sense, non-coding RNAs, may be components of oncogenic and tumor suppressor pathway.

I will now review the functions of some characterized miRNAs and their involvement in common cancer pathways (Fig 1.3). Examples of miRNA alternations in cancer such as genomic deletion or amplification, epigenetic

silencing is summarized in Table 1.

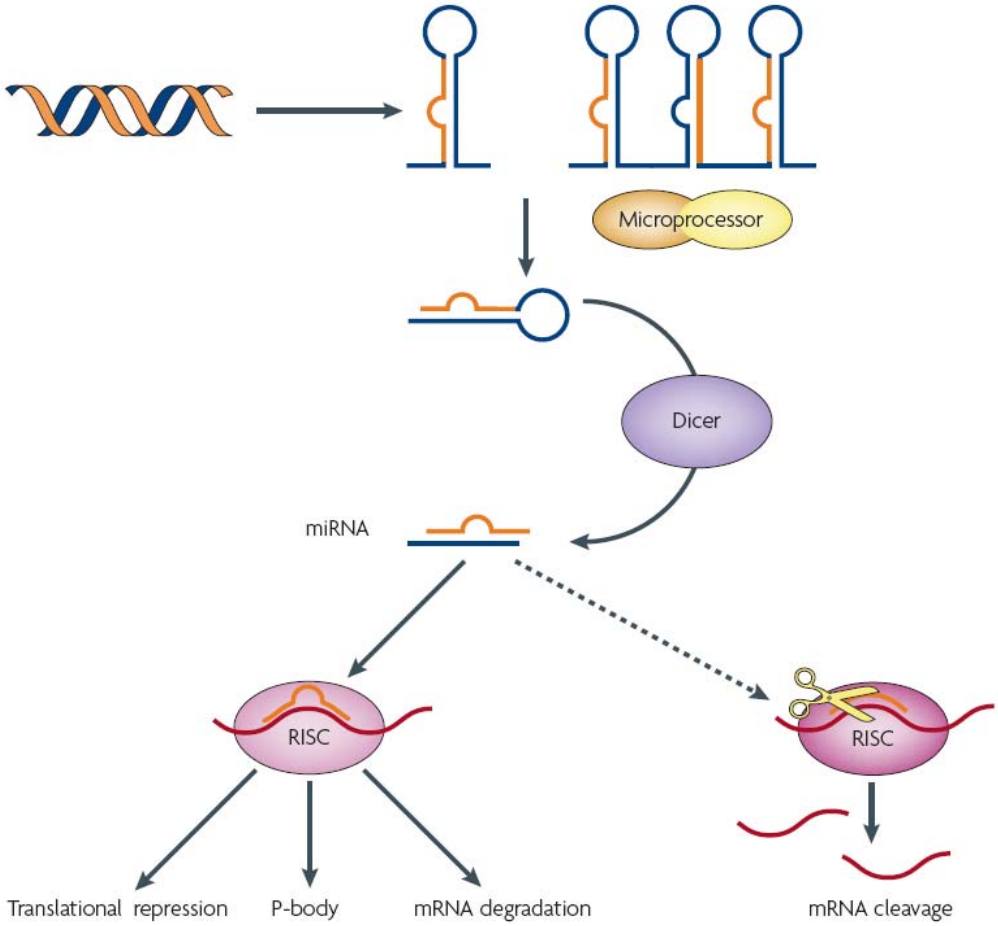

Figure 1.1 Current model of miRNA biogenesis and post-transcriptional silencing. Nascent transcripts of microRNA (miRNA) genes are processed by microprocessor into a stem-loop precursor, which is further processed by Dicer into a mature miRNA duplex, which often displays imperfect base-pairing. One strand of the miRNA duplex gets incorporated into the effector complex RISC (RNA-induced silencing complex), which recognizes specific targets through imperfect base-pairing and induces post-transcriptional gene silencing. Several mechanisms have been proposed for this mode of regulation: miRNAs can induce the repression of translation initiation, mark target mRNAs for degradation by deadenylation, or sequester targets into the cytoplasmic P-body. Apapted from He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. Nat Rev Cancer. 2007 7(11):819-22.

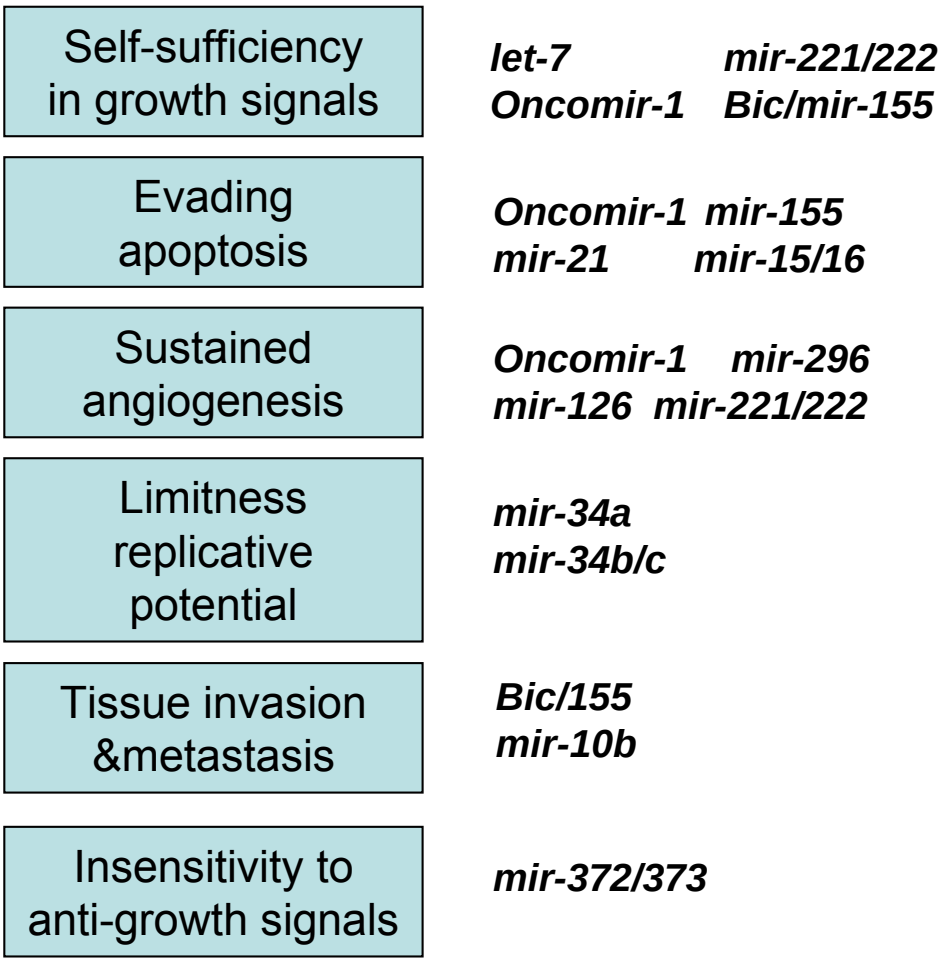

Figure 1.2 microRNA is involved in major cancer pathways. Oncomir-1 refers to 17-92b cluster. Part of the figure is adapted from (Hanahan and Weinberg, 2000).

Table 1.1 miRNAs in cancer

		Alteration in cancer	Biological effect	Target gene	Induced by	Knockout mice
Onco-genes	*mir17-92*	Amplified	Anti-apoptosis	BMI1, E2F1	Myc	premature death of B cells
	mir-155	Amplified		AID		defective B and T cells
	mir21	Over-exprss	Anti-apoptosis	PTEN PDCD4		
	mir-372/mir-373	Amplified		LATS2, CD44		
TSG	mir-15	Deletion		BCL2, Cyclin D1 WNT3A		
	let-7	Reduced Deletion	stem cell self renewal	RAS HMGA2		N/A
	mir-34a	Deletion	Cell cycle arrest, apotosis	CDK4, CyclinE2, MET, RB	p53	N/A
Angio-genesis	*mir17-92*			TSP1 CTGF		
	miR-221 miR-222			c-Kit		
	miR-126			SPRED1, PIK3R2		
	miR-296			HGS	VEGF	
Meta-stasis	miR-335	Reduced	Anti-invasion	SOX4		
	Mir-10b	High	Invasion	HOX10D	TWIST	

1.3.1 miRNAs as oncogenes

MicroRNAs that are amplified or overexpressed in cancer could act as oncogenes, and a number of putative oncogenic miRNAs have been proposed (reviewed in Medina and Slack, 2008, see also Table 1.1).

miR-17-92

miR-17-92 cluster includes six miRNAs that are processed from a single primary transcript (reviewed in Mendell, 2008). miR-17-92 was linked to cancer because its chromosomal region is frequently amplified in human B cell lymphomas (Ota et al., 2004) and a variety of other human cancers such as hepatocellular carcinoma. He et al. (2005) demonstrated that a truncated version of the cluster miR-17-19b cooperates with Eu-Myc to promote tumorigenesis in a mouse model of B cell lymphoma. They demonstrated that miR-17-19b attenuates Myc induced apoptosis in B cells.

Interestingly, miR-17-92 is transcriptionally activated by the c-Myc oncogene. The transcription factor E2F1 is an additional target of c-Myc that promotes cell cycle progression. Two miRNAs in this cluster, miR-17-5p and miR-20a, were able to negatively regulate E2F1 expression (O'Donnell et al, 2005). Another candidate *miR-17~92* target gene is the pro-apoptotic gene *Bim* (Ventura et al., 2008; Xiao et al., 2008). *Bim* encodes a Bcl-2 homology domain 3 (BH3) containing protein which interacts with BCL2 and MCL1 to act as an apoptotic activator (Hemann et al, 2005). It is a potent tumor suppressor gene in the *Eμ-Myc* model of B cell lymphoma (Egle et al., 2004). The 3′ UTR

of *Bim* contains multiple binding sites for miRNAs encoded by *miR-17~92. Bim* expression is increased in *miR-17~92* null pre-B cells and reduced in B cells from mice overexpressing *miR-17~92* (Ventura et al., 2008*).*

Transgenic mice overexpressing *miR-17~92* cluster in lymphocytes develop a lymphoproliferative disorder and autoimmunity (Xiao et al., 2008). In contrast, mice with a homozygous deletion of the *miR-17~92* locus exhibit premature death of B cells at the pro-B/ pre-B stage (Ventura et al., 2008). These data suggest miR-17-92 is relevant for survival and proliferation of B cells as well as other normal tissues (Ventura et al., 2008).

miR-155

Another notable family of oncogenic miRNAs is miR-155. *miR-155* is upregulated in several hematopoietic malignancies and solid tumors such as breast, lung, and pancreatic cancers (reviewed in Kluiver et al., 2006). The gene encoding BIC RNA (the primary transcript of *miR-155)* is a common proviral insertion site in lymphomas induced by the avian leukosis virus. The *BIC* RNA cooperates with *Myc* in inducing hematopoietic tumors. BIC can form extensive secondary structures but does not encode a protein (Tam et al., 1997). Later it was found that BIC RNA has a 145 base pair stem loop that is the precursor of *miR-155*. Mouse models with gain- and loss-of-function alleles of *miR-155* provided valuable insights into its biological function. Ectopic expression of *miR-155* in the bone marrow of mice has been reported to induce B cell leukemia (Costinean et al., 2006). *miR-155*-deficient mice have

defective B and T cells (Rodriguez et al., 2007; Thai et al.,2007; Vigorito et al., 2007). The targets of *miR-155* include the gene encoding activation-induced cytidine deaminase (AID), which allows immunoglobulin diversification by promoting somatic hypermutation and class-switch recombination in B cells.

miR-21

Chan et al (2005) demonstrated that miR-21 is upregulated in glioblastoma. An independent study that used microarray analysis to compare the expression of 245 miRNAs in glioblastoma versus normal tissues also identified miR-21 levels as being increased in glioblastoma tumours (Ciafre et al, 2005). miR-21 expression is also increased in human breast cancer samples (Iorio et al, 2005) and hepatocellular carcinoma (Meng et al, 2007). Antisense studies of miR-21 in glioblastoma cell lines showed that this miRNA controls cell growth by inhibiting apoptosis but does not affect cell proliferation, which implies an oncogenic role for this miRNA. Recent studies suggest that miR-21 down-regulates PTEN (Meng et al, 2007) and the proapoptotic protein PDCD4 (Programmed Cell Death 4) (Chen et al, 2008).

miR-372/373

miR-372/373 was identified in a genetic screen for miRNAs that cooperate with oncogenic Ras to transform primary human fibroblasts (Voorhoeve et al., 2006). These miRNAs are potential oncogenes in testicular germ cell tumors (Voorhoeve et al., 2006). Their oncogenic potential is partly due to the regulation of Lats2 tumor suppressor.

Of note, the *miR-373* miRNA was also identified in a functional screen for promoting cell migration *in vitro* (Huang et al., 2008), and its prometastatic potential has been validated in tumor transplantation experiments using breast cancer cells in which it appears to regulate CD44.

1.3.2 miRNAs as tumor suppressors

Several miRNAs have been implicated as tumor suppressors based on their physical deletion or reduced expression in human cancer. Functional studies of these miRNAs indicate that they can limit cancer cell growth or induce apoptosis (reviewed in Medina and Slack, 2008).

let-7

let-7 family is the most studied tumor suppressor miRNAs (reviewed in Roush and Slack, 2008). The human genome contains 11 *let-7* family members, organized in eight different loci. Reduced expression of members of the *let-7* family is frequently observed in human lung cancers, where they correlate with poor prognosis (Yanaihara et al., 2006). In addition, various *let-7* genes are located at chromosomal deletions in a variety of human cancers. Functionally, *let-7* represses the Ras family of oncogenes (Johnson et al., 2005), *HMGA2* (Lee and Dutta, 2007; Mayr et al., 2007) and c-Myc (Sampson et al., 2007). Finally, overexpression of *let-7* miRNAs by lentivirus can suppress tumor development in mouse models of lung cancer (Kumar et al., 2008). Mouse knockout studies have not been reported for any *let-7* family members. In a recent study, let-7 was shown to regulate self renewal and

tumorigenicity of breast tancer initiating cells (Yu et al, 2007). Ectopic expression of let-7 reduced proliferation, mammosphere formation, and tumor formation whereas antagonizing let-7 enhanced self renewal of non tumor initiating cells. Let-7 reduced H-RAS and HMGA2. Silencing H-RAS leads to reduced self renewal but not differentiation, while silencing HMGA2 enhanced differentiation but did not reduce self renewal. Therefore let-7 was suggested to regulate multiple stem cell properties by silencing more than one target gene.

miR-15a~16-1

The *miR-15a~16-1* cluster of miRNAs map to chromosome 13q14, a region that is deleted in the majority of CLLs (chronic lymphocytic leukemia) and in a subset of mantle cell lymphomas and prostate cancers (Calin et al., 2002). *miR-15a~16-1* is located in the minimally deleted region in CLL (Calin et al., 2002), and a germline point mutation (a single base change) immediately downstream of the *pre-miR-16-1* sequence has been observed in a few CLL patients (Calin et al., 2005). In addition to in B cells, the tumor suppressor activity of *miR-15a~16-1* is relevant in prostate cancer. Inhibition of *miR-15a* and *miR-16* activity leads to hyperplasia of the prostate in mice and promotes survival, proliferation and invasion of primary prostate cells *in vitro* (Bonci et al., 2008). Moreover, intra-tumoral delivery *of miR-15a* and *miR-16-1* leads to regression of prostate tumor xenografts, implicating the therapeutic potential of this miRNA cluster. The candidate targets of these two miRNAs consists

known oncogenes such as BCL2, cyclin D1, and WNT3A. Downregulation of miR-15a and *miR-16-1* is proposed to result in increased expression of these oncogenes..

miR34a/b/c

We and other labs have explored miR34 family as p53 regulated miRNAs (He et al., 2007 and references therein). This family consists of three highly related miRNAs expressed from two separate loci: *miR-34a* from chromosome 1p36 and the *miR-34b*/*miR-34c* cluster from chromosome 11q23. Reduced expression of *miR-34* has been reported in breast and non-small cell lung cancer cell lines. Our detailed studies of miR34 will be presented below.

1.3.3 miRNA and angiogenesis

Neovasculature is an essential hallmark of tumor initiation and progression. To date, several miRNAs have been implicated to play roles in regulating angiogenesis.

miR-17-92, a Myc induced miRNA cluster, was found to augment tumor angiogenesis (Dews et al. 2006). Kras-transformed mouse colonocytes lacking p53 formed indolent, poorly vascularized tumors. miR-17-92 microRNA cluster targets Tsp1 and CTGF, which are anti-angiogenic proteins and both are upregulated by K-Ras and Myc. Transduction of Kras cells with a miR-17-92 reduced Tsp1 and CTGF levels and allowed formation of larger, better-vasculatured tumors, suggesting miR-17-92 has non–cell-autonomous role in tumorigenesis by suppressing anti-antigenic signals.

Another study performed large-scale analysis of miRNA expression in human umbilical vein endothelial cells (HUVECs) and identified 15 highly expressed miRNAs targeting angiogenic factors. In particular, miR-221 and miR-222 were found to affect c-Kit expression (Poliseno et al. 2006)

Studies in endothelial cells also found microRNAs that were enriched in endothelial cells and in developing mouse embryos. miR-126 regulated the response of endothelial cells to VEGF. Knockdown of miR-126 resulted in loss of vascular integrity and hemorrhage in zebrafish embryos. miR-126 directly represses negative regulators of the VEGF pathway, including the Sprouty-related protein SPRED1 and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-beta) (Fish JE et al. 2008).

In a recent study, miR-296 was shown to increase VEGFR2 expression on endothelial cells (Würdinger T et al. 2008). miR-296 downregulates the expression of hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) and thereby inhibiting HGS-mediated degradation of the growth factor receptors VEGFR2 and PDGFRb. miR-296 is highly expressed in primary tumor endothelial cells isolated from human brain tumors compared to normal brain endothelial cells. Angiogenic growth factors or co-culturing with glioma cells elevate the level of miR-296 in human brain endothelial cells. Functionally, antagomirs targeting miR-296 were able to reduce angiogenesis in tumor xenografts *in vivo*, suggesting miRNAs involved in angiogenesis can be a therapeutic target in cancer.

1.3.4 miRNA and tissue invasion & metastasis

miRNAs have also been implicated in affecting tumor invasion and metastasis by modulating cell adhesion, migration, and invasion (Ma et al, 2007).

Ma and Weinberg showed that miR-10b is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion (Ma et al, 2007). Ectopic expression of miR-10b in non-metastatic breast tumours initiates robust invasion and metastasis. miR-10b is a direct transcriptional target of is induced by the transcription factor Twist, a known inducer of the epithelial-to-mesenchymal transition (EMT) and metastatic progression. miR-10b inhibits the anti-metastatic gene HOXD10, resulting in increased expression of the pro-metastatic gene RHOC.

Several miRNAs have been revealed as inhibitors of metastasis. *miR-126*, *miR-206*, and *miR-335* were specifically lost as human breast cancer cells develop metastatic potential (Tavazoie et al., 2008). Ecotopic expression miR-126 reduces overall tumour growth and proliferation, whereas miR-335 and miR-206 inhibits metastatic cell invasion and morphology. miR-335 regulates the progenitor cell transcription factor SOX4. Reduced expression of *miR-126* and *miR-335 is* correlated with poor metastasis-free survival of breast cancer patients.

Recently, members of the *miR-200* family of miRNAs were found underexpressed in advanced breast cancers and capable of inhibiting cell

migration and metastasis. *miR-200* family of miRNAs target the ZEB transcription factors, known inducers of the EMT, and thus reduce migration and invasiveness (Gregory et al., 2008; Park et al., 2008).

1.3.5 Global dysregulation of miRNA

In addition to roles for individual miRNAs in cancer, global alterations in miRNA expression patterns were observed in human cancer (Lu et al., 2005). miRNA expression profiling has demonstrated that most (although not all) miRNAs are underexpressed in tumor tissues compared to normal tissues (Lu et al., 2005). There are two possibilities to explain this global down-regulation of miRNAs. First, undifferentiated cells usually have a low expression of miRNAs. The reduced miRNA levels may reflect the less differentiated states of the tumor cells. For example, a significant increase in miRNA levels is observed upon induction of differentiation of the cancer cell line HL60 (Lu et al., 2005), consistent with the ability of miRNAs to reinforce transcriptional programs and to help maintain the differentiated state. Second, oncogene c-Myc, which is frequently overexpressed in cancer, is known to transcriptionally silence a wide variety of miRNAs such as Let7 (Chang et al., 2008), suggesting a potential mechanism for the global downregulation of miRNAs in transformed cells. Nevertheless, the fact that many miRNAs are bona fide tumor suppressors explains the advantage of losing these miRNAs in cancer.

Consistent with this, the suppression of key components of the miRNA

biogenesis machinery such as Dicer, Drosha or DGCR8 has been reported to promote transformation both *in vitro* and *in vivo* by reducing tumor suppressive miRNA such as miR-16 and Let-7 and increasing expression of their target oncogenes such as K-Ras and c-Myc (Kumar et al, 2007). Moreover, conditional Dicer knockout enhances the *in vivo* tumor burden in a K-Ras^{G12D} mouse model of lung cancer (Kumar et al, 2007).

miRNA profiles have been valued as diagnostic and prognostic markers of disease. For example, it is sometimes impossible to determine the tissue of origin of a metastatic tumor in patients with unknown primary tumors. Because many miRNAs display exquisite tissue specificity, miRNA profiling of these lesions might prove useful. The initial findings are encouraging, as it appears that miRNA-based classification is more efficient at identifying the tissue of origin of poorly differentiated cancers than is mRNA profiling (Lu et al., 2005; Rosenfeld et al., 2008). MicroRNA profiling of human cancer might guide the choice of the best treatment strategy by providing prognostic information. Indeed, in the two most common forms of non-small cell lung cancers (adenocarcinomas and squamous cell carcinomas), high expression of *miR-155* and low expression of *let-7* correlate with poor prognosis (Yanaihara et al., 2006). Similarly, in colon cancers, elevated expression of *miR-21* is associated with poor survival (Schetter et al., 2008), whereas in chronic lymphocytic leukemias an miRNA "signature" composed of 13 miRNAs is associated with disease progression (Calin et al., 2005). Larger scale studies

will be required to validate the usefulness of miRNA profiling in a clinical setting.

During my PhD work, I studied the biological functions of *mir-34* miRNAs in the p53 tumor suppressor network, focusing on *mir-34*'s targets, the potential role of *mir-34* as a tumor suppressor, and its loss-of function studies. Using microRNA expression profiles in MEFs from different genetic backgrounds, I identified a microRNA family, including *mir-34a, b* and *c*, as part of the p53 tumor suppressor network. Ectopic expression of *mir-34s* induced cell cycle arrest in both primary and tumor-derived cell lines by repressing cell cycle genes such as MET, CDK4 and CYCLIN E2. Furthermore, I used tetracycline controlled expression of *mir-34* in a mouse hepatocellular carcinoma (HCC) tumor model to investigate whether *mir-34* over-expression can inhibit tumorigenesis and/or tumor progression. I plan to use the HCC model to evaluate *mir-34*'s potential as a therapeutically method. Finally, to understand the endogenous function of mir-34, I generated constitutive *mir-34* knock-out alleles in mice to investigate *mir-34's* function in normal development and in cancer formation. With this mouse, we can further investigate the role of loss-of *mir-34* in tumorigenesis using different tumor models and its cooperation with distinct oncogenic lesions.

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Chapter 2

Identification of miR-34 as p53 regulated miRNA

2.1 Introduction:

p53 responds to DNA damage or deregulation of mitogenic oncogenes through the induction of cell cycle checkpoints, apoptosis, or cellular senescence (Levine et al, 2006). Mutations in p53 are often associated with aggressive tumor behavior and poor patient prognosis. The p53 tumor suppressor network has been intensively studied; however, genetic analyses long hinted at the existence of components that remained elusive. For example, although p53 is clearly a transcriptional activator, numerous reports indicated that p53 also represses the expression of specific genes either directly or indirectly. The manner in which this occurred was obscure, with both transcriptional and posttranscriptional suppression as possible mechanisms. In the latter case, the discovery of extensive networks of microRNAs (miRNAs), which act through the RNA interference pathway (RNAi), offered the possibility that p53-mediated control of miRNA expression could allow it to act indirectly to repress target gene expression at the posttranscriptional level. Increasing evidence has suggested that miRNAs are components of oncogene and tumor suppressor pathways.

A global decrease in microRNA (miRNA) levels is often observed in human cancers (Liu et al, 2005; Thomson et al, 2006), indicating that small RNAs may have an intrinsic function in tumour suppression. To identify miRNA components of tumour suppressor pathways, we compared miRNA expression profiles of wild-type and p53-deficient cells. We and others identified a family

of miRNAs, miR-34a–c, whose expression reflected p53 status (Bommer et al, 2007; Chang et al, 2007; He et al, 2007; Raver-Shapira et al, 2007; Terasov et al, 2007).

Genes encoding miRNAs in the miR-34 family are direct transcriptional targets of p53, whose induction by DNA damage and oncogenic stress depends on p53 both *in vitro* and *in vivo*. Ectopic expression of miR-34 induces cell cycle arrest in both primary and tumour-derived cell lines, which is consistent with the observed ability of miR-34 to downregulate a programme of genes promoting cell cycle progression. The p53 network suppresses tumour formation through the coordinated activation of multiple transcriptional targets, and miR-34 may act in concert with other effectors to inhibit inappropriate cell proliferation.

2.2 Results

2.2.1 Expression of miR-34 is correlated with p53 status in MEF

The p53 tumour suppressor lies at a nexus of cellular pathways that sense DNA damage, cellular stress and improper mitogenic stimulation (Fig 2.1, Levine et al, 2003). p53 integrates such signals and, in response, induces growth arrest, promotes apoptosis, blocks angiogenesis, or mediates DNA repair in a context-dependent manner (Ko et al, 1996). The importance of p53 in preventing tumour formation is indicated by the presence of mutations in the p53 pathway in nearly all cancers (Hollstein et al, 1991). Although p53 is most studied as a transcriptional activator, several reports have suggested that p53

represses the expression of specific genes (Spurgers et al, 2006). Studies of p53-mediated repression have shown that both genes that modulate apoptotic responses and genes that promote cell cycle progression can repressed by p53 (Bartel et al, 2004).

miRNAs enforce post-transcriptional silencing through the RNA interference pathway (Chen et al, 2005). p53-mediated induction of one or more miRNAs could therefore allow it to exert negative effects on gene expression indirectly. To explore the possibility that miRNAs might constitute part of the p53 tumour suppressor network, we examined miRNA expression profiles in wild-type and p53-deficient mouse embryonic fibroblasts (MEFs). Using the semi-quantitative reverse transcription-polymerase chain reaction (QRT-PCR) (Dickins et al, 2005), we measured the expression of a panel of 145 mouse miRNAs in wild-type or $p53^{-/-}$ MEFs that ectopically express various oncogenes (Fig. 2.2). miRNA expression was strongly affected by genetic alterations, because unsupervised clustering grouped MEFs according to their genotype (Fig 2.2). The expression of three miRNAs, miR-34a, miR-34b and miR-34c, was precisely correlated with p53 status (Fig. 2.3a). This raised the possibility that mir-34 genes might be regulated by p53. miR-34s belong to an evolutionarily conserved miRNA family, with single, recognizable orthologues in several invertebrate species (Fig 2.4). According to predicted gene structures, human miR-34a is located within exon 2 of its primary transcript, whereas miR-34b and miR-34c are located within intron 1

and exon 2, respectively, of the same primary transcript (Fig. 2.3b). Aside from the miRNAs themselves, the only other region of significant sequence conservation in mir-34 genes lies in their putative promoter regions (Fig. 2.3b).

2.2.2 Genes encoding miR-34 are direct targets of p53

Because expression of miR-34s was correlated with p53 status, we asked whether miR-34s were directly regulated by p53. In MEFs expressing a tetracycline-regulated p53 short hairpin RNA (shRNA), endogenous p53 activity gradually increased over 6–8 days after repression of the shRNA6. Reactivation of p53 led to significant induction of both primary (pri-) mir-34 transcripts and mature miR-34s (Fig. 2.5a and Fig 2.6). The kinetics and magnitude of induction were comparable to those of the canonical p53 target, p21 (Fig 2.6). Silencing of p53 in human tumour cell lines led to a roughly fourfold decrease in miR-34a levels.

Multiple physiological stresses can induce the accumulation of p53 protein and activate p53-mediated transcriptional programmes. DNA damage mediates p53 activation mainly through posttranslational modification (Fei et al, 2003; Giaccia et al, 1998). In a p53-dependent manner, both pri-mir-34s and mature miR-34s were induced by ionizing radiation in a variety of mouse tissues, including spleen, colon, thymus and kidney (Fig. 2.5b and data not shown). miR-34s were also induced after DNA damage in wild-type but not p53-null MEFs, with an amplitude and kinetics that closely resembled those of p21. Similarly, in TOV21G cells, a human ovarian cancer cell line, members of

the miR-34 family had one of the highest levels of induction after DNA damage among the miRNAs examined (Fig. 2.5c). Oncogene activation often induces the p53 pathway through induction of ARF (alternative reading frame) (Sherr et al, 2000). Such a response was evident in a mouse hepatocellular carcinoma model, in which tumorigenesis driven by activated Ras required continuous suppression of p53 by an inducible shRNA (Xue et al, 2007). Repression of the shRNA allowed Ras-mediated activation of endogenous p53 and resulted in the senescence of tumour cells. Under these circumstances, all three miR-34s were strongly induced, supporting their regulation by p53 *in vivo* (Fig. 2.5d). Similarly, oncogenic stress can induce miR-34a in cultured primary human fibroblasts (data not shown). One of the few conserved regions within the genes encoding miR-34 family members contains a match to the canonical p53 binding site (see Fig. 2.3b). To test p53 binding to these sites, we performed chromatin immunoprecipitation (ChIP). In wild-type MEFs, in which p53 activity was induced by DNA damage, regions of the genes encoding both miR-34a and miR-34b/c that contained putative p53 binding sites were enriched in p53 immunoprecipitates. This enrichment was absent from similarly treated p53-null MEFs (Fig. 2.5e). These same sites were previously detected in genome-wide chromatin occupancy experiments with p53, though their significance for regulation of miR-34s was not noted (Wei et al, 2006). To examine the potential of mir-34 promoters to confer p53 regulation, we inserted fragments of the mouse or human genes encoding miR-34a or

miR-34b/c upstream of a luciferase-coding region. Co-transfection of these reporters with a p53 expression vector robustly stimulated luciferase expression (Fig. 2.5f, and data not shown). Mutation of p53 binding sites in these reporters negated this induction. These findings indicate that miR-34a and miR-34b/c are direct transcriptional targets of p53.

2.2.3 miR-34 family miRNAs mediate growth arrest

Two major endpoints of p53 activation are apoptosis and growth arrest (either cell cycle arrest or senescence). The ectopic expression of either mir-34a or mir-34b/c in IMR90 cells led to substantial inhibition of growth (Fig. 2.7a). This was attributable to effects on cell proliferation: the fraction of S-phase cells decreased, and the fraction of cells in G1 and G2 increased (Fig. 2.7b). We also noted distinctive morphological alterations characteristic of cellular senescence (Fig. 2.7c), and about 60% of infected cells stained positively for a senescence marker, SA-b-Gal, at 6 days after selection (Fig. 2.7d).

Importantly, all of these effects were seen with mir-34 expression levels similar to those achieved after p53-mediated induction. Transfection of miR-34 miRNAs, but not that of miR-34s containing seed mutations, also led to G1 arrest in immortalized mouse cells and in human tumour cell lines including NIH-3T3, HCT-116, A549 and TOV21G (data not shown). These studies indicate that arrest can be induced independently of the integrity of major tumour suppressor pathways, at least in some cell lines (Fig. 2.7, and data not

shown). Ectopic delivery of miR-34a also sensitized MEFs to apoptosis in response to genotoxic stress, although the effects were not as pronounced as the growth arrest induced by this miRNA (data not shown).

2.2.4 miR-34 regulates cell cycle and DNA damage response genes

miRNAs often decrease the mRNA levels of direct regulatory targets (Lim et al, 2005). After transfection of miR-34a, b or c into a panel of four tumour cell lines, a cluster of genes was specifically downregulated at 24 h after transfection (Fig 2.8), with some genes showing significant repression as early as 10 h after transfection. These genes were highly enriched for transcripts with 39 untranslated regions (UTRs) containing complements to miR-34 seed hexamers. On the basis of functional annotation, genes involved in control of the cell cycle were strongly overrepresented among this set (Fig. 2.8). A selection of candidate targets, including cyclin E2 (CCNE2), cyclin dependent kinase 4 (CDK4) and the hepatocyte growth factor receptor (MET) (Lewis et al, 2005), were validated by western blotting (Fig 2.9a). On transfection of miR-34a into A549 and HCT116 cells, we observed the expected twofold to fourfold decrease in each target examined. To test whether regulation was direct, we fused the 39 UTRs of these selected targets to luciferase. Co-transfection with miR-34a but not miR-124a specifically decreased luciferase levels from each reporter (Fig. 2.9b). Mutations in seed complementary sites fully rescued repression for both CDK4 and MET. For cyclin E2, mutation of the single best seed complementary site had only a

partial effect (Fig. 2.9b), indicating either the presence of other relevant seed complements or a combination of both direct and indirect effects of miR-34. Silencing of these selected miR-34 targets by using siRNAs led to a substantial arrest in G1 (Fig. 2.9c), partly phenocopying activation of their upstream regulator. Ectopic miR-34 delivery caused a decrease in levels of phosphorylated retinoblastoma gene product (Rb), consistent with lowered activity of both CDK4 and CCNE2 complexes (Fig. 2.9a). Repression of CDK4 and CCNE2 has previously been noted after p53 activation in PC3 cells. Our results indicate the possibility that p53 might repress these genes indirectly by the induction of miR-34. We also noted a significant overlap between miR-34-regulated genes and those whose expression is altered after DNA damage. This was seen both for genes that increased after either miR-34 delivery or DNA damage and for those that decreased in response to either treatment. Although strong seed enrichment was seen in the mutually downregulated set, seed enrichment was not seen in the mutually upregulated set, indicating that such increases in expression might be secondary effects of miR-34.

Activation of p53 leads to the coordinated induction of multiple downstream effectors, many of which act in a partly or fully redundant manner. A classic example is p53-dependent apoptosis, which depends not only on the induction of bax (Miyashita et al, 1995) but also on puma and noxa (Villunger et al, 2003). For p53-mediated growth arrest, induction of the CDK inhibitor

p21 is clearly important. However, p21 loss does not completely negate the ability of p53 to halt proliferation (Brugarolas et al, 1995; Deng et al, 1995). This demonstrates the existence of redundant or cooperating pathways that contribute to p53-mediated arrest in G1. Recent studies have identified several p53 targets, including Gadd45a (for growth arrest and DNA damage-inducible), 14-3-3 and Reprimo, which have been proposed to collaborate with p21, primarily to trigger arrest in G2 in specific cell types. At least in some contexts, miR-34s can exert their growth inhibitory effects in the absence of p21, because HCT-116 cells lacking p21 are susceptible to miR-34. This is especially important because reports have implicated p21 in p53-mediated repression (Lohr et al, 2003).

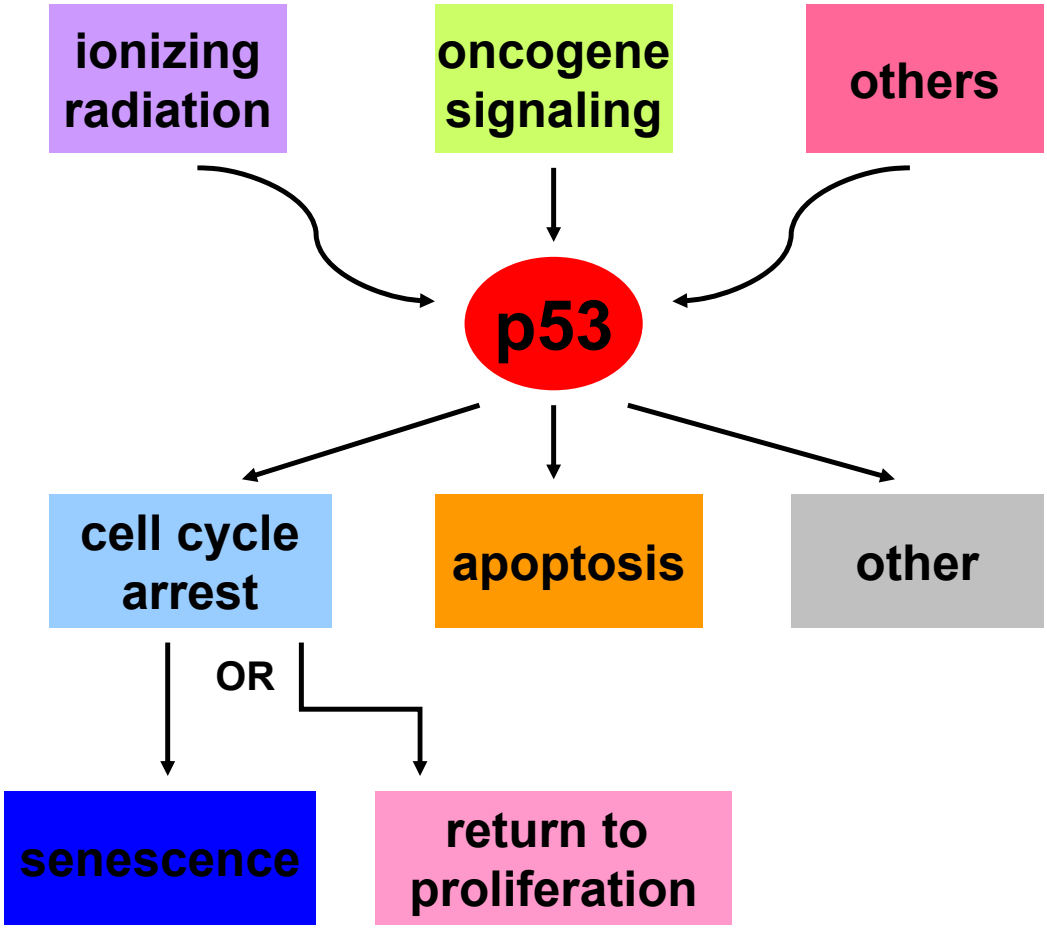
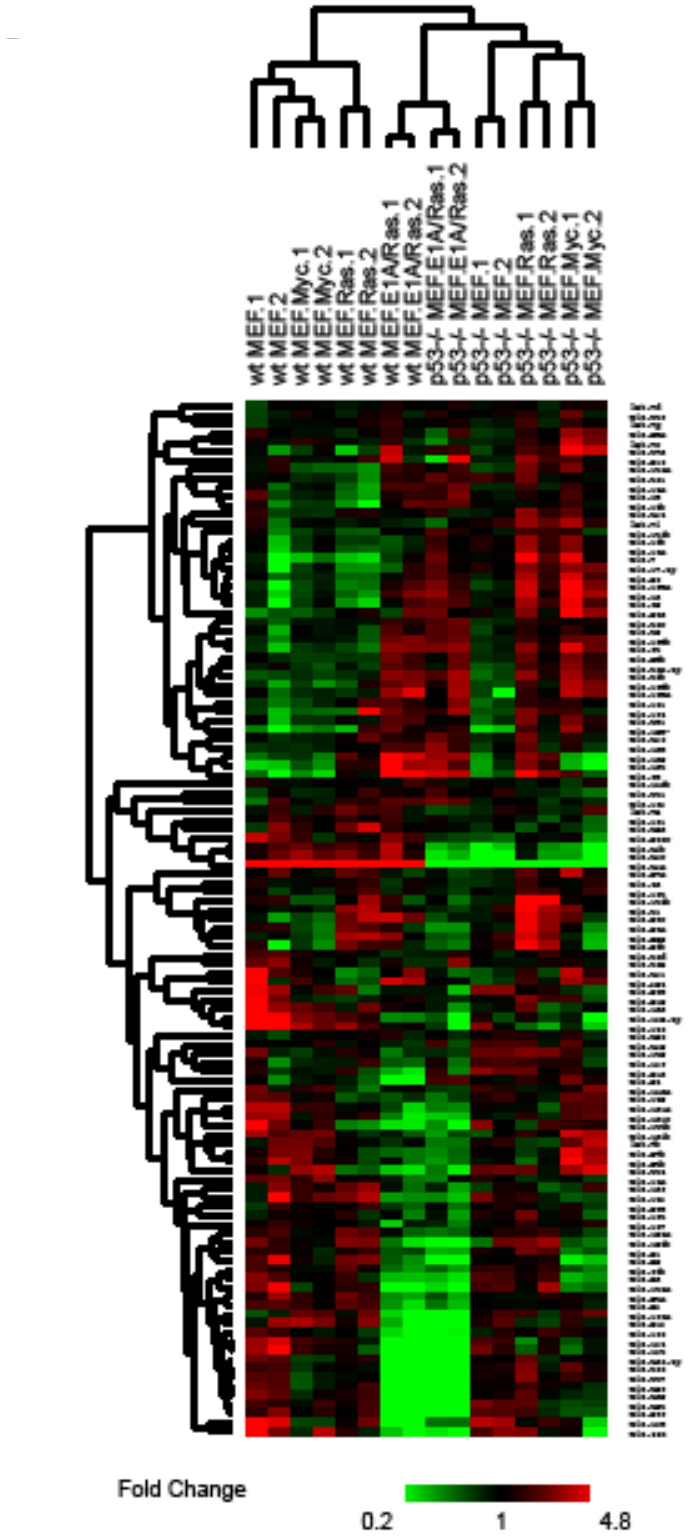


Figure 2.1 Model of p53 network. P53 responds to many upstream stress signals including irradiation and oncogene activation to activate downstream effector pathways such as apoptosis and senescence.



F gure 2.2 |miRNA profiles of engineered MEFs. The full heatmap for the unsupervised clustering of MEF lines, with the indicated genotypes,

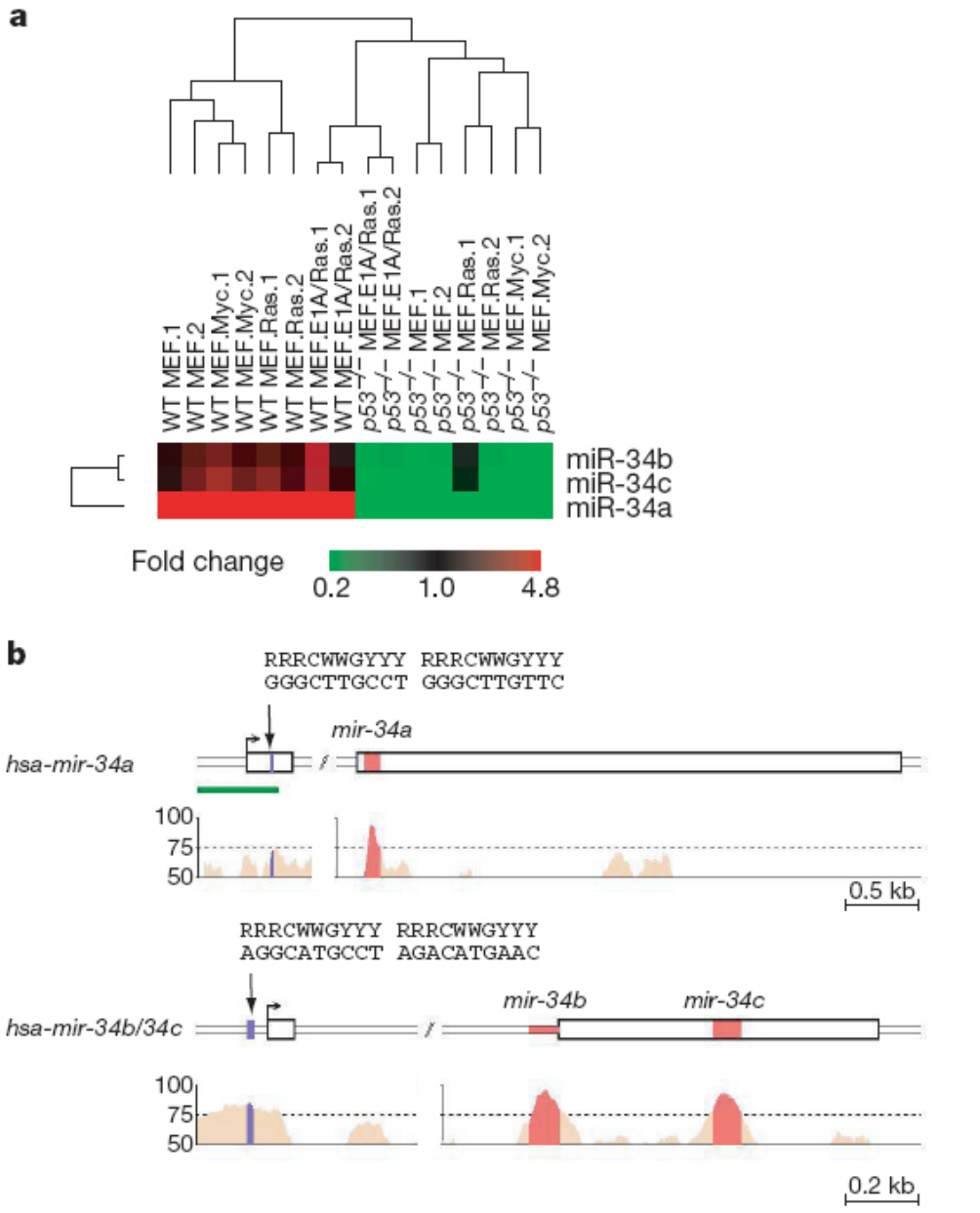


Figure 2.3 | Expression of miR-34 is correlated with p53 status in MEFs. a, An unsupervised hierarchical clustering based on miRNA expression profiles in wild-type and p53-/- MEFs with the indicated additional genetic alteration. Two independently constructed cell lines (.1 and .2) were analysed in each case. The complete heat map (linear scale) is presented in Supplementary Fig. S1. b, Predicted gene structures for human mir-34a and mir-34b/c were generated by combining information from expressed sequence tag databases, CAGE databases and 59 rapid amplification of cDNA ends. Sequence conservation between human, mouse and rat are represented as the percentage of conservation in the Vista analysis shown in the lower panel. The promoter regions of mir-34a and mir-34b/c each contain a palindromic sequence (shown in blue) that matches the canonical p53 binding site. The green bar indicates a CpG island. kb, kilobase.obtained for each size class. external GFP-tumor imaging (top panel) or direct imaging of the respective explanted tumor bearing livers (bottom panel).

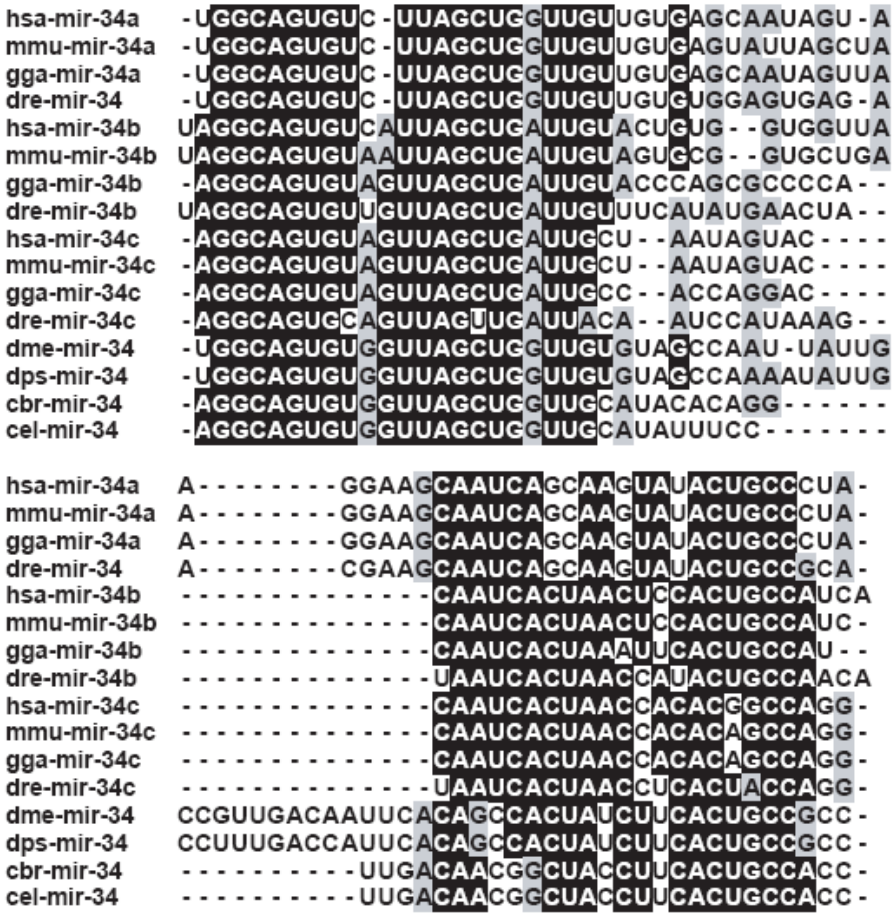


Figure 2.4 Conservation of miR-34 sequences. miR-34 represents a family of evolutionarily conserved miRNAs, with single conserved homologues in invertebrates, such as flies and worms.

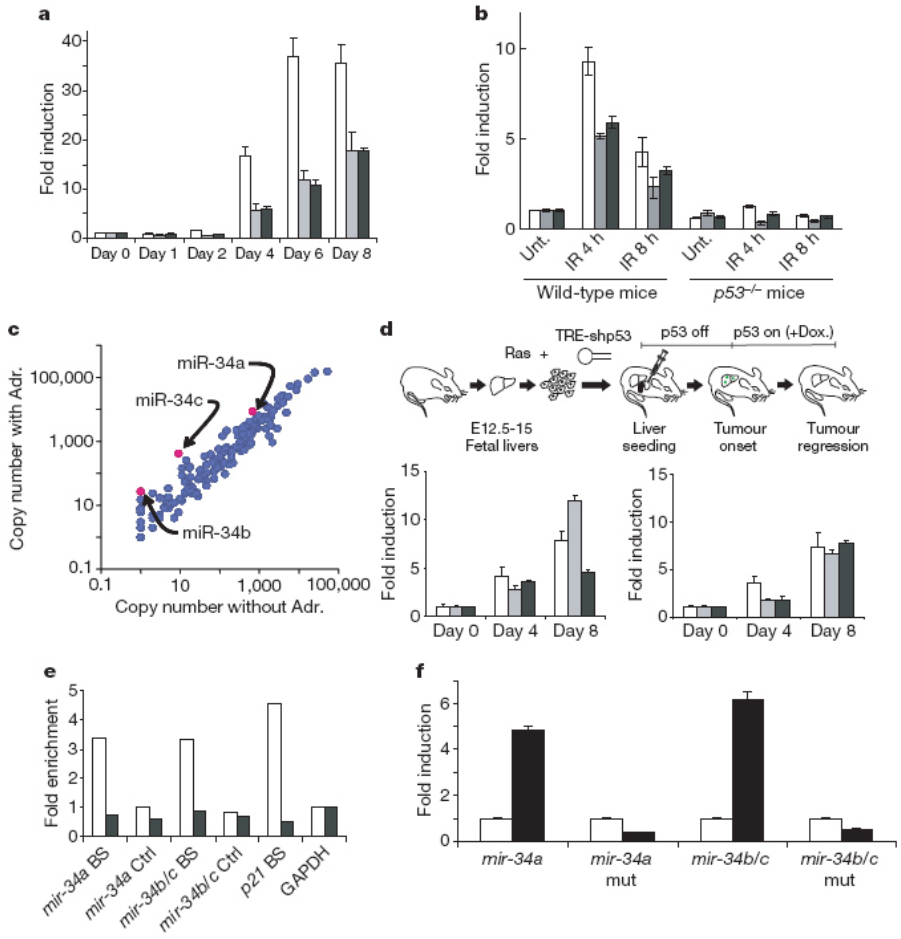


Figure 2.5 Genes encoding miR-34 are direct targets of p53. a, miR-34 levels were measured in MEFs expressing a tetracycline-repressible p53 shRNA6 at the indicated times after the addition of doxycycline. White columns, mature miR-34a; grey columns, mature miR-34b; black columns, mature miR-34c. **b,** Wild-type and p53-/- animals were subjected to 6 Gy of ionizing radiation (IR), and miR-34 levels (identified as in a) were measured in spleens by Taqman assays both before and at the indicated times after irradiation. Unt., unirradiated. **c,** A group of 191 miRNAs and selected miRNA* sequences were quantified by QRT-PCR in TOV21G cells before and after treatment with 0.1 mgml21 adriamycin (Adr.). **d**, Hepatocellular carcinomas were produced by combined expression of activated Ras and a conditional p53 shRNA. p53 suppression was relieved by treatment with doxycycline (Dox.). Tumours were harvested at the indicated times during treatment with doxycycline. Left: white columns, pri-mir-34a; grey columns, pri-mir-34b/34c; black columns, mp21. Right: column colours as in a. **e,** ChIPs were performed with p53 antibodies on wild-type MEFs (white columns) or p53-/- MEFs (black columns) treated with adriamycin. BS indicates quantification of the fragment containing the predicted p53 binding site in the mir-34a, mir-34b/c or p21 promoter regions, and Ctrl indicates a 39 fragment from the same gene. **f,** Firefly luciferase coding sequences were placed under the transcriptional control of human mir-34a or mir-34b/c promoter elements containing either wild-type or mutant (as indicated) p53 binding sites. These reporters were co-transfected with either control (white columns) or human p53 expression plasmids (black columns). Error bars indicate s.d. (n=3).

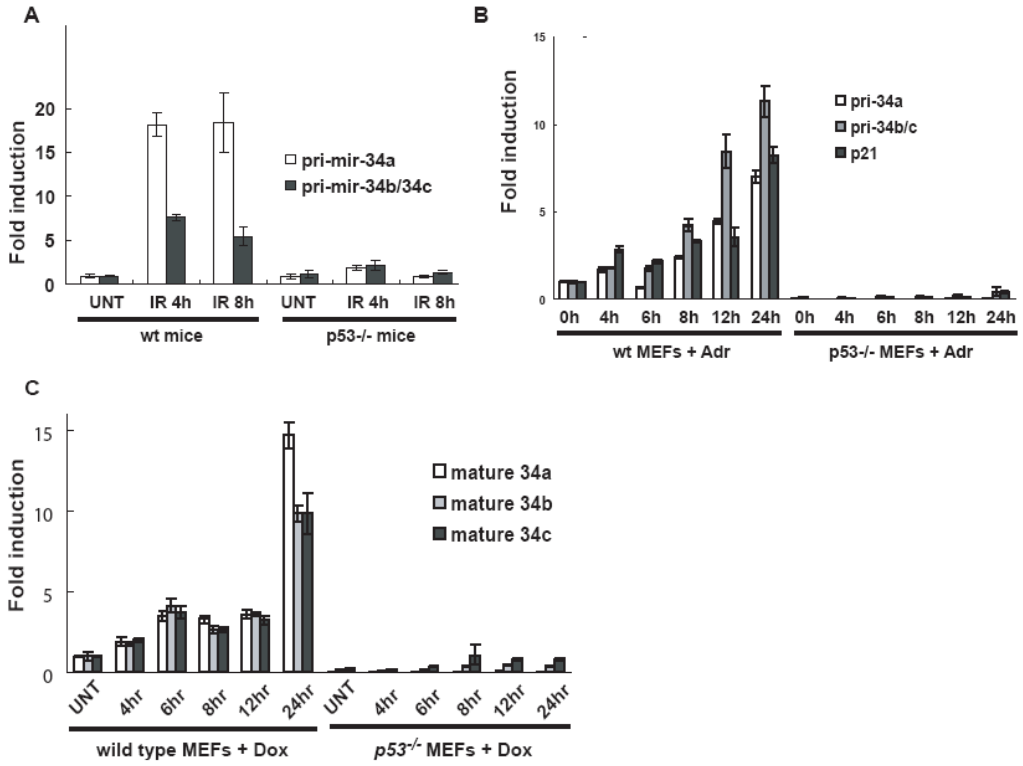


Figure 2.6 Detection of miR-34 induction in p53$^{+/+}$ background. A. Induction of miR-34 in wildtype mice (wt) but not in p53-/- mice. B. miR-34 induction by DNA damaging agent Adiamycin (Adr). p21 is a known p53 target gene. C. miR-34 mature transcript in wildtype and p53-/- MEF.

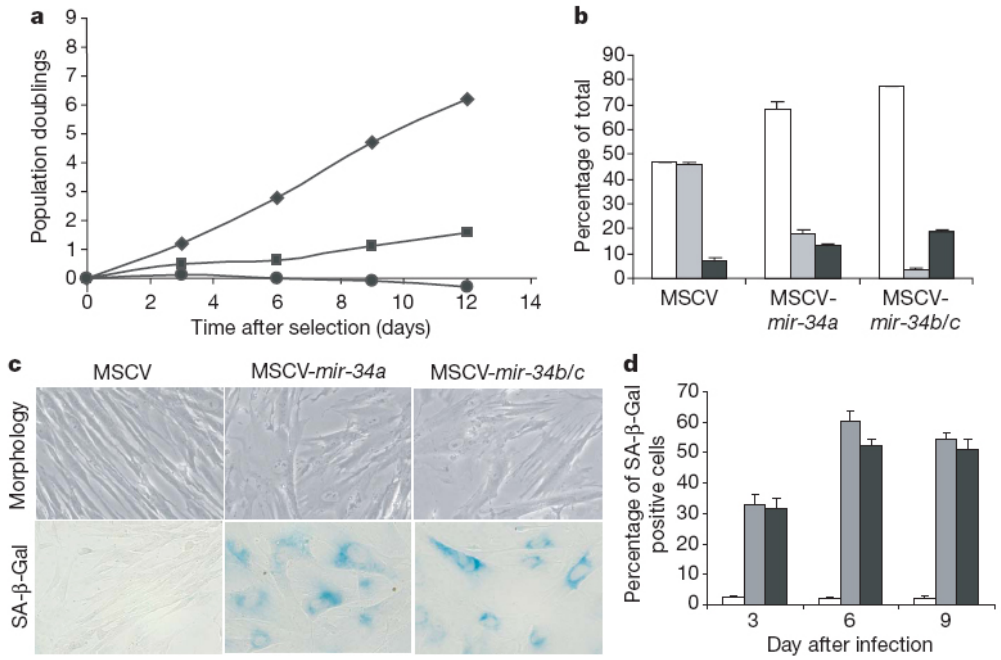


Figure 2.7 miR-34 family miRNAs mediate growth arrest in a variety of cell types. a, Proliferation of IMR90 cells was measured as cumulative population doublings after retroviral delivery of vectors directing the expression of primary miR-34a (squares), miR-34b/c (circles) or a control MSCV vector (diamonds). Measurements were initiated immediately after selection with puromycin. b, Cell cycle analysis was performed 1 day after selection with puromycin by BrdU/FACS on IMR90 cells engineered as in a. White columns, G1; grey columns, S; black columns, G2/M. c, IMR90 cells engineered to express pri-miR-34a or pri-miR-34b/c showed morphological alterations similar to those seen in senescent cells. d, Percentages of SA-b-Gal-positive cells were determined at 3, 6 and 9 days after the completion of selection with puromycin. White columns, MSCV; grey columns, MSCVmir-34a; black columns, MSCV-mir-34b/c. In all cases, error bars indicate s.e.m. (n=3).

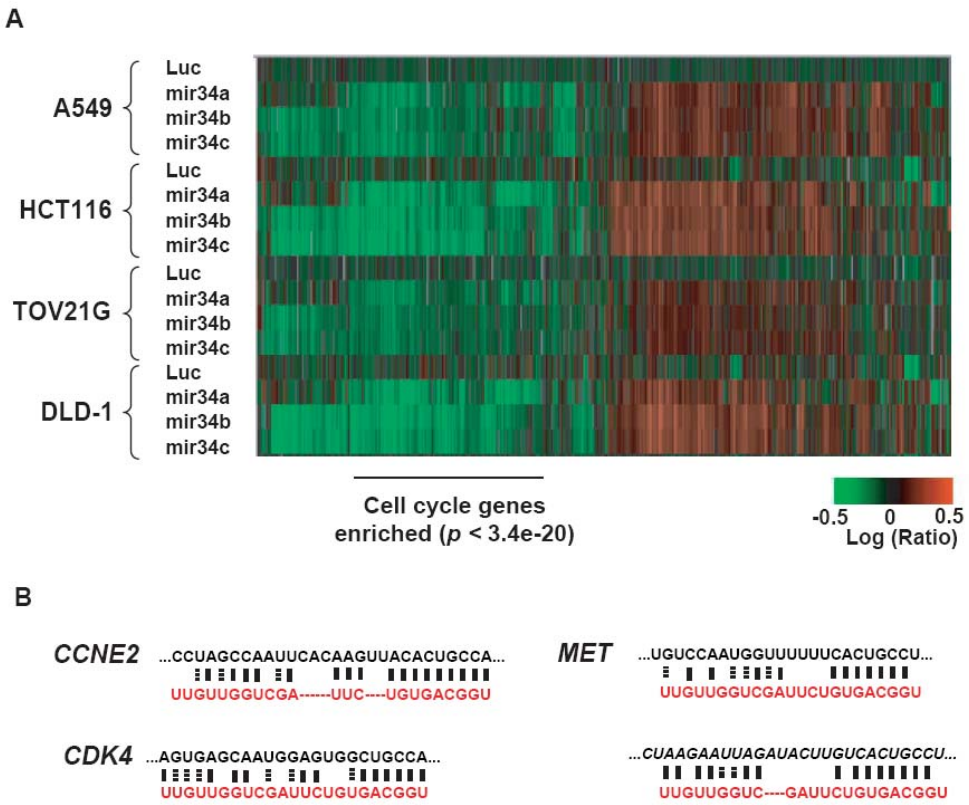


Figure 2.8 Genes regulated by miR-34. A. RNA duplexes corresponding to each mir34 family member or a control siRNA target luciferase (Luc) were transfected into A549, HCT116 Dicerex5 , TOV21G, DLD-1 Dicerex5 cells. Total RNA was isolated 24 hrs post transfection, and subjected to microarray expression analysis. Consensus expression signatures that were down-regulated by all mir-34 family miRNAs were indicated below the heat map. These consensus signatures were tested for enrichment of cell cycle regulation genes annotated with GO Biological Process terms and 3'UTR hexamer seed matches (see methods). B. Seed hexamer matches from each of the candidates examined are shown.

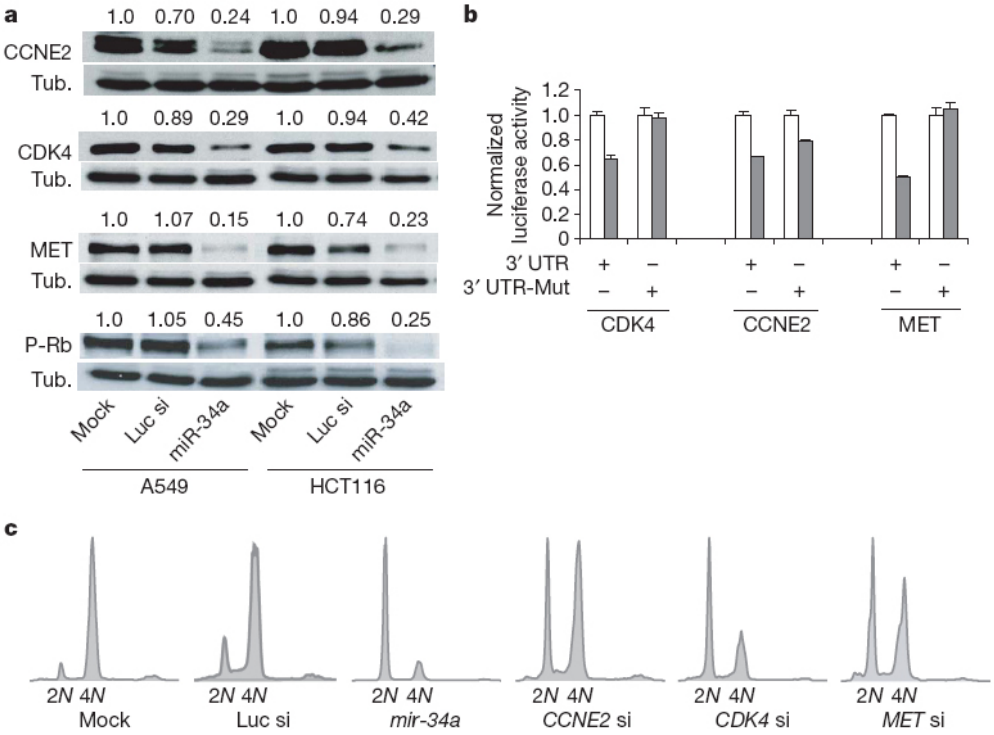


Figure 2.9 miR-34 regulates a programme of cell cycle and DNA damage response genes. a, Western blots were used to measure protein levels after miR-34 delivery for multiple candidate targets identified in the cell cycle overlapping gene set in Supplementary Fig. S6. Tub., tubulin. b, Reporter plasmids in which the luciferase coding sequence had been fused to the 39 UTR of CDK4, CCNE2 or MET, as indicated, were transfected into HeLa cells in conjunction with either miR-34a (grey columns) or miR-124a (white columns) siRNAs. Luciferase activity was normalized relative to a simultaneously transfected Renilla expression plasmid. In each case 39-UTR-Mut indicates the introduction of alterations into the seed complementary sites shown in Supplementary Fig. S8. Error bars indicate s.e.m. (n53). c, HCT116 Dicerex5 cells were transfected with siRNAs targeting CDK4, CCNE2 and MET, and cell cycle effects were analysed. The somewhat less efficacious arrest on transfection with CCNE2 siRNA could reflect a partly redundant function or less potent suppression of its mRNA.

2.3 Discussion

Our data identify the miR-34 family of miRNAs as direct targets of p53 that possess anti-proliferative potential. It is likely that miR-34s mediate this response through additive or synergistic effects of multiple targets, because many components of the cell cycle machinery are affected after the manipulation of miR-34 levels. The effects of miR-34s may also extend to the other arm of the p53 response, given a recent report and our findings (data not shown) that miR-34a can enhance apoptotic responses in some cell types22. Thus, the actual phenotypic output of miR-34 activation may vary by cell type depending on the spectrum of its targets that are available for repression In accord with their regulation by p53, comparatively low levels of miR-34s are observed in human tumours and cancer cell lines, which have a high frequency of functional p53 deficiency. Although selective pressures for miR-34 deletion in human cancers may be alleviated by frequent p53 mutations, deletion of miRNAs of the miR-34 family has been reported in several human tumours and cancer cell lines (Welch et al, 2007; Calin et al, 2004).

In fact, the human gene encoding miR-34a maps to 1p36, a locus frequently deleted in human cancers. Recently, one gene within this locus, CHD5, has been implicated in its tumour suppressive activity (Bagchi et al, 2007). CHD5 has been proposed to act upstream of p53 by regulating its expression in response to various p53-inducing stimuli. Deletions at 1p36 are

often quite large and can encompass both CHD5 and the mir-34a locus, as well as other genes. Thus, 1p36 genomic lesions might affect the p53 pathway at multiple levels, both upstream and downstream of p53 activation.

Although dozens of p53 targets have been identified in mammals, very few are evolutionarily conserved in Drosophila and Caenorhabditis elegans, both of which retain homologues of the p53 pathway (Sutcliffe et al, 2004). miR-34 is one of only 18 mammalian miRNA families (Rudy et al, 2006) that are also present in flies and worms. This raises the possibility that the link between p53 and this non-coding RNA target may have arisen early in the evolution of the p53 network and may be important in p53 function in diverse species.

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2.5 Author contributions

X.He, L.He and G.Hannon designed the study. This chapter is a joint work between Dr. Lin He (Fig 2.2-2.5, 2.8) and Xingyue He. (Fig 2.5-2.9).

Chapter 3

Conditional expression of miR34a in HCC mouse model

3.1 Introduction

p53 is a pivotal tumor suppressor gene in maintaining genomic integrity and preventing tumor initiation and maintenance (Lowe et al, 2004). As miR-34 miRNAs are regulated by p53 and potentially form an essential component of the p53 network (He et al, 2007), it is important to investigate the role of *mir-34* in both tumor initiation and maintenance *in vivo*.

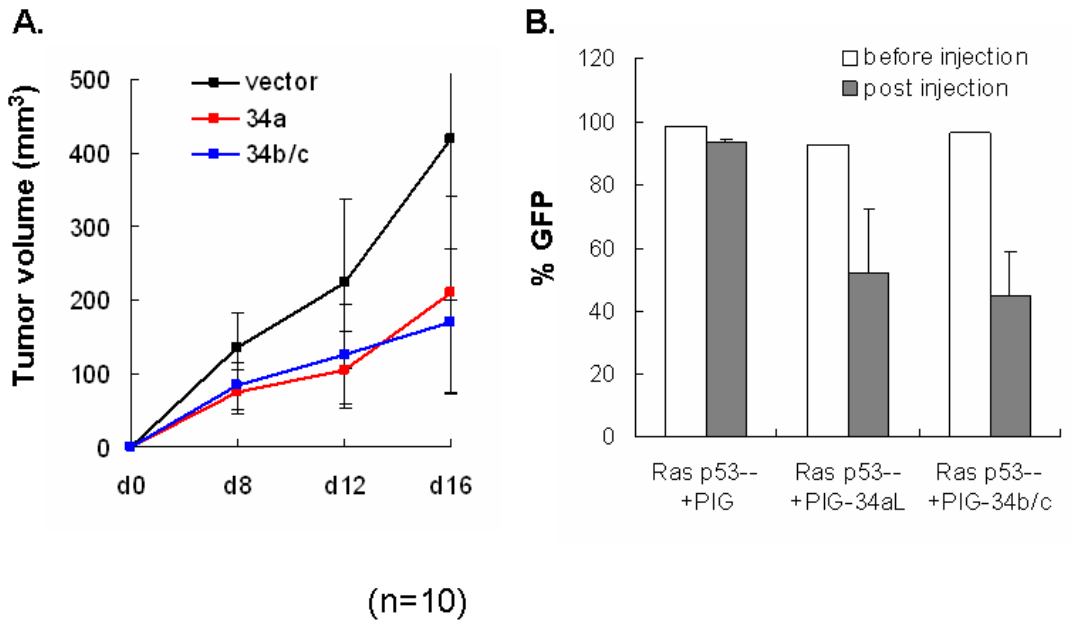
3.2 Results

3.2.1 Role of *mir-34* in tumor initiation

We and others have shown in cultured cells that mir34 over-expression leads to cell cycle arrest or apoptosis. It is worthwhile to study whether mir34 can suppress tumor growth *in vivo*, particular in p53 null tumors.

To study whether *mir-34* could suppress tumor growth, I used a mouse model of hepatocellular carcinoma (HCC) developed by Dr. Lars Zender (Zender, et al, 2006). In a pilot experiment, embryonic liver progenitor cells (hepatoblasts) from $p53^{-/-}$ ED=12.5-15 fetal liver embryonic mouse livers were transduced by retroviruses expressing oncogenic *ras* (*HrasV12*) together with empty vector or with LTR driven *mir-34a* or *mir-34b/*c cluster (Fig 3,1A). The PGK-puro-IRES-GFP cassette (PIG) in the vector allows us to select the infected hepatoblasts to obtain a pure population before injecting them into nude mice. As shown in Fig 3.1A, both mir-34a and mir34b/c clusters significantly delayed tumor growth. Although tumors in the mir-34 group eventually grow to a certain size, we showed that percentage of GFP positive

cells decreased at day 16 post injection compared to day 0 (Fig 3.1B), indicating that mir-34 retrovirus are negatively selected by the tumor. This result underlines mir-34's role as a potent tumor suppressor *in vivo*.



(n=10)

Fig 3.1. Ecopotic expression of mir34a or mir34b/c clusters resulted in delayed tumor growth. (A) P53-/-; Ras liver progenitor cells were infected with retrovirus expressing miR34 (PIG vector) and selected with 2.5ug/ml puromycin for 2 days. $3X10^6$ cells were injected in nude mice and tumor volume was measured by caliper. Error bars denote s.d. (n=10). (B) Negative selection against mir34 in the tumors. The percentage of cells retaining mir-34 retroviruses were quantified by GFP percentage expressed from the PIG vector. D16 tumors (post injection) were digested by dispase to generate single cell suspension and sorted by FACS. The decrease of %GFP post injection indicated that either some tumor cells have lost mir-34 expression or the non-infected cells escaping the puro selection have taken over in the tumor population.

3.2.2 Conditional expressing mir34 to study its role in tumor maintenance

Our preliminary data showed that constitutive expression of mir34 in tumor cells resulted in delayed tumor growth (Fig 3.1). In such an infection-selection experimental setting, the chronic expression of mir34 may lead to the production of tumor cell clones that lose mir-34 expression, therefore obscuring the real biological effect of mir34 as tumor suppressor.

To better understand mir-34's role in suppressing tumorigenesis and to investigate the potential of using mir34 in cancer therapy, we need a model that allows conditional expression of mir34.

The work from Drs. Gregory Hannon and Scott Lowe's labs has recently shown that microRNA or microRNA-based shRNAs can be conditionally expressed from tetracycline-regulated Pol II promoters (TRE) (Dickins et al, 2005; Dickins et al, 2007). Depending on whether tTA or rtTA is co-infected (Gossen et al, 1992), the TRE driven microRNA can be regulated in either a tet-off or tet-on manner. In the tet-on setting, the microRNA is not expressed in the absence of tetracycline (or its analog, Doxycycline, Dox) but is expressed upon Dox treatment. We therefore applied this methodology to conditionally express mir34 in a liver mouse model developed by Dr. Lars Zender in Scott Lowe's lab (Zender et al, 2006; Zender et al, 2008). As shown in Fig 3.2, TRE driven mir34 and rtTA were retrovirally tranduced into p53-/- liver progenitor cells (co-expressing an oncogenic Ras). The cells were selected for puromycin resistance carried on the TRE.mir34 vector and for neomycin resistance

carried on the rtTA vector to ensure purity. Selected cells were seeded into the liver of recipient mice. We tested whether tet-based systems could be used to conditionally regulate tumor suppressor function in the hepatoblast model. Wild-type liver progenitors were transduced with retroviruses expressing oncogenic ras, tTA-luciferase, and the TRE-shp53 cassette (Xue et al, 2007). Cells were seeded into livers of retrorsine conditioned mice by intrasplenic injection and tumors were allowed to form (Xue et al, 2007). Using the tet-off shRNA system, the endogenous level of mir34 can be effectively restored by doxycycline (Dox, a tetracycline analog). Upon tumor manifestation, animals can be treated with Dox or left untreated, and tumor growth monitored by bioluminescence imaging or overall survival.

I compared conditional miR-34 expression from STP and TGM vectors (Fig 3.3). Dox induced miR-34a expression level is similar in both vectors (Fig 3.3) but TGM vector supports a much higher miRNA level (Fig 3.4). I therefore used TGM vector design in the following experiments.

I selected the infected cell population with puromycin on TRE-*mir3*4 vector and neomycin on rtTA vector to ensure that the level of *mir-34* will be effectively increased upon doxycycline treatment. To ensure a homogenous mir-34 induction, I selected single cell clones and cultured them with or without Dox. We've observed two different phenotypes among the single cell clones, some undergo apoptosis after Dox treatment while the other showed a senescence phenotype. Three cell clones were tested *in vivo*. The cells were

injected into nude mice as subcutaneous tumor. Upon tumor manifestation, animals were treated with Dox or left untreated, and tumor growth was monitored by bioluminescence imaging based on the luciferase marker linked with Ras.

Clone 1 and clone 3 showed a cellular senescence phenotype upon Dox treatment (SA-b-Gal, Fig 3.5A) (Schmitt et al; 2002; Narita et al, 2003). Clone 2 showed a possible apoptosis phenotype (Fig 3.6A, Fig 3.7). It was soon apparent that all tumors halted growth as compared to the untreated tumors (Fig 3.5B, 3.6B), and some tumors even regressed after *mir-34a* activation (Fig 3.8 and 3.9). Overall, these results imply that miR-34 can act as tumor suppressors *in vivo*. Noteably, we did not observe a complete tumor regression in this experiment; some resistant clones did emerge even under prolonged Dox treatment. We reasoned that this might come from genomic instablity in the Ras;$p53^{-/-}$ cells or the breakdown of the tet-on system in some cells. Furtherwork is required to characterize the regressing or growtth arrested tumors with Dox treatment.

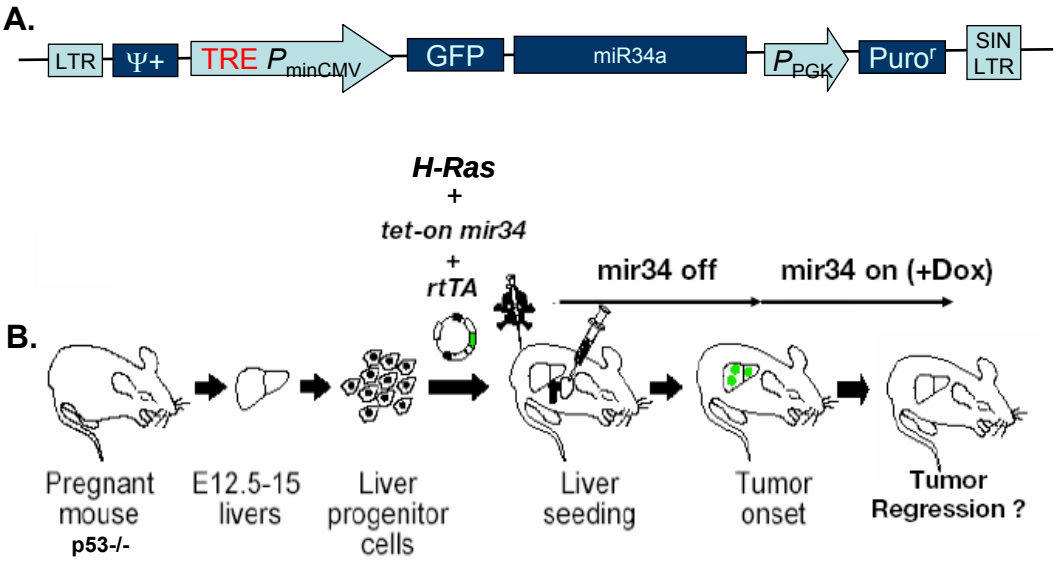


Fig 3.2. Schematic view of conditional expressing mir34 in liver cancer model. A. Vector design. B. p53 null Liver progenitor cells are infected with retroviruses harboring TRE driven *mir-34* rtTA and oncogenic Ras. Following tumor onset, mir34 expression can be induced by Doxycycline treatment.

A.

STP (from Dr. Ross Dikins)

TGM (from Prem Premsrirut)

B.

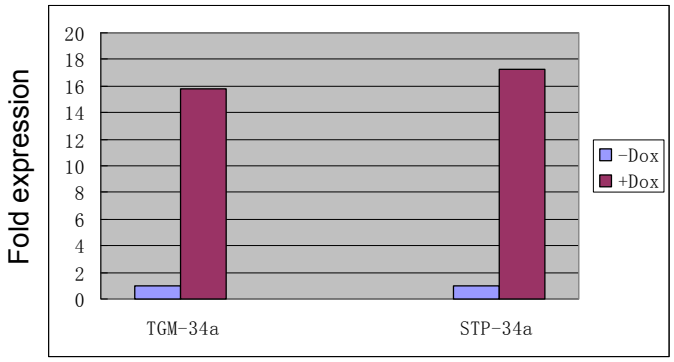


Figure 3.3. Comparing miR-34a expression from STP vector and TGM vector. (A) Vector design. (B) Expression level of miR34a was measured by miRNA RT-Q-PCR.

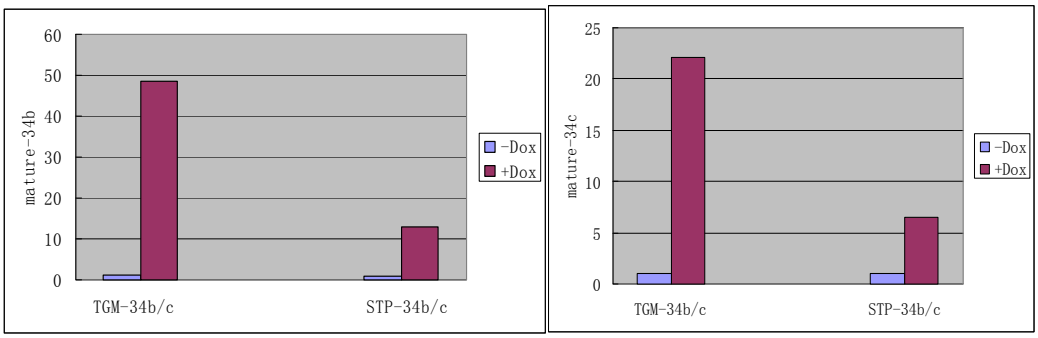


Figure 3.4. Comparing miR-34b/c expression from STP vector and TGM vector. Expression level of miR34b/c was measured by miRNA RT-Q-PCR.

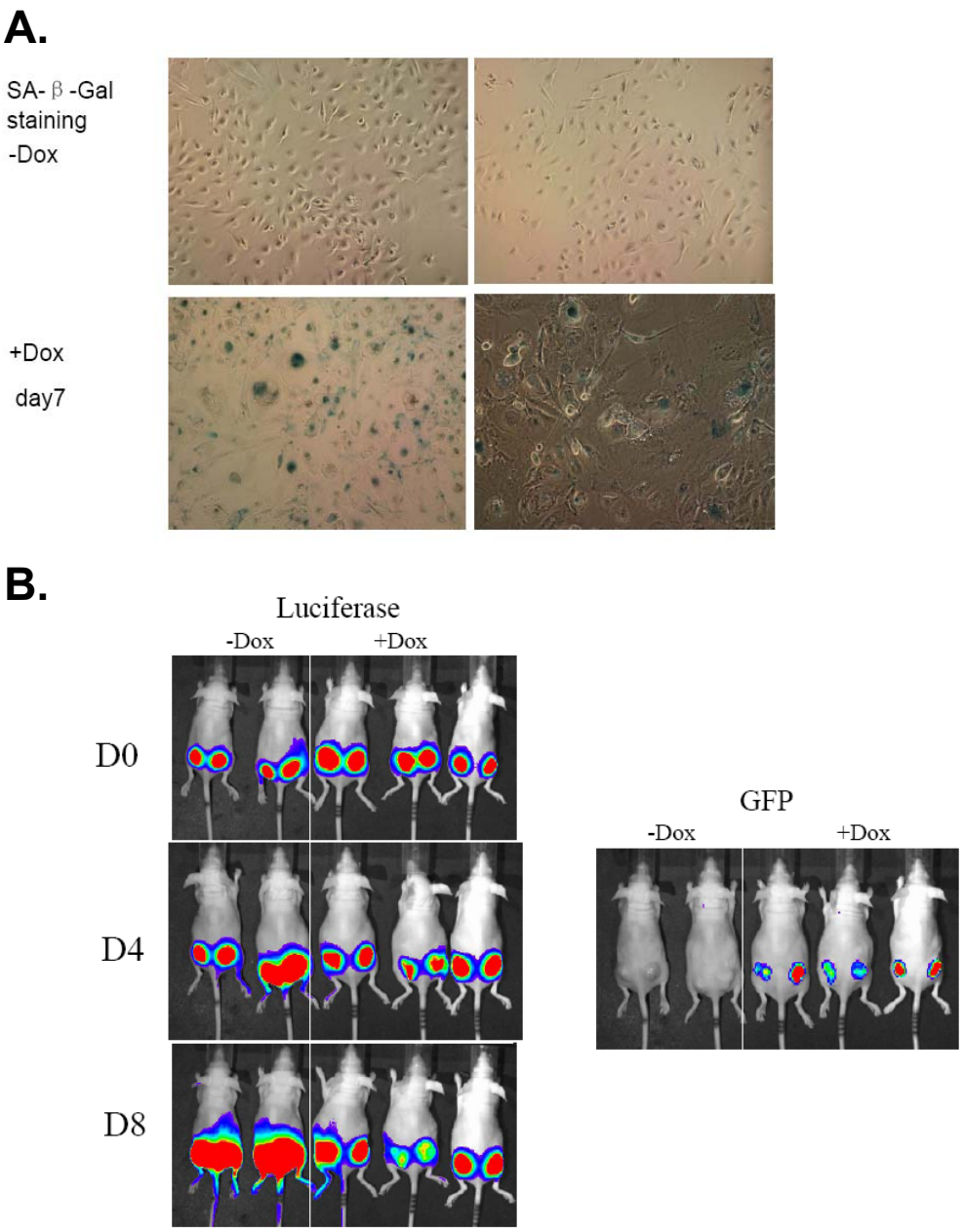


Fig 3.5. Acute induction of miR-34a results in senescence and delayed tumor regression. (Clone 1). p53 null murine liver cells were infected with Ras and tet-on mir-34a retrovirus. Single cell clones are selected and several clones are injected into nude mice. The animals are either untreated (-Dox, upper panel) or treated with Doxycyclin (+Dox, lower panel) to induce the expression of mir-34a *in vivo.* **A.** SA-b-Gal staining in cells treated or untreated with Dox. **B.** Bioluminescence pictures of representative animals. GFP imaging confirms miR-34a inductions in Dox treated tumors

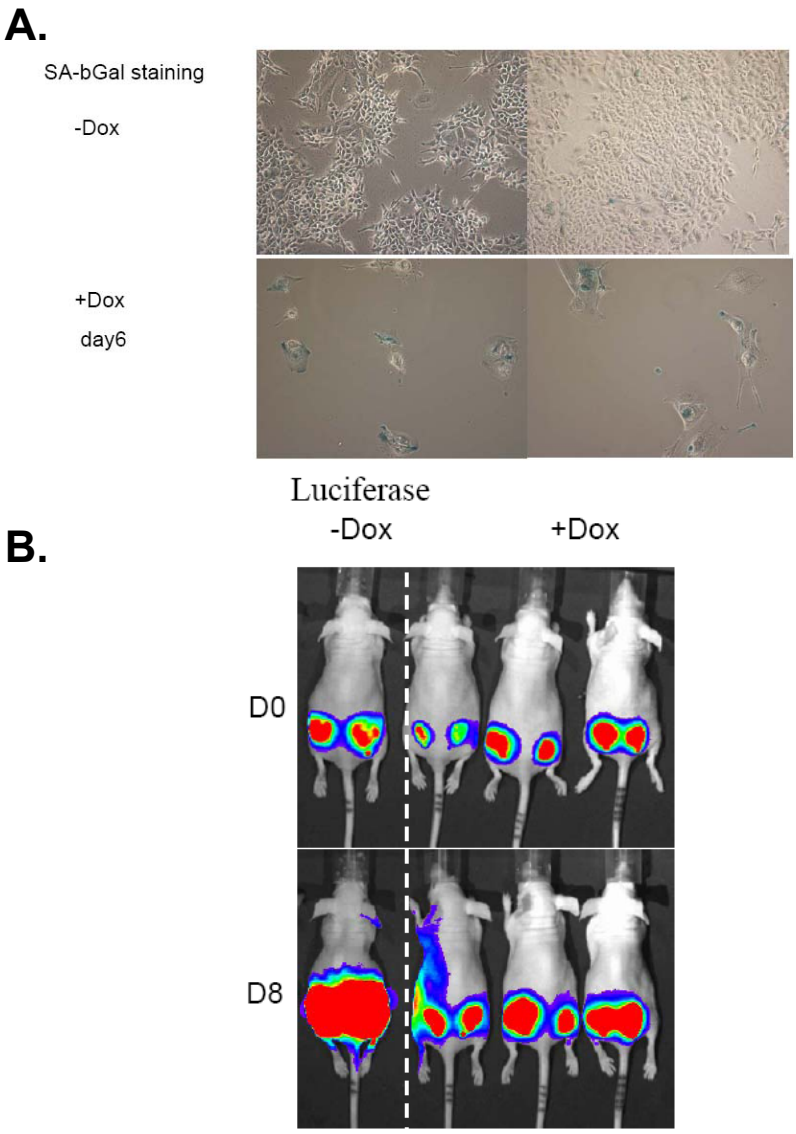


Fig 3.6. Acute induction of mir34-a results in cell death and delayed tumor regression (Clone 2). A. Clone 2 shows massive cell death upon Dox treatement. After 6 days only a few cells are left in the plate. **B.** Bioluminescence pictures of animals in each group.

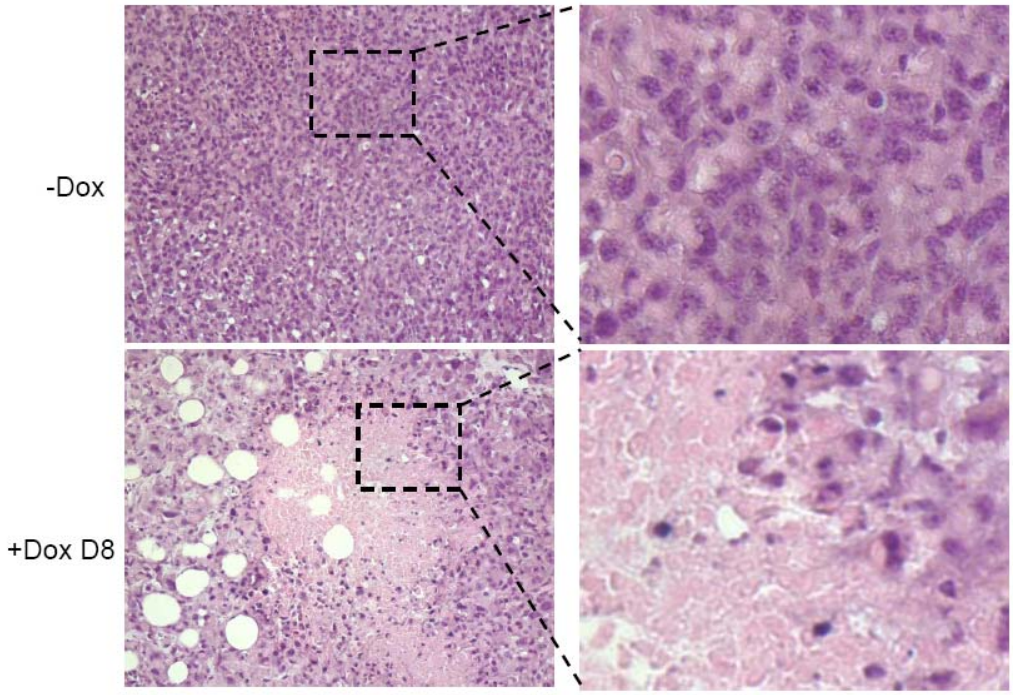


Fig 3.7. H&E staining of Dox treated tumors (Clone 2). There are putative apoptotic cells in the Dox treated tumors.

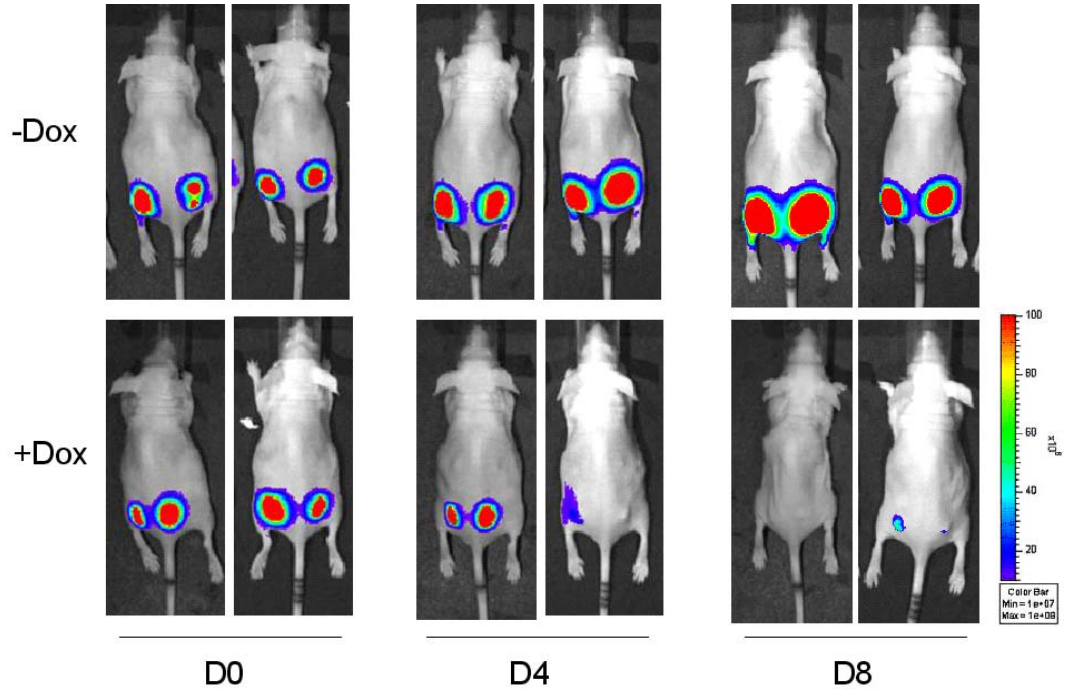


Fig 3.8. Acute induction of mir34-a results in complete tumor regression. (Clone 3)
In some clones, miR-34a induction leads to complete remission of S.C. tumors.

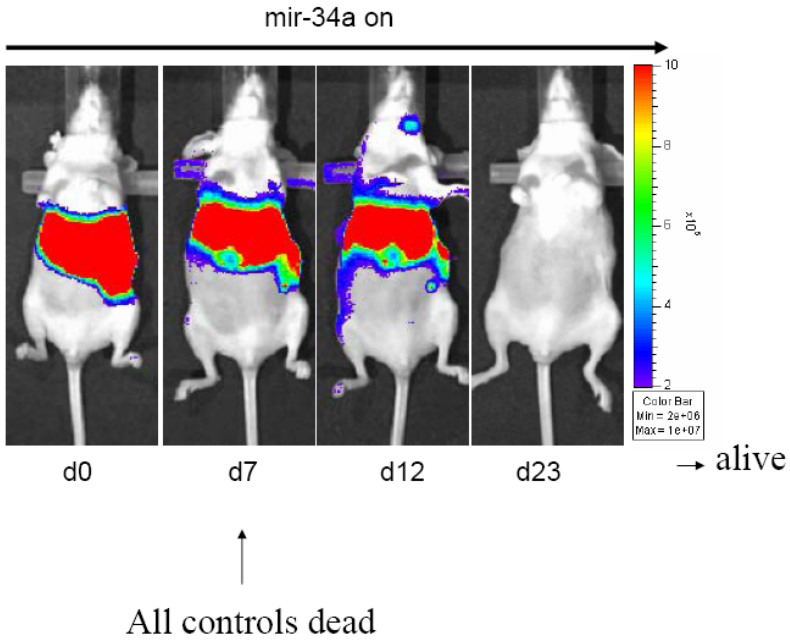


Fig 3.9. Acute induction of mir34-a results in complete tumor regression in situ (Clone 3). p53-/-;Ras cells coexpressing tet-on miR-34a were transplanted into the livers of nude mice. Dox treatment leads to complete remission of liver tumor. The treated animals are alive after 23 days whereas untreated controls die within 7 days.

3.3 Discussion

mir34 is a direct target of p53 tumor suppressor (He et al, 2007a, He et al, 2007b, He et al, 2007c). Because its expression level is correlated to p53 status, mir34 expression is compromised in $p53^{-/-}$ cells. For this reason, it's possible that *mir34* would restore p53's tumor suppressive function on cancer cells, for example, by regulating cell cycle related genes such as CDK4 and CyclinE2. We've performed preliminary studies in a clinically relevant liver cancer model and investigated whether *mir-34* could inhibit tumorigenesis and tumor progression.

Using tetracycline-inducible miRNA expression system, we showed that acute expression of miR-34a pre-miRNA lead to delayed tumor progression or even tumor regression (Fig 3.5-3.9). More effort will be required to study whether the remaining tumors are derived from tumor clones that lost miR-34a expression.

One interesting question is how the cells choose between the apoptosis/senescence programs upon mir-34a activation. We plan to measure the mir-34a induction level in different cell clones and also compare the cell lineage marker of the clones. These work will establish how mir-34a directs downstream cell death pathways in the p53 tumor suprressor network.

I plan to confirm the effective expression of mir34 *in vivo* by microRNA RT-qPCR. I will collect tumor samples at different time points and stain them for Ki67 (proliferation marker), TUNEL (apoptosis marker) and SA-b-Gal

(senescence marker). Protein or RNA samples can be quantified for mir34 targets in the tumors

The next step to study miR-34 as cancer therapy is to test the *in vivo* delivery of miR-34a siRNA into the livers of HCC bearing mice. Mir-34 clusters can be delivered by *in vivo* fectamin into liver tumors at high efficiency. This may allow potential application of mir-34 as cancer therapy in animal models.

3.4 Reference

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3.5 Author contributions

X.He and G.Hannon designed the study. TGM.miR-34a expression vector is kindly provided by Prem Premsrirut in Dr. Scott Lowe's lab. Animal facility at CSHL provided histopathological analyses.

Chapter 4

Generating miR34 knockout mice

4.1 Introduction

Our previous analysis of *mir-34*'s function reveals its role in mediating cell cycle arrest and suppressing a family of cell cycle related genes. To further explore mir-34's function in p53 mediated tumor suppression network, we tried to create both constitutive and conditional loss-of-function alleles for *mir-34s* in mice and to use these animals as a platform to characterize mir-34's role in various mouse cancer models.

4.2 Results

4.2.1 To create constitutive *mir-34* knock-out alleles in mice

As shown in Fig 4.1, we constructed the constitutive knock-out alleles of *mir-34*s in which the primary miRNA transcript is replaced by LacZ (the gene encoding β-galactosidase). First, we generated about 200 base pair (bp) homology arms flanking the *mir-34a* or *mir-34 b/c* locus by PCR from BAC or mouse genomic DNA. Second, the two fragments were ligated to a linear reporter cassette which contains a LacZ gene in tandem with a neomycin resistance gene flanked by *loxP* sites that allows positive selection in both bacterial and mouse cells. Then BACs were engineered in *E. coli* by homologous recombination between the BACs containing *mir-34a* or *mir-34b/c* locus and the ligated cassette. The modified BAC were retrieved into a plasmid vector before introduced into mouse ES cells for homologue recombination. Genotyping of ES cells was accomplished through PCR and Southern blotting. Finally, targeted ES cells were be microinjected into blastocysts by Dr. Sang Yong Kim in the CSHL animal facility.

We've generated constitutive knock-out alleles of *mir-34a* in ES cells and the genotyping of targeted C57/black6 ES cells was accomplished by Southern blotting (Fig 4.2). Cells in which miR-34a was replaced by LacZ were recovered at a rate of 60%. Correct targeted ES cell clones were microinjected into the albino (C57/B6 Tyrc-Brd) blastocysts by the CSHL animal facility. The chimeric mice were born and were bred for germline transmission (Fig 4.3). Heterozygous mir-34a deficient animals were obtained through successful germline transmission, and we crossed them to B6 mice to expand the colony. In the meantime, we targeted black6/129 hybrid ES cells with the same constitutive knock-out construct and we generated mice using the tetraploid-embryonic stem cell complementation method. In this approach, correct targeted ES cells were injected into 4n blastocysts and the ES cells give rise to epiblast and the 4n host cells give rise only to the placenta. This method allows us to study the consequences of *mir-34a* loss at an accelerated pace without the need for a chimeric intermediate. The disadvantage for this method is the lack of stable genetic background. The correctly targeted ES cell clones were recovered at a rate of 30% and injected into 4n blastocysts by the animal facility (data not shown). Heterozygous mice were obtained in the hybrid genetic background as well. We set up heterozygous to heterozygous cross to obtain mir-34a null mice (Fig 4.4).

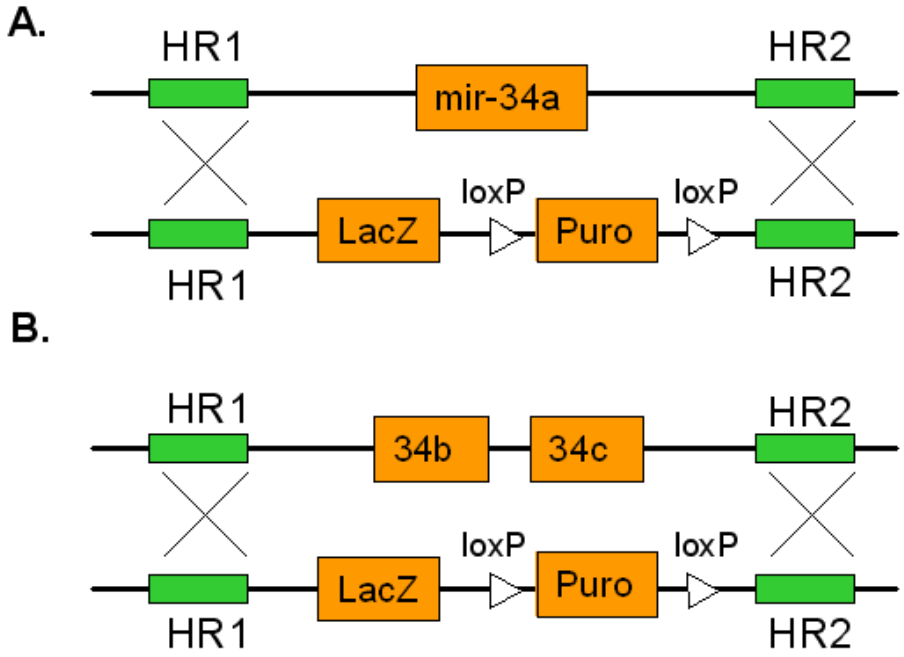

Fig 4.1. Schematic view of generating constitutive *mir-34* knockouts. Primary mir-34 transcripts will be replaced by LacZ reporter tandem with a neomycin selective marker. Homologue recombination will take place at the homology arms flanking *mir-34a* or *mir-34b/c*. HR1: homologous region 1; HR2: homologous region 2.

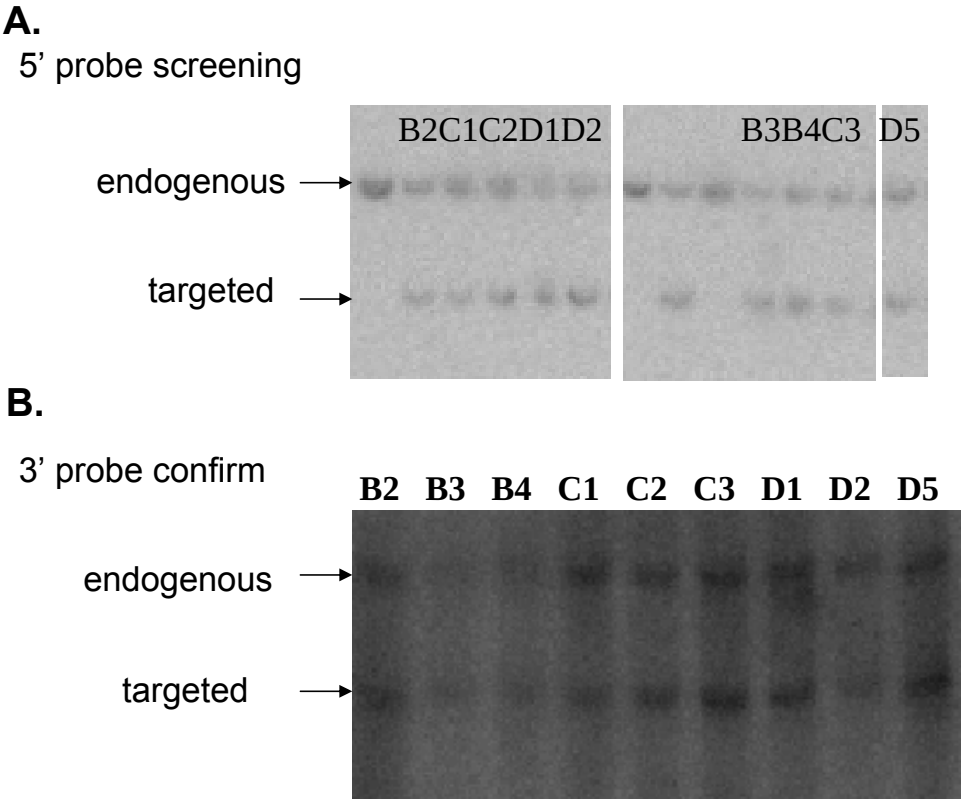

Figure 4.2: Southern blot showing correct ES cell targeting. The endogenous miR-34a allele produces a 11kb band, while the replaced lacZ allele creates a 8.3kb band.

Figure 4.3: Pictures of C57/Black6 Chimera mice

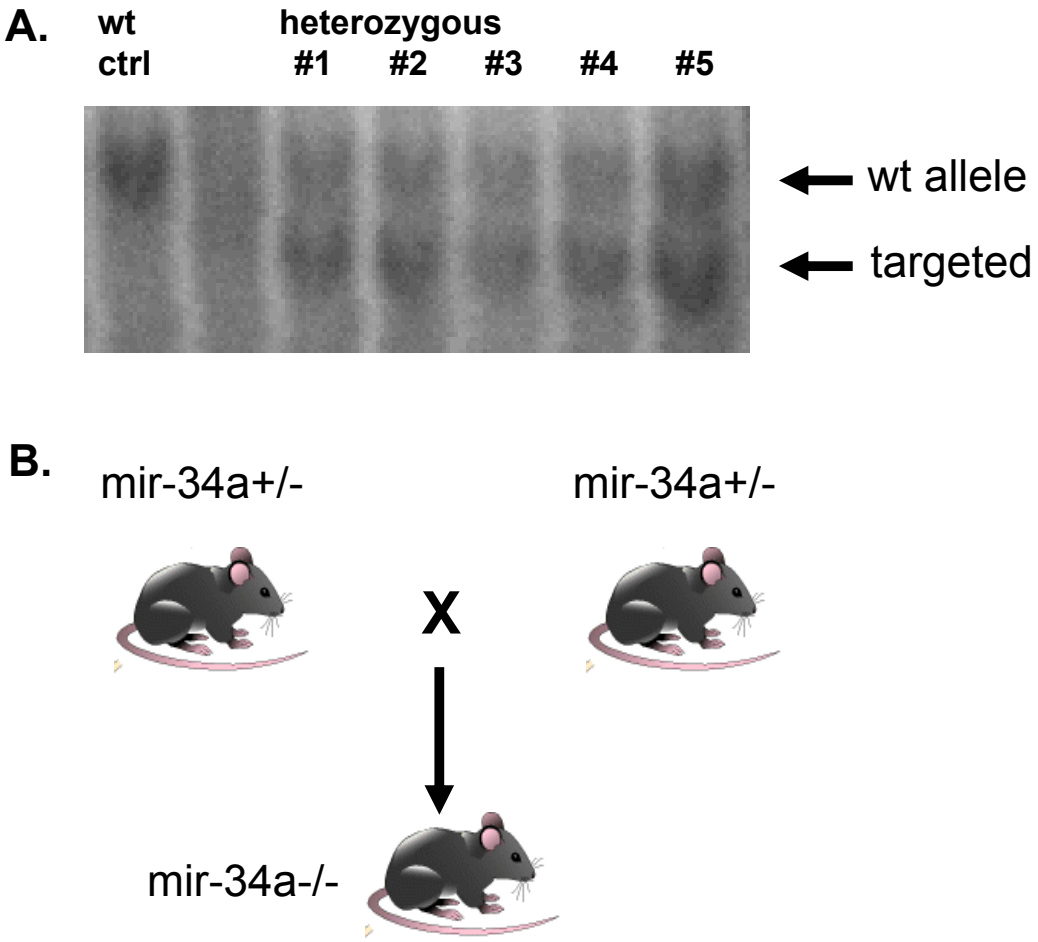

Figure 4.4: Crossing miR-34a heterozygous mice to make null mice. A. Southblot to genotype hetergzygous miR34+/- mice. B. Schematic view of het to het genetic cross.

4.2.2 Creation of conditional *mir-34* knock-out alleles in mice

We have also worked to develop conditional knockout of *mir-34a* using the Cre/loxP system in which the *mir-34a* locus have been flanked by two LoxP sites tandemly with a neomycin selective marker (Fig 4.5). The miR-34a region can be excised *in vivo* in a spatially and temporally regulated manner upon Cre recombinase activation. The conditional *mir-34* knockouts become valuable upon crossing to suitable Cre-expressing lines. For an example, crossing to Oct4-Cre, Nestin-Cre and Sca1-Cre may illuminate the role of *mir-34* in embryonic stem cells; using Rosa26-Cre-ER lines, the effects of simultaneously creating a null allele in many tissues can be studied in the adult and developing animal. Conditional knockout animals will also be useful for *ex vivo* studies since we can deliver Cre or CreER by retrovirus or adenovirus.

C57/B6 ES cells with miR-34a replaced by the conditional allele were recovered at a rate of 15%. Correctly targeted ES cells were microinjected into albino (C57BL/6 Tyrc-Brd) blastocysts and the chimeric mice were bred for germline transmission. However, we were not able to get successful germline transmission.

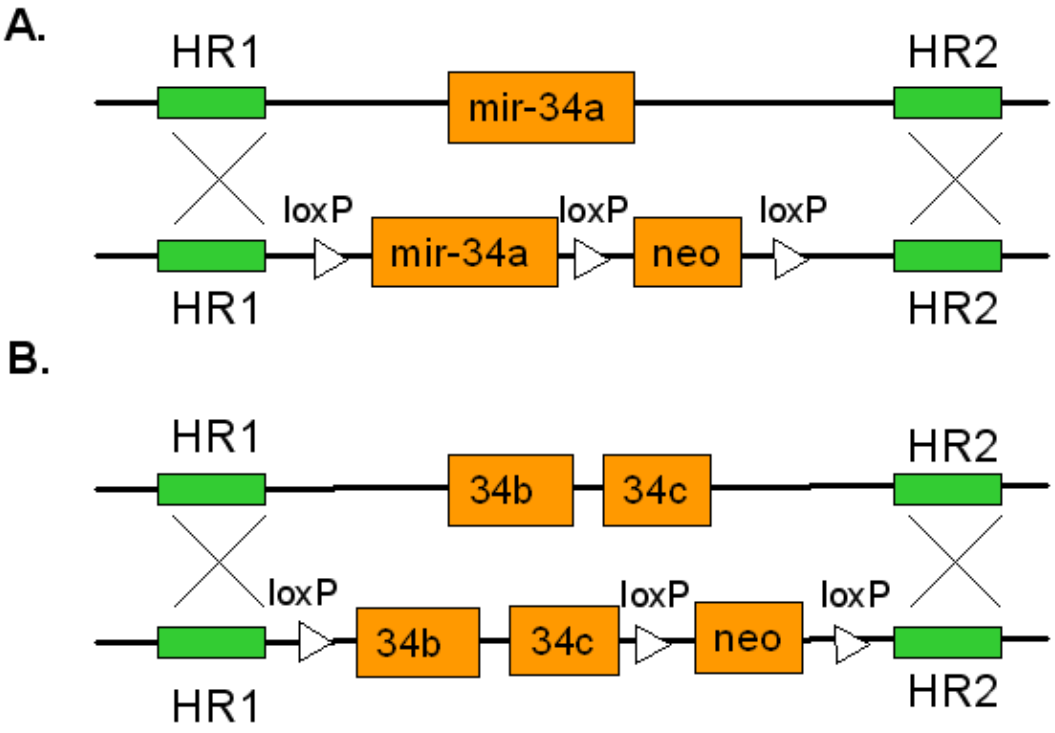

Fig 4.5. Schematic view of generating conditional *mir-34* knockouts. *mir-34* locus flanked by two *loxP* sites tandem with a neomycin selective marker will be created through homologue recombination. This engineered locus will be normal functional but become inactived upon introduction of Cre. HR1: homologous region 1; HR2: homologous region 2. region 1; HR2: homologous region 2.

4.2.3 Characterization of miR-34a$^{-/-}$ MEF

mir-34a null mice were born and showed no obvious prenatal or postnatal developmental defects. We've generated MEF (mouse embryonic fibroblast) from mir-34a null and paired wild type embryos. Southern blotting shows successful deletion of mir-34a in the nulls compared to wildtype (Fig 4.6 up-left). Similar results were obtained by genomic PCR using allelic specific primers (Fig 4.6 lower-left).QPCR analysis shows there is undetectable (ND) mir-34a mature miRNA in the knockout MEF population (Fig 4.6 right) where as mir-34b,c are expressed at normal level.

We are in the progress to characterize the phenotype of mir-34a knockout MEF. As mir-34a is induced by p53 and its overexpression resulted in cell cycle arrest or apoptosis, we hypothesized that loss mir-34a allele may lead to increased cell proliferation and protection against irradiation induced apoptosis.

Preliminary data showed that mir34-/- MEF, like the p53-/-, grow faster than wildtype MEF in the population doubling assay (Fig 4.7A). BrdU incorporation assay showed there are more S phase cells in mir-34a (Fig 4.7B) MEF.

Although many studies have shown that mir-34a plays an important role in the p53 network, mir-34a single knockout may not fully reveal its function due to redundancy with mir-34b/c. These miRNAs have exactly the same seed sequence. Meanwhile other p53 target genes such as p21 may compensate

mir-34a's loss in induction of cell cycle arrest or apoptosis upon p53 activation.

In addition to further characterizing the mir-34a null phenotype, knockout the mir-34b/c knockout (straight or conditional) will help to unveil mir-34's role in development and cancer.

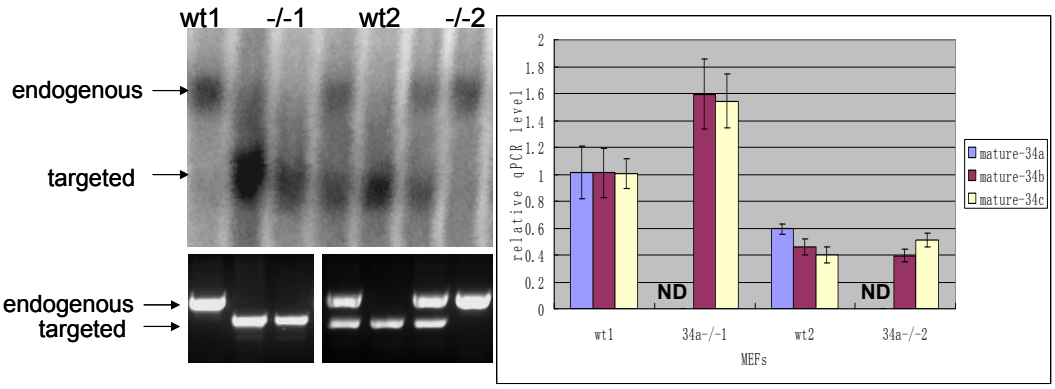

Figure 4.6 (A) Southern blot of paired wildtype and miR-34a-/- MEF. (B) PCR from genomic DNA. (C) QPCR using primers detecting mature miR-34a,b, or c. ND, not detectable.

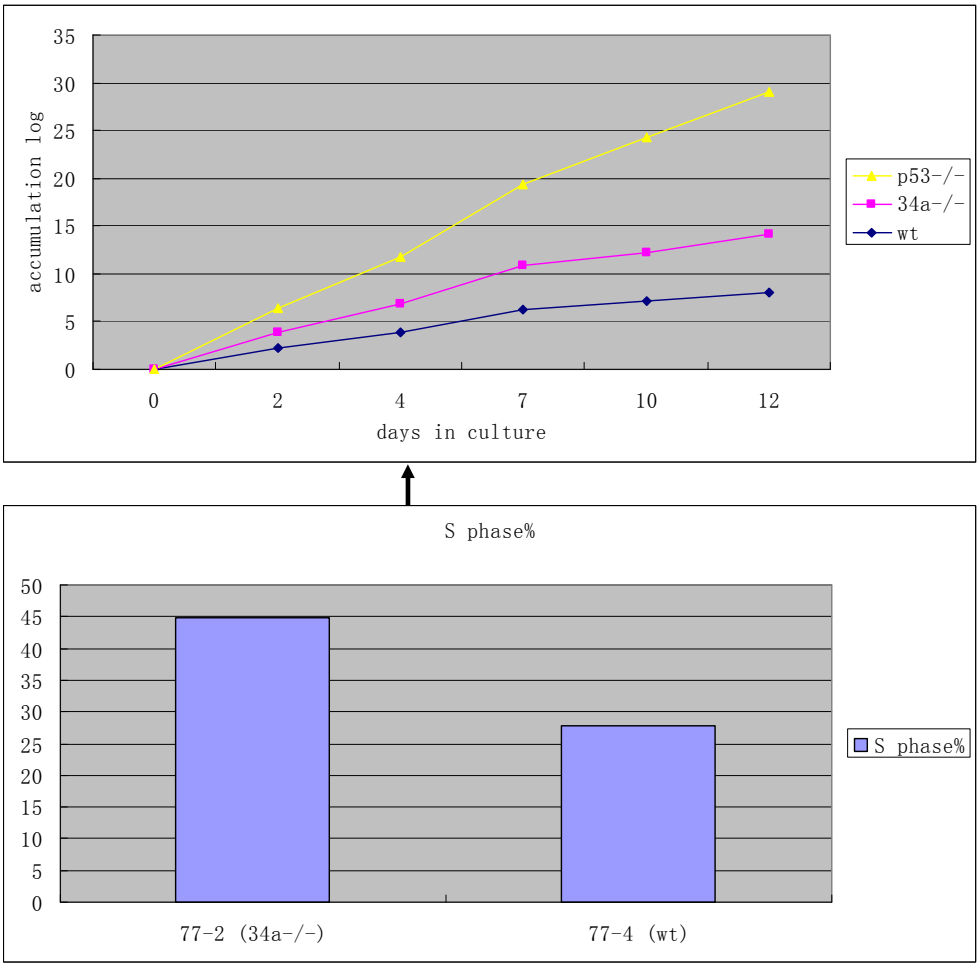

Figure 4.7: Characterization of mie-34a-/- MEF (A) population doubing (b) % of BrdU incorporation.

4.3 Discussion

As the *mir-34* knockout mice are viable and fertile, the effects of loss of *mir-34* in cancer will be examined in mouse tumor models including the HCC model as we described in chapter 3 and the LoxP-Stop-LoxP-KRas Lung cancer model. We will use both ex vivo manipulation of *mir-34* knockout stem cells and germ line approach in these mouse cancer models.

For the HCC model, we will harvest liver progenitor cells from either *mir-34* knockout or wild type embryonic livers and infect the cells with oncogenes (eg. c-Myc). The infected cells will be transplanted to recipient mouse to allow repopulation of the host liver. If *mir-34* is an important tumor suppressor in liver cancer, we expect to see accelerated tumor progression in the *mir-34* knockout cells compared to wild type control.

In the LSL-KRas model, conditional *Lox-STOPLox-Kras2* (*G12D*) mice (*LSL-Kras*) express an activating mutant *Kras* allele from its endogenous locus after Cre-mediated excision of a STOP cassette (Jackson et al, 2001; Chan et al, 2004; Tuveson et al, 2004). Non-small-cell lung cancer (adenocarcinomas) can be produced by intranasal administration of Adeno-Cre or lentiviral-Cre to the lung (Jackson et al, 2005). In addition, pancreatic cancer can be produced by crossing with pancreatic specific *Pdx-1-Cre* transgenic mice (Hingorani et al, 2003).

Mice harboring multiple genetic lesions can be produced by crossing two transgenic or knockout strains. This provides a procedure to determine

whether two genetic mutations can cooperate to promote tumor progression. For example, activated *Kras* and *Ink4a*/*Arf* deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma with similar genetics and histopathology to human pancreatic cancer (Aguirre et al, 2003). Oncogenic cooperativity was observed between $Trp53^{R172H}$ and Kras (G12D) to generate chromosomal instability and metastatic pancreatic ductal adenocarcinoma (Hingorani et al, 2005). Interestingly, *Trp53* loss or mutation strongly promotes progression of Kras (G12D)-induced lung adenocarcinomas, yielding invasive tumors that metastasize early and resemble advanced human lung adenocarcinomas (Jackson et al, 2005). We've generated the cross of our mir-34a knockout mice and the *LSL-Kras* mice, and we're still in the progress of analyzing the impact of mir-34a loss in the lung adenocarcinomas comparing to the *Trp53* loss.

These proposed experiments can establish mir34 as bona fide tumor suppressor gene in relevant mouse cancer models and provide a valuable system to study *mir-34*'s function in cancer.

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He X, He L, Hannon GJ. The guardian's little helper: microRNAs in the p53 tumor suppressor network. Cancer Res. 2007c 67(23):11099-101.

4.5 Author contributions

X.He, L.He and G.Hannon designed the study. Sangyong Kim facilitated the generation of miR-34a knockout animals. Animal facility at CSHL provided animal care.

Chapter 5

Mir library screen to identify oncogenic miRNA

5.1 Introduction

miRNAs are evolutionary conserved non-coding RNAs which regulate gene expression through post-transcriptional repression (Hannon et al, 2002; He et al, 2004). The human genome encodes >400 miRNAs. However, only a handful of miRNAs have been functionally studied. Among the miRNAs determined to be relevant to carcinogenesis, the oncogenic potential of the miR-17-92 cluster was demonstrated (He et al., 2005; O'Donnell et al., 2005). A tissue culture based genetic screen implicated miRNA-372 and miRNA-373 as oncogenes cooperating with Ras (Voorhoeve et al, 2006). However, there has not been a systematic screening approach to identify oncogenic miRNA function in mice.

The mechanism of action of miRNAs has been difficult to decipher by pure bioinformatics approaches. Several algorithms were developed to predict miRNA targets, eg. TargetScan, PicTar, and miRanda (John et al., 2004; Lewis et al., 2005; Robins et al., 2005). These programs predict dozens to hundreds of target genes per miRNA, making it difficult to directly infer the relevant cellular pathways affected by a miRNA. Furthermore, the biological effect of the downregulation of a putative target depends greatly on functional validation in a cellular context, which exemplifies the need to determine miRNA functions by *in vivo* genetic screens in well-defined model systems.

Recent studies have generated near genome-wide miRNA libraries that allow ectopic expression of any miRNA encoded in the human or mouse genome (Voorhoeve et al, 2006). Herein I performed a genome-wide *in vivo*

miRNA screen to identify oncogenic miRNAs cooperating with p53 loss and Myc in liver carcinogenesis (Zender et al, 2008). Using sequencing based positive selection, I identified and functionally validated miR-23b as a potential oncogenic miRNA.

5.2 Results

5.2.1 Cloning the genome wide mir library

We obtained two genome-wide full length miRNA library from Dr. Agami's lab (Voorhoeve et al, 2006) and Open Biosystems. I subcloned these miRNA by cutting BamHI/EcoRI (for the clones from Agami's lab) or XhoI/MluI (for the clones from Open Biosystem company) and cloning them into MSCV-PIG vector for *in vivo* study. The entire library was divided into 9 sub pools each containing ~40 miRNA (Fig 5.1).

5.2.2 Preliminary screen results

I setup the screen using the p53-/-;Myc immortalized liver cell system developed in Dr. Scott Lowe's lab (Zender et al, 2006; Zender et al, 2008, Fig 5.2). Each library pool was retrovirally infected into the immortalized liver cells and injected into nude mice as subcutaneous tumor. Among the 9 pools injected, a subset of pools significantly promoted tumor formation compared to a control shRNA (Fig 5.3), indicating these pools contain oncogenic miRNAs.

I developed a strategy to uncover scoring miRNAs from tumor genomic DNA. I amplified provirus encoded oncogenic miRNAs by PCR and cloned them into a recipient vector for high throughput sequencing (Fig 5.4A). I tested

two PCR primers complementary to the MSCV-PIG vector sequences. Primer sets #1 and #2 were able to amplify integrated miRNA from tumors (Fig 5.4B, upper panel). PIG-miR34a was used as positive control and genomic DNA from wildtype mouse tissue was used as negative control (Fig 5.4B, lower panel).

A representative sequencing result is shown in table 5.1. Among 4 individual tumors derived from pool 3, three tumors contain a substantial sequence reads for miR-23b (9.31%, 13.04% and 13.04% of the total reads respectively). Because the preinjection plasmid pool contains ~2.5% of miR-23b (pool complexity=40), this result shows a significant enrichment of miR-23b in the tumor. Therefore miR-23b is a candidate oncogenic miRNA in the screen because it is positively selected during tumor formation.

I validated miR-23b as single construct in the $p53^{-/-}$;Myc cells (Fig 5.5, p=0.01). Ecotopic miR-23b expression promoted tumor formation in this assay, suggesting it's a potential oncogenic miRNA (Fig 5.5, p=0.01). I also tested miR-27b in the assay. miR-27b is only enriched in 1/4 tumor from pool 3. In the tumor growth assay, miR-27b weakly promotes tumor growth (Fig 5.5, p=0.042).

I am still in the process analyzing the remaining injected pools. Additional enriched miRNAs will be tested similar as miR-23b.

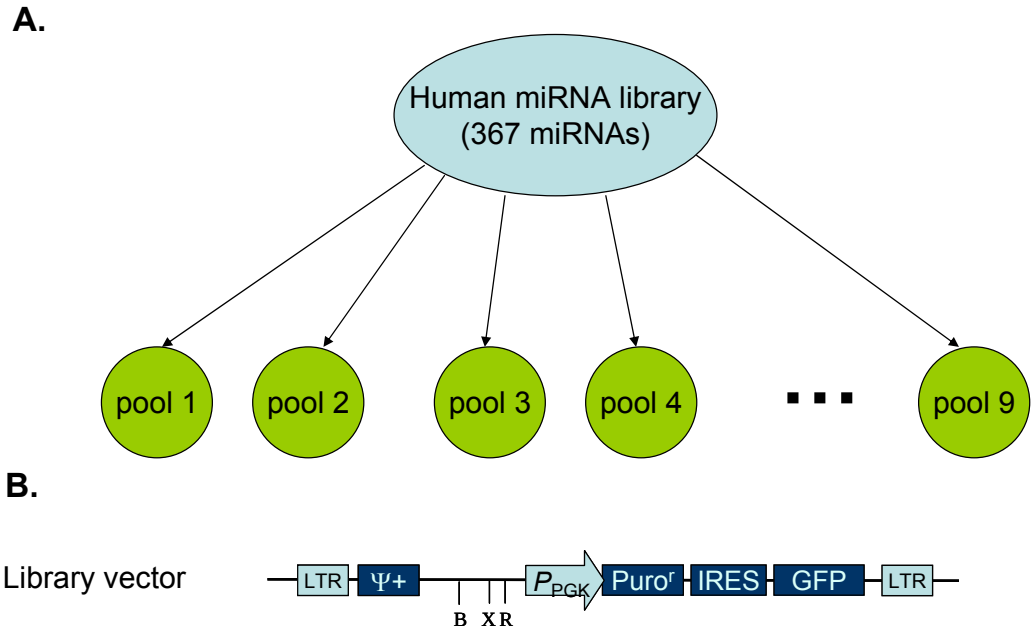

Figure 5.1. Construction of a miRNA library. A. 367 human miRNAs were subcloned into 9 library pools at pool size = 40. B. Design of the library vector. miRNAs were inserted into BglII (B) and EcoRI site (E).

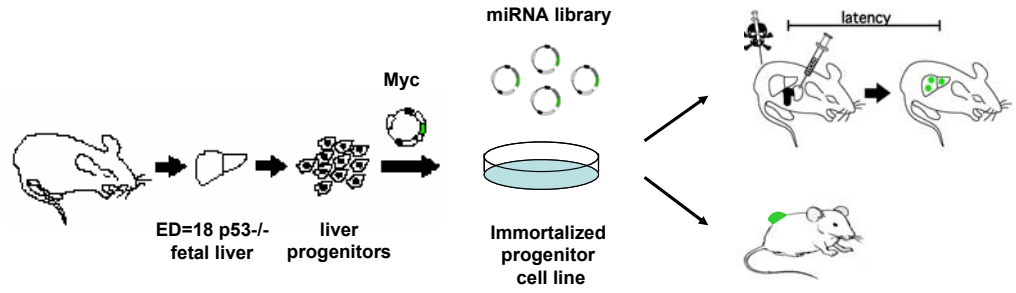

Figure 5.2. Screen setup. ED18 $p53^{-/-}$ liver progenitor cells are immortalized by transduction with a Myc-expressing retrovirus. Subsequently, the cells are infected with miRNA library pools and injected into the liver or subcutaneously to allow tumor formation.

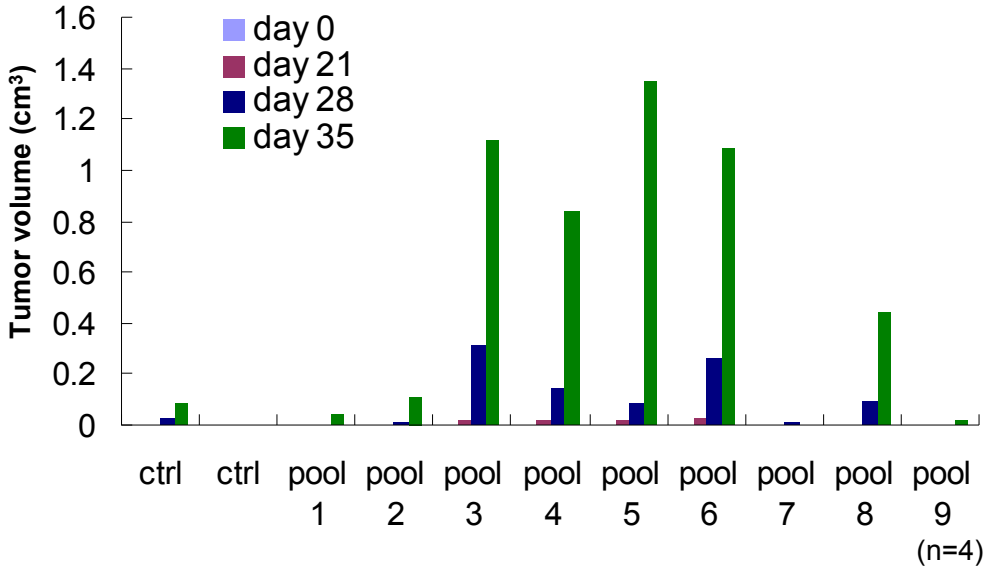

Figure 5.3. Screen results. Average volume (n = 4) of tumors derived from $p53^{-/-}$;Myc cells infected with a control shRNA (control) and 9 miRNA pools (pool size n = 40).

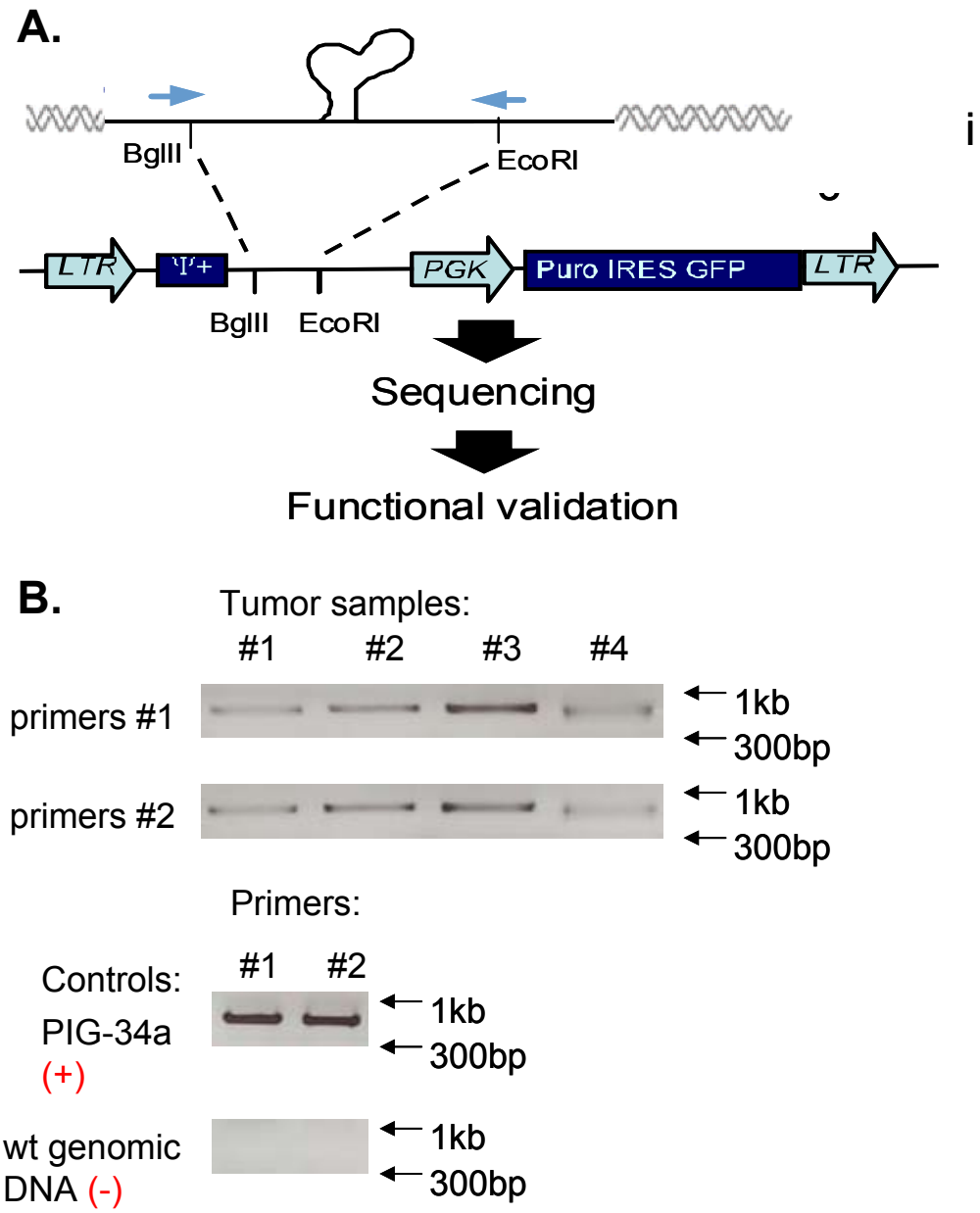

Figure 5.4. Strategy to uncover scoring miRNA from tumor genomic DNA. A. Provirus encoding oncogenic miRNA were PCR amplifided from tumor genomic DNA and cloned into a recipient vector for high throughput sequencing. B. Testing primers complementary to the MSCV-PIG vector sequences. Primer sets #1 and #2 were used to amplify integerated miRNA from four tumor (upper panel). Plasmid PIG-miR34a was used as positive control and genomic DNA from wildtype mouse tissue were used as Negative control (lower panel).

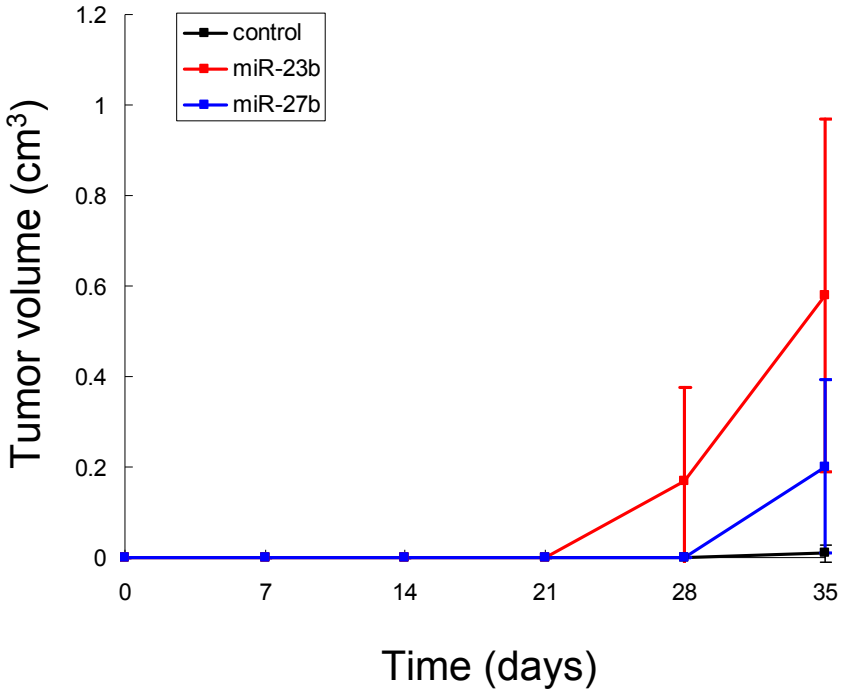

Figure 5.5. Validation of miR-23b and miR-27b as potential oncogenic miRNA. $p53^{-/-}$;Myc liver cells were infected with scoring miRNA and injected into nude mice. Error bars inducate s.d. (n=6). p=0.01 for miR-23b and p=0.042 for miR-27b (student t test).

Table 5.1. mir-23b is enriched in tumors derived from pool 3.

pool	tumor incidence	tumor ID	microRNA ID	%of sequences
3	3 of 4	2-5R	mir-25	63.04348
		2-5R	mir-30a	22.6087
		2-5R	X87	14.34783
		2-4R	mir-29b-1	31.0559
		2-4R	mir-337	14.28571
		2-4R	mir-27b	10.55901
		2-4R	mir-23b	9.31677
		2-4R	mir-342	9.31677
		2-5L	mir-346	52.17391
		2-5L	mir-320	19.11765
		2-5L	mir-23b	13.04348
		2-5L	X87	5.797101

Table 5.2. Expression levels of mir-23b and mir-27b in cancer.

Cancer type	Expression level	miRNA	Reference
Renal cancer	Down	miR-23b	O'Rourke et al., 2006
Prostate cancer	Down	miR-23b	Porkka et al., 2007
Head and neck cancer cell lines	Up	miR-23b	Tran et al., 2007
Breast, colon, lung, pancreas, prostate, and stomach cancer	Up	miR-23b	Volinia et al., 2006
Hematologic Tumor-derived cell line	Down	miR-27b	Gaur et al, 2007
Prostate cancer	Down	miR-27b	Porkka et al., 2007

5.3 Discussion

The preliminary results presented here demonstrated the feasibility of an *in vivo* genetic screen to identify oncogenic microRNAs.

This study describes a forward genetic screen for miRNAs that can promote tumorigenesis in mice. We show that a subset of pools of miRNAs were able to promote tumor formation in a mouse model of hepatocellular carcinoma. By identifying miRNAs that were enriched in the resulting tumors, we identified several candidate oncogenic miRNAs whose overexpression results in accelerated tumor formation.

The oncogenic miRNAs described here are not well characterized in cancer. Although miR-23b has been reported to be suppressed by Myc to enhance mitochondrial glutaminase (GLS) expression and glutamine metabolism glutaminase expression (Gao et al, 2009), it has been implicated to be highly expressed in some human cancers (Table 5.2). It remains interesting to functionally study how miR-23b promotes tumor formation.

miR-23b is predicted to target known of putative tumor suppressor genes such as FAS death receptor, WDR37 (WD repeat domain 37, TGFBR3 (transforming growth factor, beta receptor III), RAD17, DLG2 (discs, large homolog 2, chapsyn-110) as well as DNA damaging machinery genes such as TOP1 topoisomerase. WDR37 and RAD17 are new tumor suppressor genes identified by Dr. Lowe's lab in forward shRNA screens (Zender et al, 2008).

miR-27b also scored in our screen (Fig 5.5). miR27b is predicted to target

known tumor suppressor genes such as FBXW7 (F-box and WD repeat domain containing 7), TSC1 (tuberous sclerosis 1), SFRP1 (secreted frizzled-related protein 1) and BTG2 (BTG family, member 2). The detailed mechanisms of how miR-23b and 27b transform $p53^{-/-}$;Myc immortalized liver cells requires further study.

To further validate how miR-23b triggers tumorigenesis, I plan to identify miR-23b target genes by several approaches. I will use western blot to examine the protein levels for the candidate miR-23b targets in cell lines transfected with *miR-23b* siRNA or control luciferase siRNA or *mir-124* siRNA (which should have no effects on *mir-23* targets). To test whether regulation was direct, luciferase reporter assay will be performed. I will clone 3'-UTR of the candidate *mir-23b* target genes from genomic DNA, mutate the seed complimentary sites of *mir-23b*, and fuse the wild type and mutated 3'-UTRs separately to luciferase. The reporters will be cotransfected with *mir-23b* or control *mir-124* miRNA into 293 and Hela cell lines. If the targets are valid, the luciferase level of wild type 3'-UTR reporter will be specifically reduced upon transfection of *mir-23b*, while the repression will be attenuated when the seed complementary sequences are mutated.

The strategy outlined herein describes an approach to cancer miRNA discovery. Most current efforts to catalog cancer related miRNAs depend solely on their genomic alternation or highly expression in human cancer specimen. By incorporating our screening approach, it is possible to rapidly

filter genomic information for miIRNAs that impact cancer development *in vivo*, and all candidates can be functionally validated in various *in vitro* or *in vivo* models. Although our study used a mouse model of liver cancer, this high throughput approach can be applied to other mouse models, or to compile miRNA sub-pools that are amplified or over-expressed in human cancer. We believe that such integrative approaches will provide an effective strategy for functionally annotating the cancer miRNA genome. This strategy is also suitable for the identification of miRNAs that regulate other cellular pathways such as the DNA damage response, differentiation and chemo-resistance.

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5.5 Author contributions

X.He and G.Hannon designed the study. MSCV retroviral expression vector and mouse models are kindly provided by Dr. Scott Lowe.

Chapter 6

Discussion and Perspectives

Tumorigenesis is a multistep process that involves an accumulation of genetic lesions conferring uncontrolled proliferation, cell survival, loss of differentiation and invasive growth (Hanahan and Weinberg 2000). Previous studies have uncovered many key molecular events which lead to cancer development-activation of oncogenes and inactivation of tumor suppressors. Functional characterization of these genetic alterations not only illuminates the molecular mechanisms of tumorigenesis but also provides the molecular basis for tumor maintenance and therapy response. So far, most studies have focused on alterations in the sequence, gene structure, copy number and expression of protein coding genes which are tumor-related. However, recent studies have shown that non-coding RNAs, in particular, miRNAs, are subject to changes in gene structure and expression regulation in tumors (Voorhoeve et al, 2006). These observations have raised an intriguing hypothesis that certain miRNAs may be components of oncogenic and tumor suppressor networks that were previously unrecognized. Therefore, miRNAs have the potential to be used as new diagnostic indicators and potential therapeutic targets.

6.1 miRNA as a component of p53 tumor suppressor network

Activation of p53 leads to diverse cellular responses, including apoptosis, cell cycle arrest, blockade of angiogenesis, and activation of DNA repair (Vogelstein et al, 2000; Levine et al, 2006). The output of the p53 pathway is determined by the coordinated transcriptional activation of p53 target genes in

a context-dependent manner (Fig. 6.1). For example, p53-induced apoptosis is dependent on the induction of not only bax (Miyashita et al, 1995) but also puma and noxa (Villunger et al, 2003). The identification of the miR-34 family as p53 targets expands the repertoire of p53-regulated genes to include small RNAs. An important lesson to be drawn from this finding is the potential for small RNAs to fill roles in signaling network, which have persisted as long-standing mysteries. p53 induces growth arrest through its activation of the cdk inhibitor p21 (Levine et al, 2006). However, studies of p21- deficient MEFs indicated the existence of another pathway that worked in parallel with p21 to enforce p53-dependent G1 arrest (Brugarolas et al, 1995). Searches for protein-coding mediators of the cell cycle effects of p53 yielded several candidates, but these mainly promote G2 arrest (Levine et al, 2006). Notably, miR-34 can induce G1 arrest independently of p21 in specific cell types, raising the possibility that this small RNA may normally act in parallel with p21, filling the genetically predicted gap in this arm of the p53 pathway (He et al, 2007).

With accumulating evidence revealing the importance of miRNAs in cancer, it is now accepted that miRNAs can have tumor suppressor or oncogenic activity. For example, 13q14, a chromosomal locus deleted in >50% of B-cell chronic lymphocytic leukemias, contains two miRNAs, miR-15 and miR-16, which suppress the expression of bcl-2 and likely act as tumor suppressors (Calin et al, 2002). The miR-17-92 gene, which is amplified in B-cell

lymphomas and shows altered expression in numerous tumor types, displays oncogenic activity in a variety of models (He et al, 2005). Now, we see that miR-34s may at least participate in tumor suppression as part of the p53 network. Whether or not miR-34s are bona fide tumor suppressors in their own right awaits further study. The mir-34a gene maps to 1p36, a region of common loss in many human tumor types (Welch et al, 2007). In addition, reduced miR-34a expression is a frequent feature of both pancreatic tumors and neuroblastomas (Welch et al, 2007) and reduced miR-34b and miR-34c expression has been observed in a subset of non–small cell lung cancers (Bommer et al, 2007). In these cases, the lack of miR-34 may not simply reflect the loss of p53 as p53 is often wild-type in these tumors. Overall, accumulating evidence is leading us to remodel our notions of oncogenes and tumor suppressors to include non-coding RNAs, and as a class, these may afford new opportunities for diagnosis and treatment of human cancer.

6.1.1 Identification of p53-regulated miRNAs

The p53 network acts as a sensor for many cancer associated stress signals, including DNA damage, telomere depletion, oncogene activation, hyperactive cytokine signaling and hypoxia (Vogelstein et al, 2000). These signals are translated into effects on cell proliferation, cell death, DNA repair and angiogenesis (Vogelstein et al, 2000) through the function of p53 as a sequence-specific transcriptional regulator. Despite extensive efforts over the past three decades to link downstream targets of p53 to specific biological

effects, many puzzles remain. For example, several studies indicated that p53 could also repress the expression of target genes through a mechanism that had not been fully elucidated (Yu et al, 1999; Zhao et al, 2000). Moreover, genetic studies of p53-regulated protein-coding genes had not yet provided a complete picture of how regulation of these targets might lead to commonly observed effects of p53 activation, such as G1 cell-cycle arrest or apoptosis, in all tissue settings (Brugarolas et al, 1995). Given the potentially broad consequences of activating miRNA expression, it seemed possible that non-coding RNAs might contribute to p53 function. This prompted several efforts to search for links between p53 and miRNAs. These converged into one exciting finding — the identification of miR-34s as key p53 transcriptional targets capable of regulating cell proliferation and cell death (for review see Hermeking et al, 2009). Most studies focused on examining global miRNA expression profiles and correlating expression patterns with p53 status. We profiled miRNAs in wild-type and p53-null mouse embryonic fibroblasts (MEFs) carrying various additional oncogenic lesions (He et al, 2007). Raver-Shapira et al. studied a lung cancer cell line harbouring a temperature-sensitive TP53 allele (Raver-Shapira et al, 2007). Chang and co-workers set out to identify miRNAs whose expression increased after genotoxic stress in a p53-dependent manner (Chang et al, 2007), and Tarasov et al. launched their screen for p53-regulated miRNAs using a tetracycline-inducible TP53 allele (Tarasov et al, 2007). Using a complementary, bioinformatic approach,

Bommer and colleagues revisited a previous study of genome-wide p53 chromatin immunoprecipitation (ChIP) (Wei et al, 2006), in which all putative p53 binding sites had been attributed to their nearest protein-coding genes. In all of these studies, miR-34 family members emerged as prime candidates for p53-regulated miRNAs. First identified in Caenorhabditis elegans, mir-34 encodes an evolutionarily conserved miRNA, with single orthologues in several invertebrate species. In vertebrates, mir-34 diverged into a family of three homologous miRNAs — mir-34a, mir-34b and mir-34c. The mature mir-34a sequence is located within the second exon of its non-coding host gene, nearly 30 kb downstream of its first exon, which contains a predicted p53 binding site (He et al, 2007). Both mir-34b and mir-34c are located within a single non-coding precursor (mir-34b/c), whose transcriptional start site is adjacent to a predicted p53 binding site. Both of these p53-binding sites are evolutionarily conserved and match the consensus derived from p53-regulated protein-coding targets (Wei et al, 2006). Extensive studies were carried out, both *in vitro* and *in vivo*, to validate the regulation of miR-34 family miRNAs by p53. Both exogenous and physiological stresses are able to induce miR-34 expression in multiple cell culture systems and animal tissues in a p53-dependent manner. The induction of miR-34s by p53 doesnot rely on de novo protein synthesis (Raver-Shapira et al, 2007), but does depend on having intact p53 binding sites within their putative promoter regions (He et al, 2007; Raver-Shapira et al, 2007). The kinetics and magnitude of miR-34 induction is

comparable to that observed for the canonical p53 target, the cyclin-dependent kinase (CDK) inhibitor p21 (Tarasov et al, 2007), as is the approximate binding affinity of p53 for mir-34 promoters as measured by ChIP. Together, these studies provide compelling evidence that miR-34 miRNAs are bona fide p53 targets. Thus, p53 acts as a tumour suppressor by both positively and negatively regulating gene expression; the negative regulation occurs, at least in part, through the positive effects of p53 on the expression of noncoding RNAs.

6.1.2 miR-34 mimics the effects of p53

Given a solid connection between miR-34 and p53, studies quickly shifted focus to answering one key biological question — is miR-34 sufficient and/or necessary for any of the biological outcomes elicited by p53? So far, these have mainly probed two of the best-studied p53 outputs, growth arrest and apoptosis. The effects of simulating miR-34a activation, either through delivery of synthetic mature miRNAs or through ectopic expression of miRNA precursors, were examined in various biological systems. In most cases, key p53 effects were recapitulated in a context-dependent manner. Overexpression of miR-34a in primary fibroblasts and in certain tumour cell lines produced a significant cell-cycle arrest, evident by an increase in the G1 population at the expense of the S-phase population (He et al, 2007). It is worth noting that when miR-34 is ectopically overexpressed in IMR90 human lung fibroblast cells, ~60% of the infected cells exhibit morphological and

molecular alterations characteristic of cellular senescence. In a different set of cell types, mostly tumour cell lines, the observed effect of miR-34a overexpression was an increased apoptotic response, although the degree of cell death varied. Interestingly, in the case of HCT116 colon cancer cell lines, the predominant effect of miR-34a overexpression was growth arrest at 48 hours after transfection, but apoptosis at 72 hours after transfection (Chang et al, 2007; He et al, 2007). Mature miR-34b and miR-34c are nearly identical to miR-34a and have similar biological activities in several proliferation assays (Bommer et al, 2007; He et al, 2007). Given the potential of miRNAs to recognize many targets through imperfect base pairing, the pleiotropic effects of miR-34 may simply reflect the different spectrum of target mRNAs available in a given system, and this may represent an interesting theme within miRNA-mediated regulatory pathways. Although the pro-apoptotic effects of miR-34a are modest, miR-34a is essential for p53-mediated apoptosis in some settings. Direct support for a crucial role of miR-34a in p53-induced cell death came from the study by Raver-Shapira et al., in which inhibition of miR-34a by LNA (lockednucleic acid) oligos strongly attenuated p53-dependent apoptosis in U2OS cells in response to genotoxic stress. LNA oligos are locked in the 3′ endo conformation, thus increasing their hybridization energy. This property is exploited in the use of these agents as competitive inhibitors of miRNA activity. It is worth noting that in that study, the pro-apoptotic effect of miR-34a overexpression, albeit in MCF7 and H1299 rather than U2OS cells, was

relatively mild. In addition, loss of mir-34a in mouse embryonic stem cells dampened the apoptotic effects of differentiation stimuli such as addition of retinoic acid and withdrawal of leukaemia inhibitory factor (LIF) (Bommer et al, 2007). These studies did not resolve the question of whether miR-34 is essential for p53-mediated G1 arrest. Given the redundancy in both cell-cycle regulatory pathways and in the miR-34 family, addressing this issue will probably have to wait until genetic lesions in all three mir-34 homologues are created.

6.1.3 Mechanisms of miR-34 action

miRNAs act by inhibiting gene expression. Thus the precise mechanisms by which miR-34 contributes to p53 activity can be revealed through identification of its regulatory targets. Both bioinformatic and experimental approaches have been used to address this issue (Lewis et al, 2005), and consistent with the predicted p53–miR-34 circuit, some miR-34-regulated genes are repressed after p53 activation (Spurgers et al, 2006). Microarray analysis showed that the induction of miR-34s led to the downregulation of hundreds of mRNAs, which were enriched for cell cycle regulators. Collectively, these were also enriched for mRNAs that could bind miR-34 seed regions, and several individual genes, including CDK4, CDK6, cyclinE2 and E2F3 have been experimentally validated as miR-34 targets by western blotting. In most cases, a direct regulatory relationship was established by fusing the 3′ untranslated region (UTR) of each candidate target (containing the miR-34

seed complementary region) to luciferase and demonstrating miR-34-induced repression (He et al, 2007). Unlike proliferation genes, antiapoptotic genes as a whole are not enriched in the miR-34-repressed set or in bioinformatics predictions of miR-34 targets. However, the anti-apoptotic protein BCL2 is downregulated by miR-34 in several cell types, consistent with a role for miR-34 in p53-mediated apoptosis (Bommer et al, 2007). In classic studies of miRNA function in worms and flies, it was often true that one or few miRNA targets could account for the regulation of a specific biological process (Lee et al, 1993; Wightman et al, 1993). Although the suppression of any of several miR-34 targets can mimic its biological effects, it seems most likely that collective regulation of multiple genes by miR-34 is responsible for the full range of its physiological effects.

6.1.4 miR-34 in the midst of the p53 pathway

The CDK inhibitor p21 was recognized more than a decade ago as a key mediator of p53-induced growth arrest. However, disruption of p21 in mice failed to completely negate this output of the p53 pathway (Brugarolas et al, 1995). This predicted the existence of other essential players in this process, which had not been discovered after more than a decade of searching. A possible explanation for this failure is the exclusive focus on protein-coding genes, as these new results have raised the possibility that noncoding RNAs cooperate with protein-coding genes in various p53 effector pathways. The placement of a miRNA in the p53 pathway may also help to explain another

longstanding mystery. An examination of p53-responsive transcriptional programmes revealed a large number of genes that are quickly repressed upon p53 activation (Yu et al, 1999; Zhao et al, 2000). It now seems likely that at least some of these observations can be explained as secondary effects of the induction of repressive small RNAs. These recent reports raise the question of whether there might be other miRNAs that connect to p53 — either as downstream effectors or as regulators of p53 or its modifiers. Searches for p53-regulated miRNAs predated the recent studies of miR-34 (Xi et al, 2006). As many as a dozen miRNAs exhibit expression patterns indicative of p53-dependent regulation. Several different studies have generated largely non-overlapping sets of miRNA as candidate p53 targets, possibly owing to the differences in the biological systems studied and the detection methods used. A small number of miRNAs, including miR-26a and miR-182, were identified in multiple independent studies and are interesting candidates for further investigation (Chang et al, 2007). Strikingly, the interplay between p53 and miRNAs may not be limited to a purely linear relationship. Overexpression of miR-372 and miR-373 can bypass senescence induced by oncogenic Ras, which depends on p53 (Voorhoeve et al, 2006). This effects achieved by suppression of a p53 target, LATS2 (Voorhoeve et al, 2006), which not only inhibits proliferation, but also forms a positive-feedback loop with p53 (Tam et al, 2006).

6.1.5 More microRNAs join the p53 network

We are only beginning to understand the role of non-coding RNAs in cancer. The discovery that a conserved family of miRNAs is central to a crucial tumour-suppressor pathway may reflect ancient connections between noncoding RNAs and the regulation of developmental and physiological decisions, whose disruption can lead to tumour development. We identified miR-34 as a tumor suppressive component downstream of p53. Recently, more miRNAs have been found to be involved in the p53 network. miR-192 and miR-215 were identified as p53 induced miRNAs (Song et al, 2008; Georges et al, 2008). In addition, miRNAs are found to be at levels upstream of p53. For example, microRNA-125b is a novel negative regulator of p53 (Minh et al, 2009). Minh et al, demonstrated that miR-125b, a brain-enriched microRNA, downregulates of p53 depending on the binding of miR-125b to a microRNA response element in the 3'UTR of p53 mRNA. Overexpression of miR-125b represses the endogenous level of p53 protein and suppresses apoptosis in human neuroblastoma cells and human lung fibroblast cells. In contrast, knockdown of miR-125b elevates the level of p53 protein and induces apoptosis in human lung fibroblasts and in the zebrafish brain. These results demonstrate that miR-125b is an important negative regulator of p53 and p53-induced apoptosis. These observations suggestion in addition to miR-34a, many miRNA may play important roles in modulating the function of p53 tumor suppressor network.

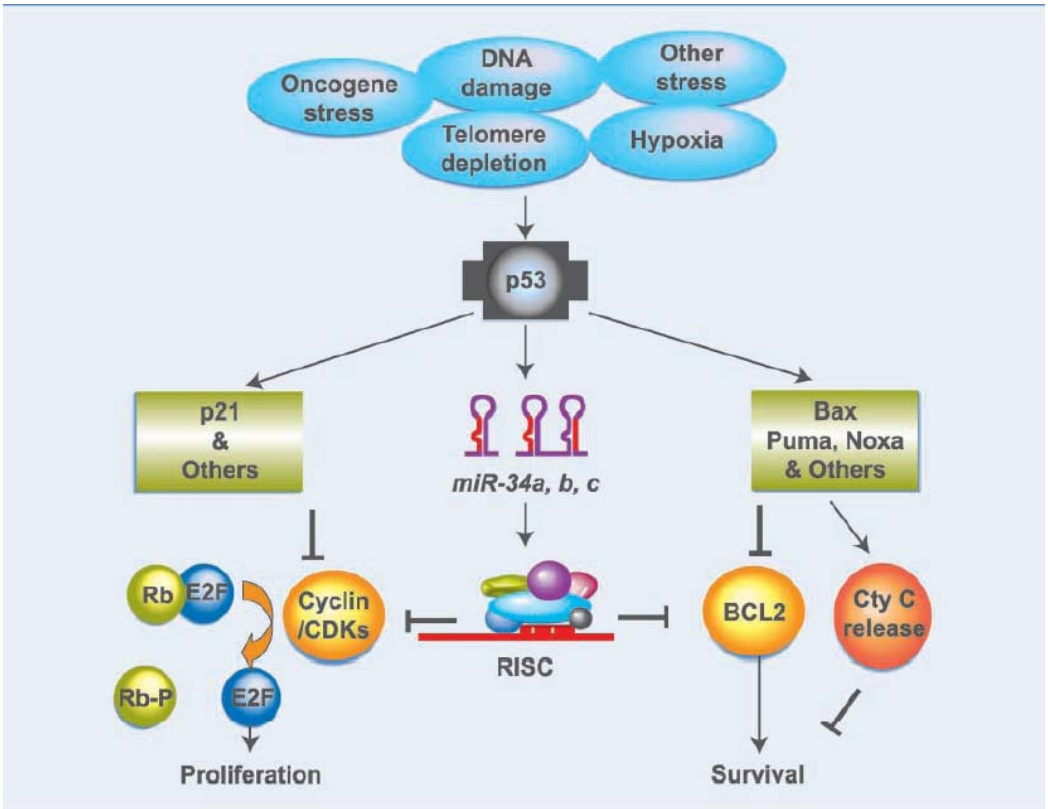

Figure 6.1. A model of the p53–miR□ 34 network in regulating cell proliferation and cell death. miR□34 is a direct transcriptional target of p53, which in turn downregulates genes required for proliferation and survival. Along with other p53 targets, such as p21 and BAX, miR□34-family miRNAs promote growth arrest and cell death in response to cancer related stress. ATM, ataxia talangiectasia mutated; ATR, ataxia telengiectasia and RAD3-related; CDK, cyclin-dependent kinase; CHK, checkpoint kinase

6.2 miR-34s regulate many biological pathways

A constant challenge of studying miRNA function is to determine the target genes regulated by a particular miRNA. A combination of bioinformatics and experimental approaches is often performed to identify miRNA targets. Recent literature and bioinformatics prediction suggest that miR-34 may regulate many biological pathways in addition to modulating cell cycle or apoptosis genes.

6.2.1 Experimentally validated miR-34a targets

We and others determined that miR-34a regulates cell cycle genes (CDK4, CCNE2, MET). Downregulation of these cell cycle genes lead to G1 arrest or cellular senescence (He et al, 2007). Recently, additional miR-34a target genes were identified such as E2F3 and Bcl2 (Table 6.1) (Hermeking et al, 2009). The level of miR-34 expression and cellular genetic context may affect the decision between apoptosis and cell-cycle arrest. Moreover, miR-34 is validated to regulate key oncogenes such as c-Myc and N-Myc.

c-MYC, which is an essential oncogene in many cancer types, is a direct target of miR-34b/c (Leucci et al, 2008; Kong et al, 2008). c-Myc is a multifunctional transcription factor that plays a role in cell cycle progression, apoptosis and cellular transformation. It regulates transcription of specific target genes. Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas, including Burkitt lymphoma. Loss of miR-34b/c will

relieve its suppression on c-Myc level and result in accumulated c-Myc level.

N-MYC, which is often deregulated in brain tumors, is a direct target of miR-34a (Cole et al, 2008). N-Myc is a member of the MYC family and encodes a protein with a basic helix-loop-helix (bHLH) domain. This protein is located in the nucleus and must dimerize with another bHLH protein to bind DNA. Amplification of this gene is associated with a variety of tumors, most notably neuroblastomas.

An intriguing study suggests that miR-34a positively feeds back to p53 (Yamakuchi et al, 2008). miR-34a was shown to target SIRT1 mRNA leading to translational repression of SIRT1. SIRT1 is an NAD-dependent deacetylase, which has been shown to inhibit several pro-apoptotic proteins. Yamakuchi et al confirmed the targeting of SIRT1 mRNA by miR-34a. They showed that SIRT1 increases p53 acetylation on lysine 382 after miR-34a expression. This was associated with increased transcriptional activity of p53 and increased apoptosis. Their data suggest the regulation of SIRT1 by miR-34a is part of a positive feedback loop that leads to further activation of p53, once it has been activated (Hermeking et al, 2009). In addition, our data show that miR-34a also down-regulates Mdm4 at the protein level (data not shown). Mdm4 is a p53 regulator and inhibits p53 transcriptional activity by binding to p53 transcriptional activation domain (TAD). Our data indicate another positive feedback loop which can further activate p53 by its target mir-34a.

Table 6.1 Validated miR-34a targets (Hermeking et al, 2009).

Gene	Down-regulate by miR-34a,b,c	Function	Cancer related
Bcl2	miR-34a	Apoptosis	Y
CDK4	miR-34a	G1 arrest	Y
CDK6	miR-34a,b	G1 arrest	Y
CCND1	miR-34a	G1 arrest	Y
CCNE2	miR-34a	G1 arrest	Y
CREB	miR-34b	Inhibition of proliferation	N.D.
DLL1	miR-34a	Notch signaling	Y
E2F3	miR-34a miR-34c	Inhibition of proliferation, senescence	Y
MET	miR-34a,b,c	G1-arrest, inhibition of invasion and migration	Y
c-Myc	miR-34b,c	G1 arrest	Y
N-Myc	miR-34a	G1 arrest	Y
SIRT1	miR-34a	Increased p53 acetylation and activation	N.D.

6.2.2 Additional miR-34a targets predicted by bioinformatic algorithms

Several published algorithms for predicting miRNA targets are widely used to identify candidates for experimental validation. The core of such prediction is the use of experimentally confirmed miRNAs and targets as a learning matrix to summarize rules for base-pairing, for free energy and for sequence conservation at the target sites. Most algorithms require perfect or nearly perfect base pairing between the 8bp 5' seed sequence of a miRNA and its complementary site at the target mRNA's 3'UTR. These prediction programs yield a list of mRNAs that are potential miRNA targets (Table 6.2) in addition to the experimentally validated miR-34a targets, thereby shedding new light on the diverse biological functions of miR-34.

As shown by Table 6.2, I examined the commonly used miRNA target prediction database, including TargetScan, miRanda and Pictar-VERT, for predicted *mir34* targets that have a well demonstrated function in promoting cell cycle progression and other biological pathways. Those targets predicted by TargetScan, a program developed by David Bartel and Chris Burge, have received the most extensive experimental validation. I collect a list of candidate miRNA targets for *mir-34* from these prediction algorithms, selecting those with a high probability score and well-characterized biological functions (Table 6.2). Some of the predicted targets suggest novel functions of miR-34a that requires further investigation.

Table 6.2 More miR-34a targets found by multiple prediction tools.

Gene	MIRANDA	TARGETSCAN	PICTAR-VERT	Cancer related	Down-regulate by miR-34a,b,c
DLL1	Y	Y	Y	Y	N.D.
NOTCH1	Y	Y	Y	Y	Y
NOTCH2	N	Y	Y	Y	N.D
JAG1	Y	Y	Y	Y	N.D
PHGDH	Y	N	N	ND	Y
PGM1	Y	N	Y	ND	N.D.
MLLT3	N	Y	Y	Y	N.D
MAP2K1	N	Y	Y	Y	Y

miR-34a and NOTCH pathway

The Notch signaling network is an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells (Allenspach et al, 2002). In Drosophilia and human, notch interaction with its cell-bound ligands (delta, serrate) establishes an intercellular signaling pathway that plays a key role in development. NOTCH protein is cleaved in the trans-Golgi network, and presented on the cell surface as a heterodimer to function as a receptor for membrane bound ligands, and play multiple roles during development and cancer.

DLL1 is a validated miR-34a target gene (Bommer et al ,2007). DLL1 is a human homolog of the Notch Delta ligand and is a member of the delta/serrate/jagged family. It plays a role in mediating cell fate decisions during hematopoiesis. It may play a role in cell-to-cell communication.

Interestingly, miR-34a may also regulate other genes in the NOTCH pathway. NOTCH1 is downregulated after ectopic miR-34 expression (Hermeking et al, 2009). Although experimental evidence of miR-34a targets NOTCH1 3' UTR is lacking, all three databases indicate NOTCH1 has matched 3'UTR sequences complementary to miR-34a seed sequence (Table 6.2). NOTCH1 encodes a member of the Notch family. Members of this Type 1 transmembrane protein family share structural characteristics including an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats, and an intracellular domain consisting of multiple, different domain

types. Notch family members play a role in a variety of developmental processes by controlling cell fate decisions. Similarly, NOTCH2 and JAG1 are also predicted miR-34a targets (Table 6.2). NOTCH2 is a family member of NOTCH gene. JAG1 the ligand for the receptor notch 1. Mutations in NOTCH pathway have been revealed in human cancer. Jagged 1 signalling through notch 1 has also been shown to play a role in hematopoiesis.

As miR-34a can potentially down-regulate several important genes (eg, DLL1) in the NOTCH pathway, it will be interesting to further study miR-34's role in regulating NOTCH signaling.

miR-34a and metabolic enzymes

In our microarray data, a metabolic enzyme PHGDH is consistently downregulated in miR34a/b/c transfected cells (data not shown), suggesting miR-34a may modulate cancer cell metabolism. PHGDH (3-Phosphoglycerate dehydrogenase) catalyzes the transition of 3-phosphoglycerate into 3-phosphohydroxypyruvate, which is the first and rate-limiting step in the phosphorylated pathway of serine biosynthesis, using NAD^+/NADH as a cofactor. MIRANDA predicts PHGDH as a potential miR-34a target gene (Table 6.2).

A second enzyme, PGM1 (Phosphoglucomutase-1**)**, is also a predicted miR-34a target. This enzyme catalyzes the transfer of a phosphate group between the 1- and 6-positions of glucose. If PGM1 and PHGDH can be experimentally validated as miR-34a targets this will link miR-34a to the

regulation of glycolysis and other relevant metabolic pathways.

6.3 Loss of miR-34 in cancer

Deletions of mir-34 family miRNAs have been reported in human cancers. Mir-34a is located within 1p36, a region of frequent heterozygous deletion in many tumour types (Versteeg et al, 2005). The chromodomain protein CHD5, another candidate tumour suppressor located within 1p36, is capable of activating p53 through ARF (encoded by CDKN2A). Thus, the loss of 1p36 may affect the integrity of the p53 pathway both upstream and downstream of p53. Minimal deletions containing mir-34b and mir-34c have also been found in breast and lung cancer (Calin et al, 2004), which is consistent with significant reduction of miR-34b/c expression in non-small-cell lung cancer cell lines (Bommer et al, 2007). In human tumours, the selective pressure to lose miR-34s may be relieved by frequent mutation of p53. Thus, genetic alterations in mir-34s are more likely to occur in tumour types that contain wild-type p53. For example, Welch and colleagues reported that mir-34a is frequently deleted or downregulated in neuroblastoma cell lines (Welch et al, 2007; Gaur et al, 2007).

In addition to deletion of miR-34 genomic locus, low expression of miR34 and its promoter methylation has also been also reported in cancer. The expression of miR-34a was low or undetectable in pancreatic cancer cell lines (Chang et al, 2007) and the expression level of miR-34b was decreased in non-small cell lung cancer (Bommer et al, 2007). More recently, the epigenetic

inactivation of miR-34a was detected in cancer cell lines and also in primary melanoma (Lodygin et al, 2008). In addition, CpG methylation of miR-34b/c was found in colorectal cancer (Toyota et a, 2008) and in squamous cell carcinoma (Kozaki et al, 2008). Furthermore, miR-34a expression is downregulated in rat models of liver cancer (Tryndyak et al, 2009). Taken together, inactivation of the miR-34 is a common event in human cancer, suggesting miR-34 may be a relevant tumor suppressor.

6.4 miR-34 loss of function animal models

To date, miR-34 knockout mouse models are not available. As shown in the results section, I have generated miR-34a germline knockout animals (see chapter 4). As miR-34a, b, c can have redundant functions in suppressing their target genes, it will be important to knockout all three miRNA members to obtain a tumor prone phenotype. Moreover, p21 pathway may compensate the cell cycle arrest phenotype rendered by miR-34 loss. This may further confound the phenotype of miR-34a animals. A compound cross to generate miR-34a; p21 double knockout mice may be required to dissect miR-34's role as an important tumor suppressor gene.

6.5 Genome wide oncogenic miRNA screen

Our initial oncogenic miRNA screen identified miR-23b and miR-27b as candidate oncogenic microRNAs (Chapter 5). Although miR-23b has been reported to be suppressed by Myc to enhance mitochondrial glutaminase (GLS) expression and glutamine metabolism glutaminase expression (Gao et al,

2009), miR-23b can have oncogenic function in a context dependent manner by regulating different set of target genes. The predicted miR-23b target genes include known tumor suppressor genes such as FAS death receptor, TGFBR3 and DLG2. Functionally validation of these potential targets is needed to establish the mechanism how miR-23b functions as an oncogenic microRNA.

6.6 Future perspective -- miRNA as cancer therapy

Large-scale expression studies of miRNA profiles in multiple human tumor types have revealed that miRNA signatures are correlated with the developmental lineage and differentiation status of various tumors. Moreover, miRNA signatures can be used to identify certain poorly differentiated tumors, many of which were difficult to classify based on mRNA profiles (Lu, et al, 2005). Such findings suggest an unexpected potential of miRNAs as diagnostic tools, and possibly as a tool to stratify patients with selective miRNA alterations for targeted therapies using miRNA or miRNA anatogists.

Recent advance in *in vivo* delivery of synthetic miRNA or its antagonists (eg, LNA) suggest mIRNA can be applied as cancer therapy. With accumulating evidence revealing the importance of miRNAs in cancer, it is critical to explore miRNA's value as novel therapeutical targets and/or diagnosis markers. Since sequence-specific miRNA or miRNA antagonists can be delivered *in vivo* to nearly all tissues except the brain (Krutzfeldt, et al, 2005), it's technically possible to inhibit certain oncogenic miRNAs (miR-17-92, miR-21,etc) or to deliver a tumor suppressive miRNA into tumor cells (let7.

miR-34a) to suppress tumor proliferation. We've shown that constitutive or conditional expression of miR-34a by retroviral vector can effectively delay liver tumor progression (Chapter 3). Our preliminary data also show that *in vivo* delivery of miR-34a mature siRNA duplex can delay tumor growth of Ras driven murine liver tumor with p53 deficiency. This data implicate the possibility of tumor suppressor miRNA as potential therapeutic tools. The fact that $p53^{-/-}$ tumors respond to miR-34a suggest that miR-34a, as a p53 target gene, can serve as a rescue molecule to treat tumors with p53 deficiency.

If future studies can demonstrate the efficacy of miRNAs as anti-cancer treatment agents, miRNA will join the small molecule chemical drugs to provide targeted therapies to treat cancer.

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Chapter 7

Material and methods

Quantification of miRNAs with real-time PCR.

TaqMan MicroRNA assays were used to quantify the level of mature miRNAs as described previously. In miRNA profiling experiments, each reverse transcriptase (RT) reaction contained 3.75 ng of purified total RNA, 50nM stem–loop RT primer, 13RT buffer, dNTPs (each at 0.25 mM), 3.33Uml21 MultiScribe reverse transcriptase and 0.25uml21 RNase inhibitor (Applied Biosystems). The reactions were incubated for 30 min at 16 degreee, 30 min at 42 uC, and 5 min at 85 uC. Real-time PCR reactions for each miRNA (10 ml volume) were performed in quadruplicate, and each 10-ml reaction mixture included 2 ml of diluted RT product (1:2 dilution), 5 ml of 23TaqMan Universal PCR Master Mix, 0.2 mM TaqMan probe, 1.5 mM forward primer, and 0.7 mM reverse primer, respectively (Applied Biosystems). Reactions were incubated in an Applied Biosystems 7900HT Fast Real-Time PCR system in 384-well plates at 95 uC for 10 min, followed by 40 cycles at 95 uC for 15 s and 60 uC for 1 min. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence exceeds the fixed threshold of 0.2. Primary transcripts level of mir-34a and mir-34b/c were determined with the SuperScript III SYBR Green One-Step qRT–PCR system (Invitrogen). Primers that amplify the mir-34a pri-miRNA, mir-34b/c pri-miRNA and the control *b*-actin mRNA were designed with Primer Express software, v. 2: hsa-pri-mir-34a forward primer, 5'-CCTCCAAGCCAGCTCAGTTG-3'; hsa-pri-mir-34a reverse primer, 5'-TGACTTTGGTCCAATTCCTGTTG-3'; hsa-pri-mir-34b/c forward primer,

5’-GCTCTTTGTCCCTCCTGCTAGA-3’; hsa-pri-mir-34b/c reverse primer, 5’-GTGGGCGGTCCCTGAAG-3’; mmu-pri-mir-34a forward primer, 5’-CTGTGCCCTCTTGCAAAAGG-3’; mmu-pri-mir-34a reverse primer 5’-GGACATTCAGGTGAGGGTCTTG-3’; mmu-pri-mir-34b/c forward primer, 5’-GGCAGGAAGGCTCCAGATG-3’; mmu-pri-mir-34b/c reverse primer, 5’-CCTCACTGTTCATATGCCCATTC-3’. The ratios of RNA species in each sample were determined in triplicate with the use of an ABI 7900HT TaqMan sequence detector following the standard curve method.

Hierarchical clustering for miRNA expression profiling.

Expression of 145 mouse miRNAs was characterized and the data were subjected to a series of adjustment and filtering before hierarchical clustering. Assays with Ct values greater than 35 were treated as 35, and low-expressing miRNAs were then removed from the analysis if their average Ct values across the samples were between 34 and 35. Four miRNAs (miR-30d, miR-148b, miR-320 and let-7d) that were least variable among the 16 samples used in this study were selected as internal references, and DCt between the Ct of each miRNA and the average of these four references for each sample were calculated. Data from each of the resulting 115 miRNAs were median-centred, and both miRNAs and samples were clustered by using the average linkage method under the correlation similarity metric.

Chromatin immunoprecipitation.

Wild-type MEF and $p53^{-/-}$ MEF cultures were grown to 70–80% confluence and

then treated with doxorubicin (0.5 mgml21) for 24 h. After being washed with PBS, cells were crosslinked with 1% formaldehyde for 15 min at room temperature. Crosslinking was stopped by the addition of glycine to 125mM final concentration. Cells were washed twice with cold PBS and then harvested in lysis buffer first (20mM Tris-HCl pH8.0, 85mM KCl, 1mM EDTA, 0.5mM EGTA, 0.5% Nonidet P40, and protease inhibitors), and the pellets were then dissolved in nuclear lysis buffer (50mM Tris-HCl pH 8.0, 10mMEDTA,1% SDS, and protease inhibitors). Samples were sonicated to generate DNA fragments less than 500 base pairs in length. Before immunoprecipitation, nuclear extracts were precleared at 4 uC with 50 ml of 50% Protein A–Sepharose slurry for 30 min. p53 antibody (CM5; Novocastra) was then added to form complexes with p53 protein and associated chromatin. These immunocomplexes were recovered with Protein A–Sepharose beads (Upstate), and the associated DNA was purified by extraction with phenol/chloroform. The enrichment across the putative p53 binding sites at both miR-34a and miR-34b/c were then tested by real-time PCR analysis. Primers 5'-CAGCCTGGAGGAGGATCGA-3' and 5'-TCCCAAAGCCCCCAATCT-3' were used to amplify the mir-34a promoter regions containing the putative p53 binding sites; primers 5'-GTTGATCCTGCCCACAGTTACTAGA-3' and 5'-ATTAAAACATGAGTCTCCCTGGTCTCT-3' were used to amplify the mir-34b/c promoter regions containing the putative p53 binding sites. Two sets of primers designed to amplify the 39 end of the gene encoding the miR-34a

precursor and the gene encoding the miR-34b/c precursor, respectively, were used as controls.

Proliferation and cell cycle analysis

Primary IMR90 cells and MEFs were infected with MSCV retroviral vectors directing the expression of mir-34a or mir-34b/c from the long terminal repeat promoter. Infected cells were selected by puromycin for 2 days, left to recover for 12 h and then used for a variety of growth assays. Growth curves were measured by determining accumulative population doublings over a course of 12 days after puromycin selection. At day 1 after selection, IMR90 cells were labeled with Bride for 6 h, and G1, S and G2/M populations were measured by the Bride APC flow kit (BD Biosciences). SA-b-Gal staining was conducted for IMR90 cells at 3, 6 and 9 days after selection with puromycin. Cells were fixed with 0.5% glutaraldehyde in PBS for 15 min at room temperature. After being washed with PBS/MgCl2 (1mMMgCl_2, pH 6.0), cells were stained in X-Gal (1 mg/ml X-Gal, 0.1M $K_3Fe(CN)_6$, 0.1M $K_3Fe(CN)_6$.3H2O, 1mM $MgCl_2$, in PBS, pH 6.0). Tumour-derived cell lines including A549, HCT116 and HCT116 $p21^{-/-}$ cells were transfected with luciferase siRNA GL-3 or miR-34a–c siRNA duplexes (100 nM). At 24 h after transfection, the cells were treated with nocodazole (100 ng ml21) for 16–20 h. Cell cycle distributions were measured by staining with propidium iodide, followed by FACS.

Microarray profiling.

Cells were plated 24 h before transfection. HCT116 DicerEx5 cells were

transfected in six-well plates with duplexed RNAs with the use of Lipofectamine 2000 (Invitrogen). DLD-1, HeLa, TOV21G and A549 cells were transfected by using SilentFect (Bio-Rad). Duplexes were used at final concentrations of 100nM for all cell lines. RNA was isolated 6–24 h after transfection, and microarray analysis was performed as described28.

Gene set analysis methods.

miRNA-regulated transcripts were identified in microarray gene expression signatures using a P-value cut-off (P,0.01). miRNA downregulated transcripts were defined by the intersection of downregulated transcripts in all the lines tested. Downregulated transcripts were tested for enrichment relative to a background set with the use of the hypergeometric distribution. miRNA target regulation was measured by enrichment of transcripts containing miRNA hexamer seed strings (stretches of six contiguous bases complementary to miRNA seed region nucleotides 1–6, 2–7 or 3–8) in transcripts having annotated 39 UTRs. Biological function was categorized by enrichment of transcripts from Gene Ontology Biological Process functional categories (http://www.geneontology.org/). The set of genes on the microarray was used as a background set.

Cell lines. Wild-type MEFs and $p53^{-/-}$ MEFs were isolated from embryonic day (E)13.5 embryos. HCT116, HCT116 $p53^{-/-}$, HCT116 $Dicer^{ex5}$, HCT116 $p21^{-/-}$ and DLD-1 $Dicer^{ex5}$ cells were provided by B. Volgelstein's group. HEPG2, A549, 3T3 and TOV21G calls were acquired from ATCC. HEPG2, A549 and

TOV21G cells were engineered to contain an empty vector or a vector expressing a hairpin targeting human p53. For these cells, the p53 shRNA was a 19-mer stem–loop cloned under the control of a human H1 promoter29 and shuttled into the pLenti6 Block-It vector (Invitrogen). The engineered lines were generated by stable transduction with packaged lentiviral particles. The knockdown level of p53 was about 95%.

Western analysis.

Antibodies againstCDK4(c-22; dilution 1:1,000; Santa Cruz), CCNE2 (dilution 1:1,000; Cell Signaling), MET (25H2; dilution 1:1,000; Cell Signaling) and phospho-Rb (dilution 1:2000; Santa Cruz) were used in western analysis in accordance with the manufacturer's instruction. Tubulin (Sigma) was blotted for a loading control, as well as for subsequent quantification.

miR-34-mediated suppression reporter assays.

The 39 UTRs from human CDK4, CCNE2 and MET were amplified from human genomic DNA (Promega) and individually cloned into pEntr/D (Invitrogen) by directional TOPO cloning. Seed regions were mutated to remove all complementarity to nucleotides 1–7 of miR-34s by using the QuickchangeXL Mutagenesis Kit (Stratagene). Both wild-type and mutant 39 UTRs were transferred into pGL3-TK (Promega) with the use of Gateway cloning (Invitrogen). HeLa cells were cotransfected with reporter constructs and miRNAs (miR-34a or miR-124a) in the form of siRNAs using LT1 and TKO (Mirus). pRLTK (Promega) was also transfected as a normalization control.

Cells were lysed 24 h after transfection, and ratios between firefly luciferase and Renilla luciferase activity were measured with a dual luciferase assay (Promega).

Promoter reporter assays.

The putative promoter regions of human mir-34a and mir-34b/c were amplified from genomic DNA and cloned into pGL4 vector (Promega). Mutagenesis of p53 binding sites was performed with the QuickchangeXL mutagenesis kit (Strategene). HCT116 p53-/- cells were transfected with the heterologous reporter vectors and pRL-TK along with either the pLPC-p53 vector or pLPC control vector. Ratios of firefly luciferase to Renilla luciferase activity were determined with a dual luciferase assay (Promega) 24 h after transfection.

SA-β-gal assays.

Detection of SA-β-gal activity was performed as described before at pH=5.5 [35]. Sections (10μm) of snap frozen tumor tissue were fixed with 1% formalin for 1 minute and stained for 12-16hrs. Tumor bearing livers were fixed with 4% formalin overnight, washed with PBS and stained for 4hrs. Cultured cells were fixed with 4% formalin for 5 minutes and stained for 10hrs.

Generation of immortalized liver progenitor cell lines

Isolation, culture and retroviral infection of murine hepatoblasts were described recently (Zender et al., 2005; Zender et al., 2006). Liver progenitor cells from ED=18 p53-/- fetal livers were infected with MSCV based retroviruses expressing Myc–IRES-GFP or Myc-IRES-Luciferase and two immortalized cell

lines were derived.

Doxycycline (Dox) treatment

Doxycycline (BD) was refreshed in cell culture medium (100ng/mL) every 2 days. Mice were treated with 0.2mg/mL Dox in 0.5% sucrose solution in light-protected bottles. Dox was refreshed every 4 days.

***in vivo* bioluminescence imaging.**

Bioluminescence imaging was performed on anaesthetized animals using a Xenogen imager. 200μL luciferin salt (Xenogen, 15 mg/mL in PBS) was injected into mice (i.p.) 10-15 minutes before imaging. Exposure time was 30 seconds for animals and 10 seconds for explanted livers.

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Appendix

Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation

DNA methylation has important functions in stable, transcriptional gene silencing, immobilization of transposable elements and genome organization[1]. In Arabidopsis, DNA methylation can be induced by double-stranded RNA through the RNA interference (RNAi) pathway, a response known as RNA-directed DNA methylation[2]. This requires a specialized set of RNAi components, including ARGONAUTE4 (AGO4)[3-6]. Here we show that AGO4 binds to small RNAs including small interfering RNAs (siRNAs) originating from transposable and repetitive elements, and cleaves target RNA transcripts. Single mutations in the Asp-Asp-His catalytic motif of AGO4 do not affect siRNA-binding activity but abolish its catalytic potential. siRNA accumulation and non-CpG DNA methylation at some loci require the catalytic activity of AGO4, whereas others are less dependent on this activity. Our results are consistent with a model in which AGO4 can function at target loci through two distinct and separable mechanisms. First, AGO4 can recruit components that signal DNA methylation in a manner independent of its catalytic activity. Second, AGO4 catalytic activity can be crucial for the generation of secondary siRNAs that reinforce its repressive effects. RNA-directed DNA methylation (RdDM) involves a class of siRNAs about 24 nucleotides (nt) in length, which are believed to confer sequence specificity on the process. Arabidopsis has evolved a set of RNAi components that are specialized for RdDM, including

Dicer-like 3 (DCL3), RNA-dependent RNA polymerase 2 (RDR2), RNA polymerase IV and ARGONAUTE4 (AGO4)[3,4,7,8]. Mutations in these proteins can lead to decreased accumulation of siRNAs, decreased AGO4 stability9, and decreased DNA methylation at many endogenous loci including transposons and repetitive elements[3–8,10]. It is highly probable that RNA polymerase IV, RDR2 and DCL3 are components of the siRNA biogenesis machinery. AGO4 is the prime candidate for the component of the effector complex that directs DNA methylation as guided by siRNAs. We therefore tested whether AGO4 exists in a complex with siRNAs *in vivo*. We generated an Arabidopsis Landsberg erecta (Laer) transgenic line expressing a tandem affinity purification (TAP)- tagged AGO4 protein. This protein was recovered from whole plant extracts[11] and its associated RNAs were examined by SYBR-gold staining. TAP-AGO4 was associated predominantly with small RNAs about 24 nt in length (Fig. 1a). Parallel examination of AGO1 complexes showed prominent small RNAs about 21 nt in length[12,13] (Fig. 1a). Northern blotting revealed the binding of AGO4 to siRNAs originating from known transposons, specifically AtSN1 (ref. 14), AtMu1 (ref. 15) and a repeated sequence, MEA-ISR (ref. 16) (Fig. 1b), whose methylation is known to be controlled by RNAi. To obtain a more complete catalogue of the small RNAs associated with AGO4, we cloned and subjected them to sequencing as described[17]. For comparison, the total small RNA population, ranging from 18 to 28 nt, and small RNAs associated with AGO1 were also sequenced. In all,

74,390 sequences were obtained for the whole population (total), and 55,497 and 193,167 sequences were obtained from AGO4 and AGO1 complexes, respectively. As a quality control, we mapped all candidate small RNA sequences to the Arabidopsis genome and found that 51,294 (total), 21,198 (AGO4) and 152,088 (AGO1) sequences perfectly matched at least one location. Only small RNAs passing this quality assessment were used in further analyses.

Small RNA sequences from the total population showed two discernible peaks at 21 and 24 nt. Most AGO4-associated RNAs were 23–24 nt in length, whereas AGO1-associated small RNAs were almost exclusively 21 nt (Fig. 1c); 10,058 (47%) of the AGO4-associated RNAs matched repetitive sequences in the genome, whereas only 15% of total and 3% of the AGO1-associated RNAs were repeat derived. Such repeats comprise 17 of the 18 different types documented in Repbase[18], with the top 30 families accounting for more than 50% of all matches. Genomic matches to AGO4-associated small RNAs were particularly dense in pericentromeric regions, reflecting their high concentration of repetitive sequences (Fig. 2). Although we cannot unambiguously determine whether a given small RNA was derived from a particular repeat copy, previous studies indicate that AGO4 complexes can act in trans to direct RdDM at matching loci[5,6]. One must therefore consider these plots as reflecting sites of possible action rather than sites of possible origin.

In all, 91% of AGO1-associated small RNAs matched known microRNAs

(miRNAs), a result consistent with the demonstrated role of AGO1 in miRNA-mediated control of plant development[12,13,19]. Only a small fraction of AGO4-associated RNAs (3%) matched microRNAs. However, a subset of the microRNAs found in AGO4 complexes showed preferential association with this protein. This implies selectivity in how individual microRNAs are processed and passed to specific AGO-containing RNA-induced silencing complexes (RISCs). Moreover, it implies that some microRNAs might need to act in concert with AGO4, perhaps in the nucleus, rather than with AGO1 in the cytoplasm. Many AGO4 -associated small RNAs were also derived from the sense or antisense strand of genes, pseudogenes and intergenic regions, indicating that AGO4 might also have a previously unrecognized general role in regulating gene expression. ATREP2 (a Helitron-like DNA transposon with dispersed repeats in the genome) and SIMPLEHAT2 (a DNA transposon) both matched abundant small RNAs that are associated with AGO4. Both the levels of these small RNAs and non-CpG methylation at these loci were reduced by genetic lesions in the AGO4 pathway (in RDR2, DCL3 and AGO4). Taken together, our data indicate that siRNAs associated with AGO4 direct it to target loci, where it can promote non-CpG (CpNpG and CpHpH) methylation. It is not known how siRNAs act at target loci to direct RdDM. Opposing classes of models involve either pairing between an siRNA and a target DNA (RNA–DNA recognition) or pairing between an siRNA and a nascent RNA transcript (RNA–RNA recognition)[20]. However, methylation of PHABULOSA can be

directed by a miR165/166 target site that crosses an exon–exon junction, strongly supporting an RNA–RNA recognition model[21]. A crucial question is whether this siRNA–RNA pairing leads to transcript cleavage and whether such cleavage functions in silencing[22]. Alignment of Arabidopsis AGO4 with known Slicers, human Ago2 (ref. 23) and Arabidopsis AGO1 (refs 12, 13), revealed the presence of the catalytic Asp-Asp-His (DDH) triad in all three proteins[24] (Fig. 3a). To test its catalytic potential directly, we incubated TAP-purified AGO4 with an RNA transcript containing a sequence complementary to identified ATREP2 siRNAs. The target RNA was cleaved by wild-type AGO4 protein (Fig. 3b) but not by mutants containing changes in essential catalytic residues (D660A, D742A and H874A substitutions, respectively; referred to hereafter as DDH mutants), despite similar expression levels and siRNA-binding capacity (Fig. 3c). We could similarly detect target cleavage on incubation of AGO4 complexes with targets for two AGO4-interacting miRNAs, miR172 and miR390. To determine whether catalysis was important for RdDM, we turned to a system of epialleles that could be tracked through an obvious visual phenotype. SUPERMAN is required for proper floral development; when its activity is decreased, plants show an increased number of stamens (an average of ten in comparison with the normal six) and incompletely fused carpels (SUP phenotype). In addition to genetic mutants, there are also SUP epialleles (Clark Kent or clk)[25]. When the clk-3 epiallele is placed in an AGO4-null background (clk-3/ago4-1, obtained

from the Arabidopsis Biological Resource Center), most plants are wild-type (Fig. 4a), although some (20–30%) do retain the SUP phenotype. This indicates that there might be complex regulation of the locus and that SUP silencing in a subset of plants might be maintained in the absence of AGO4. However, when the clk-3/ago4-1 plants were viewed as a population, CpNpG and CpHpH methylation at SUP were decreased substantially (Fig. 4b) in comparison with clk-st (a stabilized Clark Kent allele in the presence of wild-type AGO4)[4]. AGO4or each DDH mutant was expressed under the control of the AGO4 promoter in clk-3/ago4-1 plants. Pooled samples from about 30 primary transformants (T1 generation) showed that all proteins were expressed at similar levels. Essentially all T1 plants transformed with wild-type AGO4 complemented ago4-1 and displayed the SUP floral phenotype, whereas those with the empty vector did not (Fig. 4a). Intriguingly, all T1 plants transformed with AGO4DDHmutants also recovered the SUP phenotype (Fig. 4a). Bisulphite sequencing of about 30 pooled seedlings of each genotype showed that non-CpG methylation was restored to approximately normal (clk-st) levels (Fig. 4b). Although the regulation of SUP is likely to be complex, an intact AGO4 pathway reinforced silencing at the locus in a manner that did not depend on siRNA-directed RNA cleavage.

Examination of additional loci revealed a more complex picture (Fig. 5). At AtMu1, non-CpG methylation decreased in ago4-1 plants4 and was fully rescued by AGO4 or its DDH mutants. However, at three other loci, namely

MEA-ISR, ATREP2 and SIMPLEHAT2, wild-type AGO4 restored non-CpG methylation to normal levels, but the DDH mutants showed greatly decreased potency. For example, at MEA-ISR, introduction of AGO4 into ago4-1 mutants led to a 3.2-fold increase in CpNpG and a 20.9-fold increase in CpHpH methylation. The DDH mutants had from no effect to a roughly 2.1-fold increase in CpNpG and a 2.2-fld to 4.2-fold increase in CpHpH methylation. Thus, the requirement in RdDM for AGO4 catalytic potential varies with the locus. We next probed the correlation between non-CpG methylation, siRNA production and the effect of inactivating the AGO4 catalytic site. At AtMu1, a locus where DDH mutants complemented methylation defects efficiently, loss of AGO4 had no effect in itself on the abundance of AtMu1 siRNAs, and these species were not increased on expression of any AGO4 variant (Fig. 6). We were not able to examine the effect of DDH mutations on the accumulation of SUP siRNAs because these were below detection limits, as reported previously4. For the three repetitive elements for which the wild-type and DDH mutants showed differential effects, another pattern was observed. In all cases, siRNAs were substantially decreased in ago4-1 mutant plants. Expression of wild-type AGO4 generally restored siRNA levels, but the DDH mutants had much less pronounced effects (Fig. 6). For example, in the MEA-ISR locus, siRNAs in ago4-1 mutants decreased to 18% of wild-type levels.

Wild-type AGO4 restored this to 87% of normal, whereas the DDH mutants rescued siRNAs to only 27–38%. In comparison, siRNA was not decreased in

ago4-1 as described previously, and the introduction of either catalytic or non-catalytic AGO4 had no effect on overall siRNA abundance. Thus, the catalytic activity of AGO4 was important both for efficient siRNA production and for non-CpG methylation at some loci.

At others, where AGO4 loss had little effect on overall siRNA levels, AGO4 loss could still affect non-CpG methylation. However, in these cases a lack of catalytic potential was of little importance to the ability of ectopically expressed AGO4 to restore non-CpG methylation. All of these conclusions were based on multiple independent T1 transgenesis studies and bisulphite experiments (up to five each), and all results were confirmed with two individual T2 transgenic lines for each construct.

Our data indicate that AGO4 can have two distinct functions in RdDM. First, AGO4 can direct chromatin remodelling factors to a target locus, probably through interactions between siRNAs and a nascent transcript. For this process, the catalytic activity of AGO4 is not required. Thus, given a source of siRNAs, the non-catalytic activity of AGO4 would be sufficient to sustain methylation and repression.

Second, AGO4 has a function in which catalysis is required for efficient siRNA production. Cleavage may trigger the recruitment of RDR2-containing complexes to synthesize a double-stranded RNA using the cleaved transcript as template, with subsequent processing by DCL3 producing secondary siRNAs. This is reminiscent of the production of Arabidopsis trans-acting

siRNAs, which is initiated by the cleavage of their precursor RNA by a miRNA-directed RISC.

Other, currently mysterious, mechanisms must also promote siRNA production from heterochromatic loci, because in this model an existing siRNA or miRNA would be required to initiate the cycle. siRNA accumulation and non-CpGDNAmethylation of AtMu1, and by inference at SUP, are much less dependent on the catalytic activity of AGO4. This could simply indicate that another AGO protein functions redundantly with AGO4 at these sites[4]. Indeed, it was shown that DNA methylation at AtMu1 is also controlled by AGO1 (ref. 10). It also remains possible that AtMu1 and SUP might represent a subset of AGO4-dependent loci in which the role of siRNAs is less important, particularly considering that SUP siRNAs have yet to be detected.

Our results reveal a potentially general property of Argonaute proteins. A single Argonaute may simultaneously serve as a catalytic engine of RNA cleavage and as a flexible platform for the assembly of multiprotein complexes that trigger cleavage-independent repression. For AGO4, both of these functions act within a single silencing pathway to contribute to the management of repetitive sequences in the Arabidopsis genome (Fig 7).

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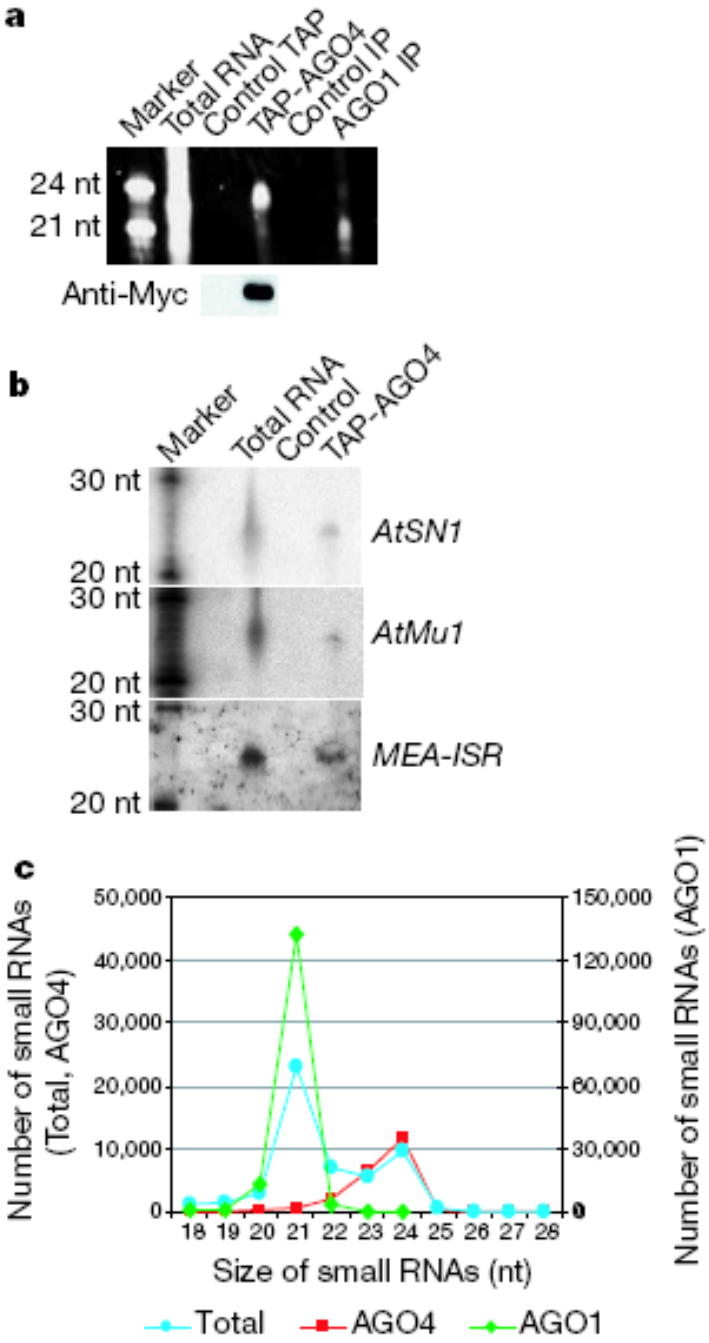

Figure 1 A catalogue of AGO4-associated small RNAs. a, SYBR-gold was used to reveal small RNAs in total Arabidopsis RNA, TAP-AGO4 complexes, AGO1 complexes and control purifications (upper panel, as indicated). Western blotting with anti-Myc antibody detected AGO4 in the TAP-AGO4 purification but not in the control purification (lower panel). IP, immunoprecipitation. b, Northern blotting was used to detect small RNAs in total RNA, TAP-AGO4 complex and control purifications with the indicated probes. Radioactive RNAs of known sizes were included as markers. c, Size distribution of total (cyan), AGO4-associated (red) and AGO1-associated (green) small RNAs. The sets of redundant small RNAs were used to generate a histogram quantifying the number of sequences obtained for each size class. external GFP-tumor imaging (top panel) or direct imaging of the respective explanted tumor bearing livers (bottom panel).

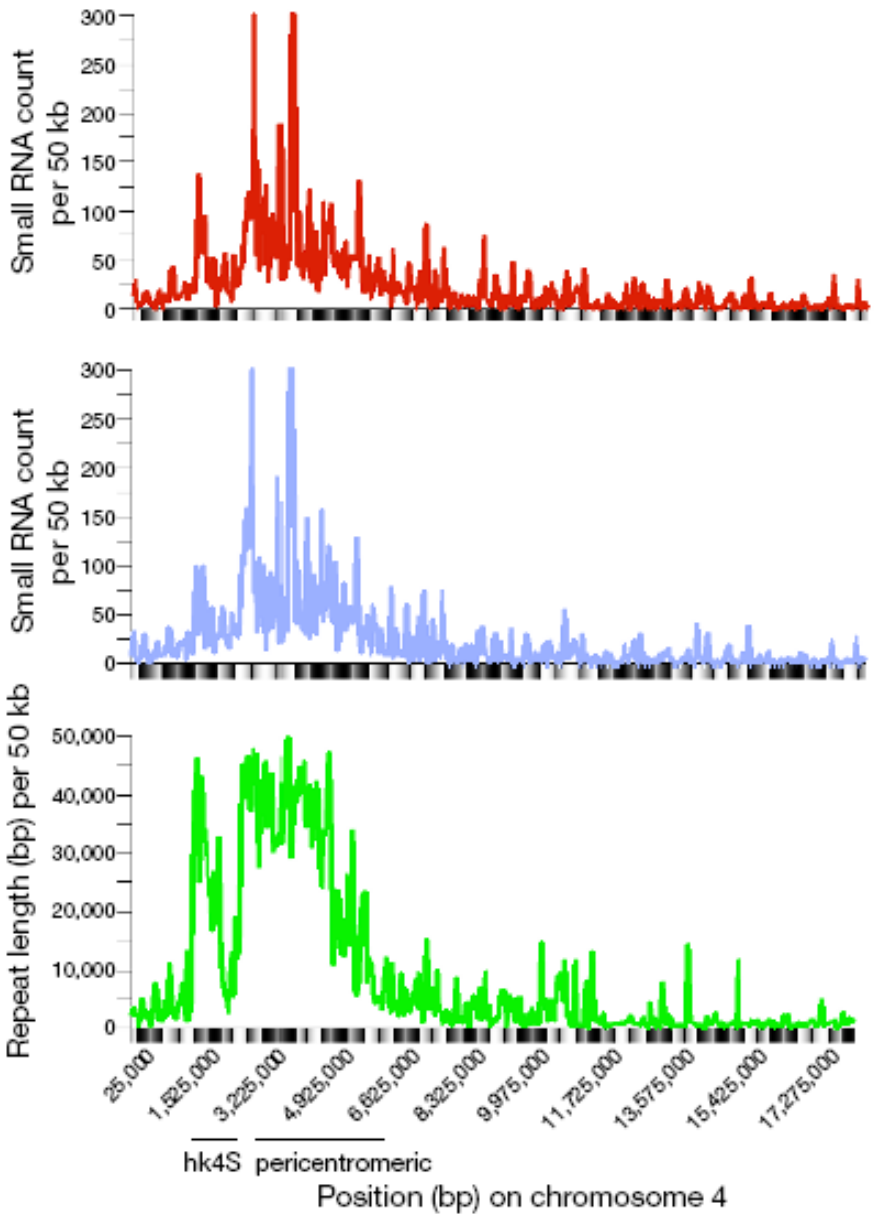

Figure 2 Chromosome-wide density analysis of the AGO4-associated small RNAs on chromosome 4. The density of small RNAs with perfect matches in the direct strand (upper panel) and the complementary strand (middle panel), and the density of repeats (presented as the total length of repeats (bp); lower panel) in a 50-kilobase sliding window, are plotted. The positions of the pericentromeric region and the heterochromatic knob hk4S are marked.

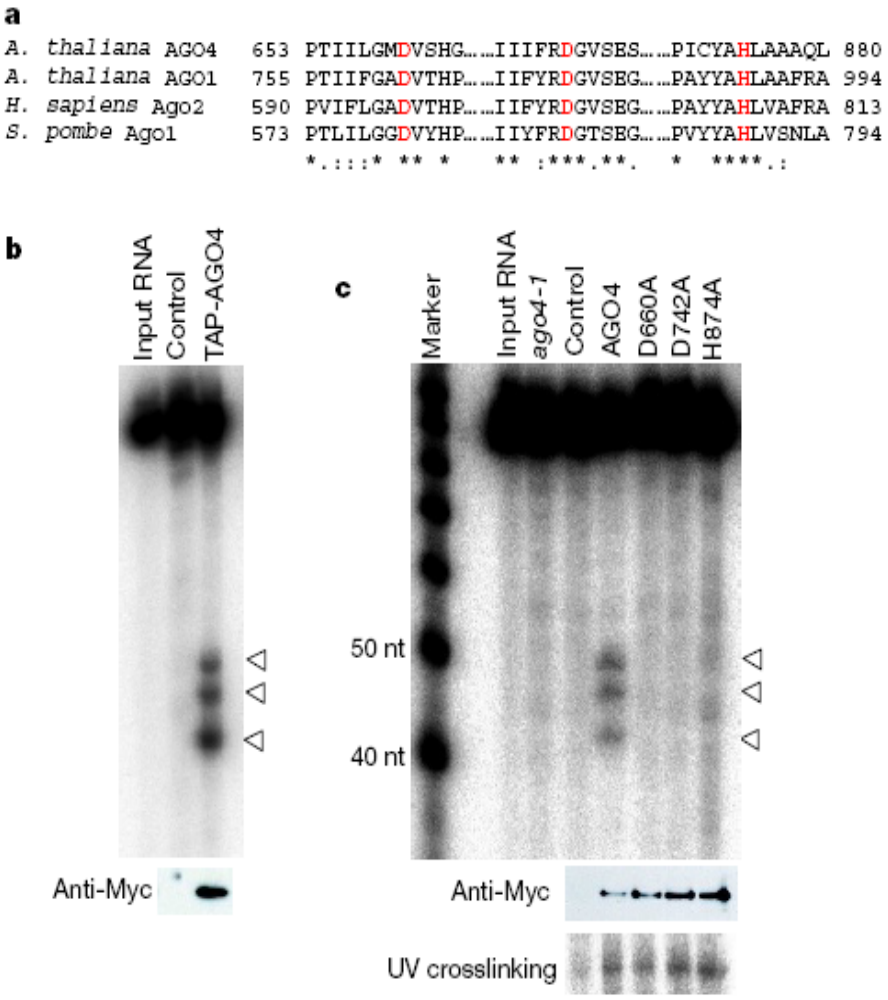

Figure 3 AGO4 is a Slicer. a, A partial alignment of the PIWI domains of Arabidopsis AGO4, AGO1, human Ago2 and Schizosaccharomyces pombe Ago1 is shown. The residues forming the catalytic DDH motif are shown in red. The degree of similarity is indicated under the alignment: asterisk indicates identity in all sequences; colon indicates conservative substitutions, and full point indicates semi-conservative substitutions. The starting and ending positions of the sequences are as labelled. b, A 32P-caplabelled synthetic target RNA containing recognition sites for cloned ATREP2 siRNAs was incubated with TAP-purified AGO4 or a control purification. Positions of 59 products of cleavages guided by three endogenous ATREP2 siRNAs are indicated by the arrows (upper panel). Western blotting with anti-Myc antibody detected AGO4 in the TAP-AGO4 purification but not in the control purification (lower panel). c, The synthetic target RNA was incubated with immunopurified Myc-tagged AGO4 wild-type and DDH mutants (top panel). Decade RNA markers are shown for reference. Western blotting with an anti-Myc antibody detected AGO4 in the immunoprecipitates but not in the control purification (middle panel). AGO4 and control immunoprecipitates, as indicated, were incubated with single-stranded 32P-labelled 24-nt siRNAs bearing photoreactive dT residues at the two 39 positions12. Mixtures were irradiated with ultraviolet (UV) as described in Methods (bottom panel).

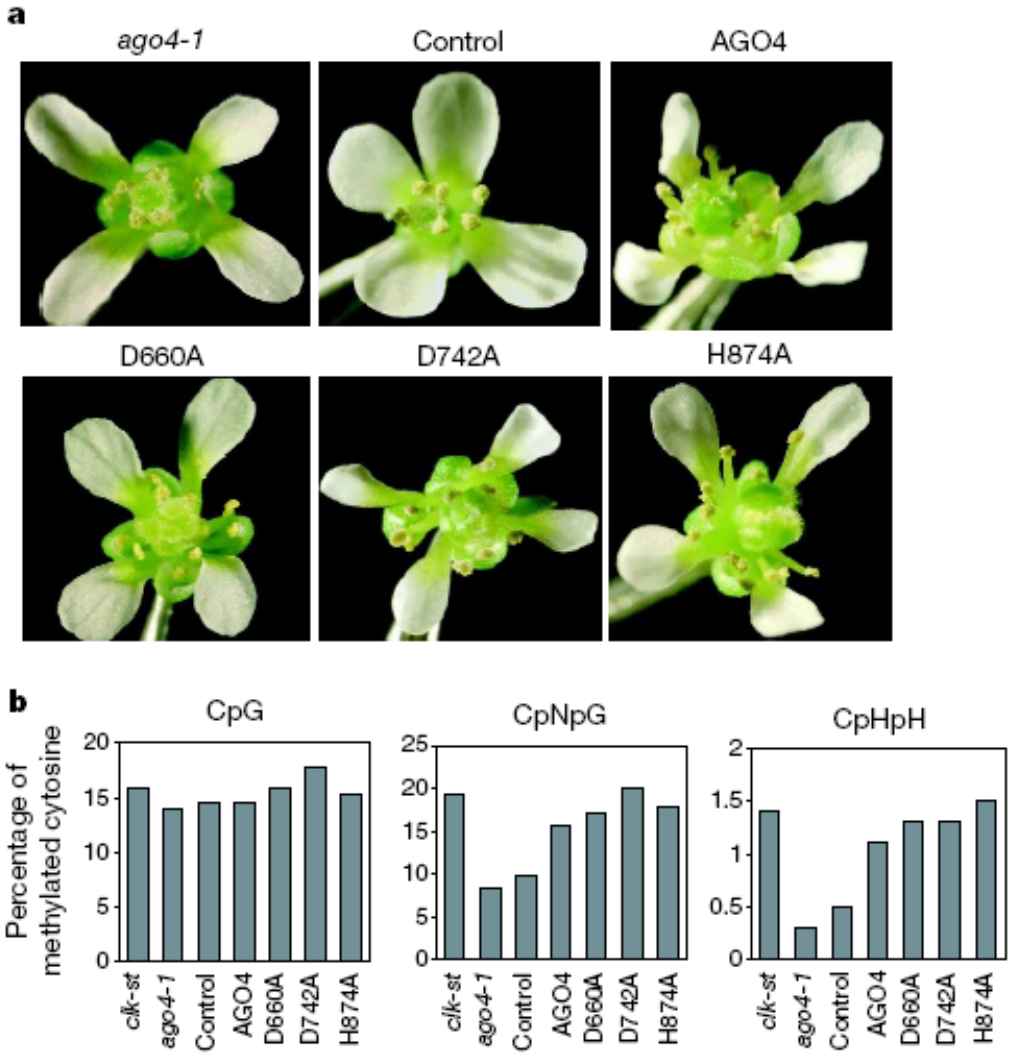

Figure 4. Slicer activity is not required for non-CpG methylation and silencing at the SUP locus. a, Representative flowers from parental clk-3/ ago4-1 and plants of the same genotype transformed with vectors as indicated. About 70–80% of the clk-3/ago4-1 plants and plants transformed with empty vector have wild-type flowers with six stamens and two fused carpels, with the remainder having the SUP phenotype (about ten stamens and three incompletely fused carpels; see the text). Essentially all flowers from plants transformed with AGO4 and DDH mutants display the SUP phenotype. b, CpG (left), CpNpG (centre) and CpHpH (right) methylation of the SUP gene was analysed by bisulphite sequencing of genomic DNA prepared from pooled T_1 seedlings. Data from two complete biological replicates were combined. The methylation level is shown by the percentage of methylated cytosine in all sequenced clones. The data in Supplementary Table S7 were used to generate the histograms.

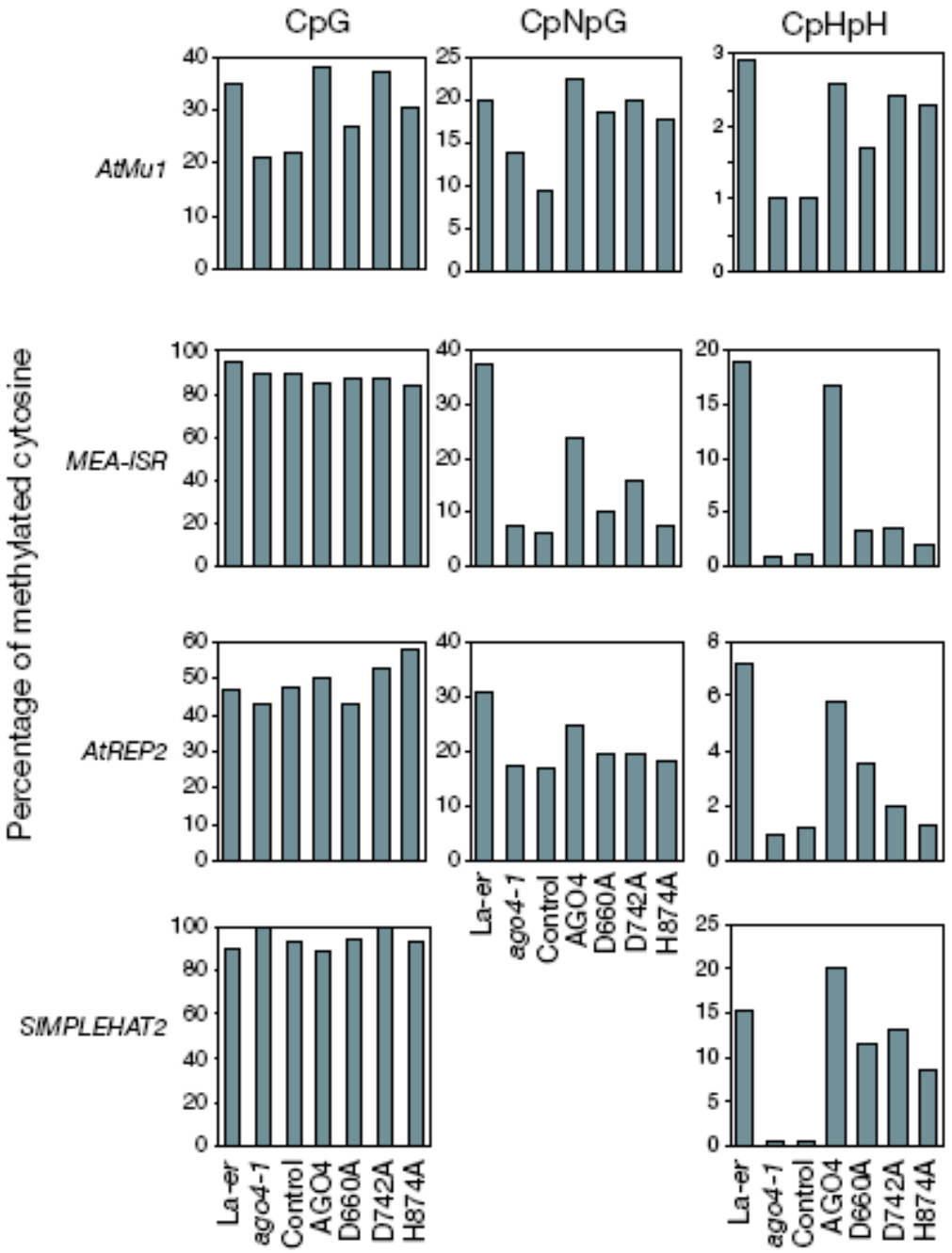

Figure 5 Distinct effects of Slicer activity on DNA methylation and siRNA accumulation at endogenous repeats. a, CpG (left), CpNpG (middle) and CpHpH (right) methylation at AtMu1, MEA-ISR, ATREP2 and SIMPLEHAT2 loci were analysed by bisulphite sequencing. Data from two complete biological replicates were combined. Methylation levels are shown by the percentage of methylated cytosines in all sequenced clones.

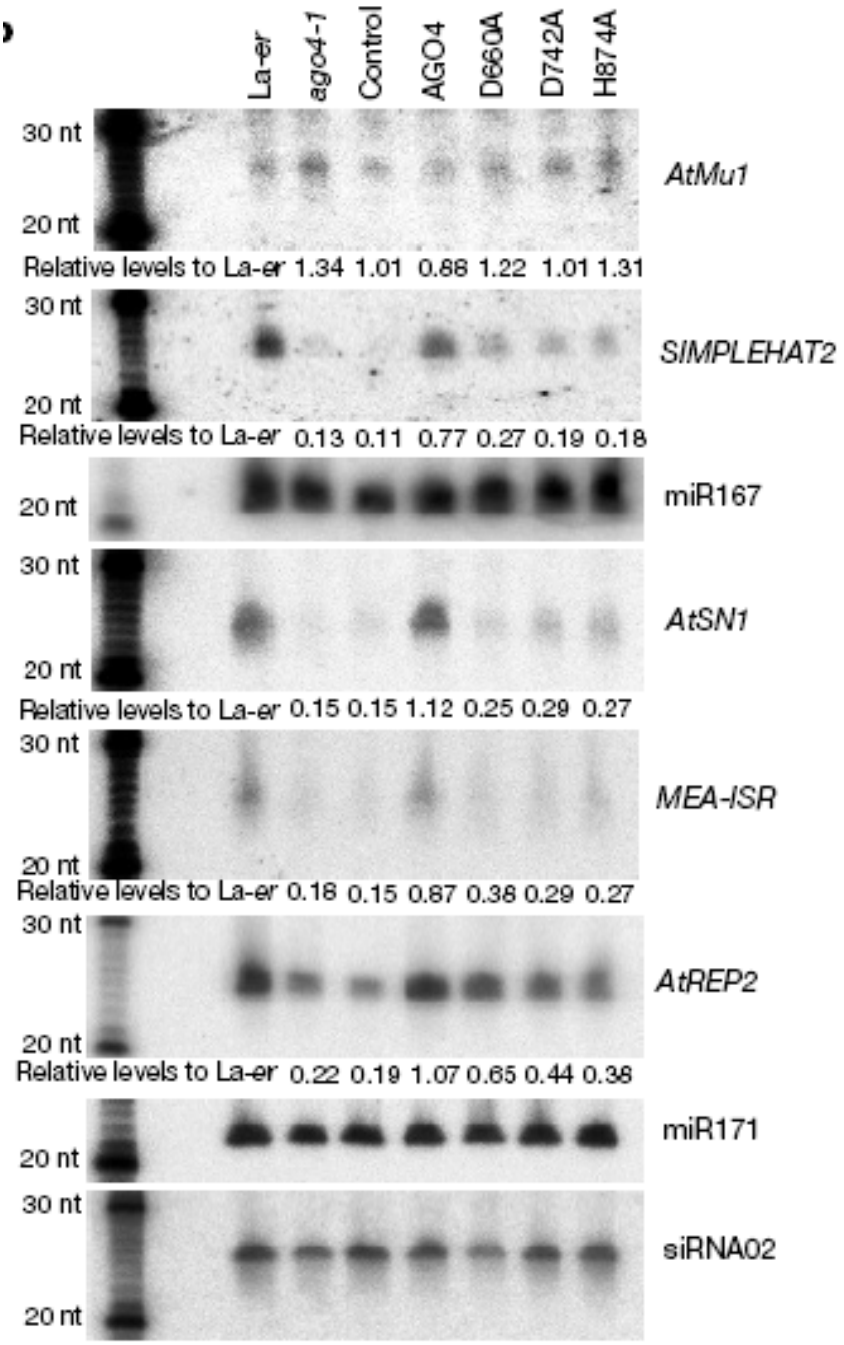

Figure 6. Northern blotting was used to analyse siRNAs derived from AtMu1, SIMPLEHAT2, AtSN1, MEA-ISR, ATREP2 and siRNA02 in RNA prepared from the indicated pooled T_1 plants. miR167 and miR171 were used as loading controls. The siRNA signals were normalized relative to miR167 (for AtMu1 and SIMPLEHAT2) or miR171 (for other siRNAs), and the relative levels were calculated by comparison with those in La-er RNA.

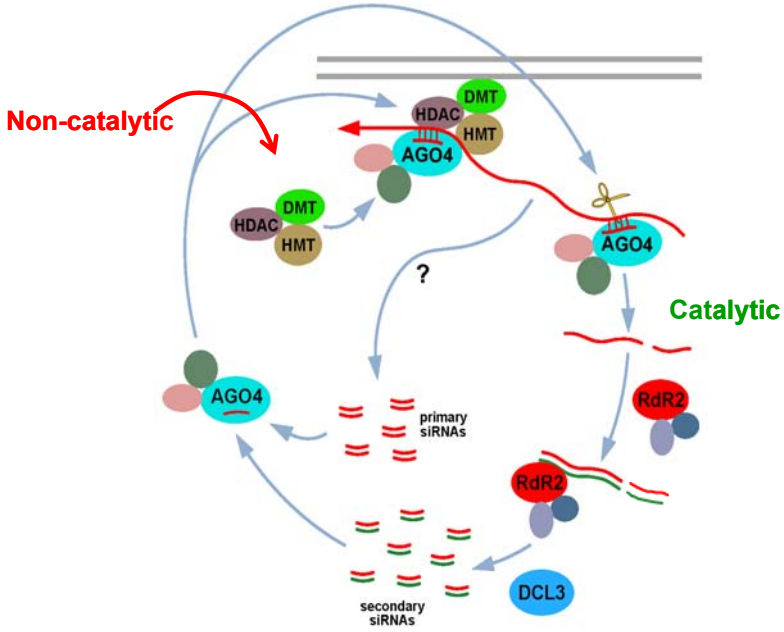

Figure 7. A model.