MicroRNAs in cellular transformation and tumorigenesis

by

Madhu S. Kumar

B.A. Chemistry and Biochemistry
University of Pennsylvania, 2004

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Signature of Author_____

Madhu S. Kumar
Department of Biology
April 10, 2009

Certified by_____

Tyler Jacks
David H. Koch Professor of Biology
Thesis Supervisor

Certified by_____

Stephen P. Bell
Professor of Biology
Chair, Biology Graduate Committee

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ABSTRACT

MicroRNAs (miRNAs) are a novel class of small (approximately 23 nucleotides long), highly conserved, non-coding RNAs that function by broadly regulating gene expression. In animals, this regulation is achieved via interaction with target messenger RNAs (mRNAs), largely through their imperfect base pairing with the 3' untranslated regions (3' UTRs) of these target transcripts. Through this imperfect base pairing, miRNAs induce a repression of mRNA translation that is frequently coupled to enhanced turnover of the targeted transcript. This miRNA-mediated repression is highly related to that of RNA interference (RNAi), in which small non-coding RNAs exhibit perfect base pairing with target mRNA transcripts, leading to the endonucleolytic cleavage and degradation of these targeted mRNAs. Computational algorithms have been designed to predict putative miRNA binding sites within mRNAs. Using these predictions, it has been suggested that more than half of all mRNAs within mammals are under the control of miRNAs.

Some of the earliest discovered miRNAs (characterized by genetic studies in the nematode *Caenorhabditis elegans*) were found to control the proliferation and differentiation of the cells in which they were expressed. As altered control of proliferation and differentiation frequently manifest in cancer in mammals, it was suggested that miRNAs might contribute to the development of tumorigenesis. In fact, several miRNAs are frequently deleted or amplified in human cancer and miRNA expression profiling studies have shown widespread reductions in steady-state miRNA levels in human cancers relative to normal tissue. These observations have implied a role for miRNAs in tumorigenesis. However, there is a paucity of functional studies demonstrating a role for miRNAs in oncogenic transformation.

In the studies described below, we first provide strong evidence for the global loss of miRNAs in human cancers functionally enhancing cellular transformation and tumorigenesis. This enhanced transformation only occurred within tumor cells, suggesting that inhibiting miRNA biogenesis would not be sufficient to induce tumor formation. Moreover, we demonstrate that inhibition of miRNA processing in cancer must be incomplete, as *Dicer1*, a component of the miRNA processing pathway, suppresses tumorigenesis via haploinsufficiency. Finally, we examine the role of a specific miRNA family, the let-7 family, in the development of non-small cell lung cancer by showing that let-7 can suppress tumorigenesis via inhibition of its targets K-Ras and HMGA2. Taken together, these findings offer promise, not just for understanding the relationship of miRNAs and cancer, but for developing therapeutic agents against the disease.

Thesis Supervisor: Tyler Jacks
Title: David H. Koch Professor of Biology

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CHAPTER 1: Introduction

Portions of this chapter were originally written in a review "MicroRNAs and cancer" by Andrea Ventura, Madhu S. Kumar, and Tyler Jacks, submitted to *Current Opinion in Genetics and Development*.

MicroRNAs (miRNAs)

Overview

MicroRNAs (miRNAs) are a novel class of short non-coding RNAs that regulate gene expression through homologous base pairing with target mRNAs in their 3' untranslated regions (3' UTRs), resulting in both translational repression and enhanced mRNA turnover (Bartel, 2004; Filipowicz et al., 2008). Based on recent computational algorithms, there are thousands of predicted miRNA targets, implying that miRNAs function to broadly control gene expression (Bartel, 2009). In particular, many miRNA targets have roles in cellular differentiation, apoptosis, and proliferation, processes commonly altered during tumorigenesis. Thus, miRNAs have been ascribed a role in tumorigenesis. Here I will describe the initial discovery of miRNAs, current knowledge of miRNA biogenesis and function, and the genetic consequences of broadly and specifically inhibiting miRNAs in mammals.

MiRNA discovery

MiRNAs were initially characterized as genes whose mutation altered larval developmental timing in the nematode, *Caenorhabditis elegans* (*C. elegans*). The initial mutation, *lin-4*, was described to encode not a traditional protein-coding gene, but instead a set of small RNAs, the larger of which (approximately 61 nucleotides) was predicted to be a stem-loop precursor for the other (approximately 21 nucleotides) (Lee et al., 1993). In addition, it was shown that this small RNA product was predicted to base pair with the 3' UTR of lin-14, which

led to reductions in Lin-14 protein levels without changes in the levels of the lin-14 transcript (Lee et al., 1993; Wightman et al., 1993). This led to the idea that the lin-4 RNA regulated developmental timing by inhibiting translation of the Lin-14 protein via base pairing with the lin-14 3' UTR. As a short RNA species whose expression was regulated precisely during development, lin-4 was initially labeled as a small temporal RNA (stRNA).

While the discovery of a second stRNA, *let-7* (discussed in greater detail below), suggested that there were several small RNAs that regulated developmental decisions in *C. elegans*, it was not clear whether such RNAs existed in other organisms (Reinhart et al., 2000). This issue was resolved in two ways. First, it was shown that orthologs of *let-7* exist in a variety of animal species as a small RNA (Pasquinelli et al., 2000). Second, a series of papers described the isolation, cloning, and sequencing of small RNA populations from a variety of different animal species (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

These studies used polyacrylamide gel electropheresis of RNA from a variety of invertebrate and vertebrate cell lines and tissues to separate the small (~20 nucleotides long) RNAs from the bulk RNA population. After adaptor ligation to 5' phosphate groups, these small RNAs were amplified, sequenced, and mapped to their corresponding genomes. In contrast to the idea that much of this small RNA would exist as degradation products of the more abundant cellular RNAs, most of these RNAs mapped to intergenic sequences and were non-randomly distributed. Furthermore, these sequences were much more highly conserved across species than other non-protein coding regions of the genome. Moreover, most of these small RNAs were surrounded by sequences that were predicted to create stem loop structures, as seen in the *lin-4* RNA, suggesting their specific biogenesis. This allowed for the eventual prediction of miRNA genes within a given genome sequence (Lim et al., 2003a; Lim et al., 2003b). Overall, these

initial observations provided powerful evidence for the broad relevance of these small RNAs to molecular biology.

Using similar approaches, the work of many groups has led to the discovery of thousands of different miRNAs from a variety of organisms (Griffiths-Jones et al., 2008). More recently, the development of large-scale, massively parallel sequencing technologies has allowed for more profound sequencing of such small RNA libraries (Rusk and Kiermer, 2008). Such methods have not only allowed for the "deep sequencing" and discovery of new miRNAs in complex, multi-lineage tissues and organisms, it has allowed for the discovery of novel small RNA species, like piRNAs and 21U-RNAs, whose production within reproductive organs is required for fertility in a variety of animals (Aravin et al., 2006; Girard et al., 2006; Lau et al., 2006; Ruby et al., 2006; Vagin et al., 2006). While each class of small RNAs share common sequence elements, they are not of a defined sequence, as seen for miRNAs. Overall, massively parallel sequencing has allowed for the discovery of both novel miRNAs and additional small RNA populations.

While such biochemical approaches have the potential to discover new small RNAs, it is likely that even the most current massively parallel sequencing technologies will miss small RNAs that are expressed in extremely rare sub-populations. An example of this type of small RNA missed by cloning is *lsy-6*, which controls the development of left/right neuronal asymmetry in the nematode (Johnston and Hobert, 2003). It was isolated as a mutant, similar to *lin-4* and *let-7*. However, it is expressed within a single neuron in *C. elegans*, where it controls the lineage fate of the cell. As *lsy-6* is expressed in a single cell in the worm, it is by definition less abundant than most miRNAs. In fact, even current massively parallel sequencing technologies are limited in their ability to detect the *lsy-6* small RNA product during small RNA

cloning from the whole worm. Thus, genetic approaches serve a vital complement to cloning techniques in the discovery of functionally relevant miRNAs.

MicroRNA biogenesis

MicroRNA maturation involves a series of regulated processing events from a primary transcript (Figure 1.1). Within the genome, miRNAs are mapped as hairpin structures that exhibit significant sequence conservation across related species; in fact, such requirements have been applied in the development of the program MiRscan, which predicts miRNA genes in various species (Lim et al., 2003b). In most cases, miRNAs are synthesized as RNA polymerase II transcripts, with 7-methylguanosine caps and 3' polyadenylation similar to mRNAs (Lee et al., 2004). After synthesis, these primary miRNA transcripts, or pri-miRNAs, undergo a first round of processing by the RNAse III-like enzyme Drosha and its dsRNA binding partner, DGCR8/Pasha, to produce a pre-miRNA hairpin (Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003). While the majority of miRNAs are intergenic, approximately one-quarter of miRNAs map within the introns of protein-coding genes and these intronic miRNAs undergo Drosha processing in tandem with mRNA splicing (Kim and Kim, 2007). While this occurs in the majority of intronic miRNAs, a subset (labeled "mirtrons") exist as a pre-miRNA intron whose splicing produces the pre-miRNA hairpin (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Overall, in the nucleus, miRNA precursors are synthesized and undergo an initial round of processing to produce pre-miRNAs.

After this initial processing, the pre-miRNA hairpin is subsequently exported from the nucleus to the cytoplasm via Ran-GTP and Exportin-5 (Lund et al., 2004; Yi et al., 2003). After

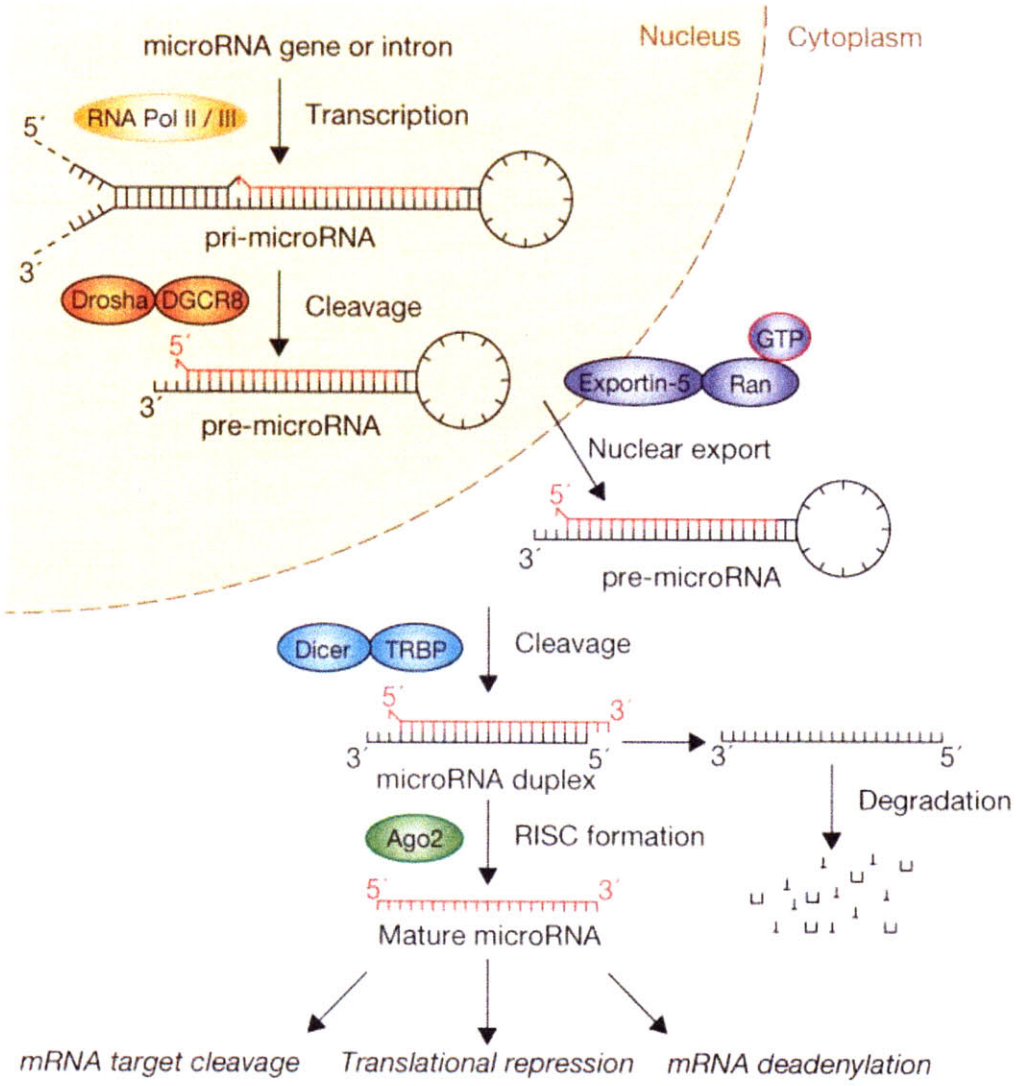


Figure 1.1 The miRNA processing pathway. MiRNAs are initially transcribed by either RNA polymerase II or III to produce a primary miRNA transcript, or pri-miRNA. In the nucleus, the pri-miRNA is converted by Drosha and DGCR8 into a pre-miRNA hairpin. This pre-miRNA hairpin is exported from the nucleus via Exportin-5 and Ran-GTP to the cytoplasm, where it undergoes a second round of processing by Dicer and TRBP into a miRNA duplex. This duplex is then unwound, and the mature miRNA associates with the Argonaute family of proteins to produce the RNA induced silencing complex, or RISC, where it engages in regulation of target mRNA transcripts. Adapted from (Winter et al., 2009).

cytoplasmic export, the pre-miRNA undergoes a second round of processing by the complex of Dicer and TRBP to produce the miRNA duplex (Chendrimada et al., 2005; Haase et al., 2005; Hutvagner et al., 2001). This complex interacts with the Argonaute proteins to produce the mature miRNA-Argonaute protein complex (with some evidence that Argonaute family members themselves cleave the pre-miRNA to produce a second intermediate), which acts with additional factors to produce the RNA-induced silencing complex, or RISC, and subsequently regulate target mRNA expression (Diederichs and Haber, 2007; Gregory et al., 2005; Matranga et al., 2005; Rand et al., 2005).

MiRNA function

Once miRNAs are loaded onto RISC, they can interact with target mRNAs and regulate their expression in a specific way depending upon their base pairing. If a miRNA exhibits complete sequence complementarity with a target mRNA, it will engage in mRNA degradation due to specific endonucleolytic cleavage of the transcript by Argonaute-2 (Liu et al., 2004; Song et al., 2004). This process was originally described by Andrew Fire, Craig Mello and colleagues as dsRNA-mediated genetic interference in *C. elegans* and was labeled RNA interference (RNAi) (Fire et al., 1998). This is the mechanism by which plant miRNAs regulate their target transcripts (Bartel, 2004). In contrast, the preponderance of animal miRNAs function through imperfect base pairing with a target transcripts to engage in translational repression and mRNA turnover that does not require Argonaute-catalyzed cleavage (Valencia-Sanchez et al., 2006). Frequently, this translational repression is coupled to turnover of the target transcript due to its deadenylation/decapping and subsequent degradation (Bagga et al., 2005; Behm-Ansmant et al.,

2006). In fact, recent proteomics analyses of miRNAs and their targets suggest that targets significantly repressed at the protein level largely undergo these effects at the level of mRNA degradation (Baek et al., 2008; Selbach et al., 2008).

The mechanism of translational repression by miRNAs of target mRNAs remains controversial. Initial studies suggested that miRNAs regulate translation at the level of initiation (Humphreys et al., 2005; Pillai et al., 2005). These results were largely based upon two types of experiments: the delivery of target mRNAs containing internal ribosome entry sites that bypass distinct stages of translation initiation, and the tethering of RISC to target mRNAs via RNA stem loop structures that would associate with Argonaute proteins fused to RNA binding proteins that bound these stem loops. Using these techniques, both groups observed that bypassing the early requirements of translational initiation, such as recognition of the 7-methylguanosine cap, mitigates miRNA-mediated translational repression.

In contrast, other reports argued that miRNA-mediated translational repression occurs post-initiation (Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). In these studies, target mRNAs were examined via polysome analysis of either endogenous mRNAs or expression of synthetic miRNA targets from DNA plasmids. Using these systems, they observed association of target mRNAs with polysomes in both the presence and absence of miRNA, which should not occur if miRNAs inhibited translation initiation. Through the application of nonsense suppressor tRNAs, it was posited that miRNA-mediated translational repression occurs via early ribosome drop-off during elongation. Due to the differences in the mRNA target delivery to cells (transfection in the former set of experiments, expression from intracellular DNA in the latter), it is difficult to directly compare the findings from these studies. Further complicating matters, a controversial report suggested that miRNAs can actually promote target translation during

quiescence (Vasudevan et al., 2007). In sum, while it is clear that animal miRNAs can function through translational repression of target transcripts, the mechanism behind this translational repression is still contested.

A further wrinkle in the analysis of miRNA-mediated translational repression is found by the effect of miRNAs on the association of mRNAs with intracellular RNA regulatory bodies called processing bodies (P-bodies). These cytoplasmic foci are sites of storage for untranslated mRNA and are believed to regulate gene expression. Interestingly, it has been observed that RISC associates with mammalian P-bodies, mRNAs enter P-bodies in a miRNA-dependent manner, and that P-body component GW182 is an important regulator of miRNA function (Jakymiw et al., 2005; Liu et al., 2005; Sen and Blau, 2005). In contrast to these findings, other data shows RISC associating in conditions of cellular stress instead with a different class of cytoplasmic RNA foci called stress granules, and that this association with stress granules, and not P-bodies, occurs in a miRNA-dependent manner (Leung et al., 2006). Though distinct on the contribution of different cytoplasmic RNA foci to miRNA-mediated repression, these findings propose a role for altered cytoplasmic localization of target mRNAs in response to miRNA-mediated repression.

While the above mechanistic studies of miRNA function leave many questions unanswered, much is known about the *cis* elements of miRNAs and target mRNAs required for translational repression. In particular, while imperfect base pairing is a hallmark of miRNA-mediated translational repression, base pairing at specific sites appears vital for this repression. This region, which encompasses nucleotides two through seven (and frequently nucleotide eight) of the miRNA, has been termed the "seed" region, and mutation of this site within a target largely abolishes miRNA-mediated repression (Doench and Sharp, 2004; Lewis et al., 2003).

Additionally, this seed sequence regulates both translational repression of target transcripts and their turnover, as shown by ectopic expression of a miRNA leading to enrichment for mRNAs containing the miRNA's seed sequence among the set of down-regulated transcripts; also, "off-target" down-regulation of mRNAs in response to a given siRNA is believed to be due to this seed sequence-mediated turnover (Birmingham et al., 2006; Lim et al., 2005).

Furthermore, additional predictive analysis of miRNA binding sites have created additional rules for enhanced miRNA-mediated repression: AU sequence richness near the miRNA binding site, closeness to other known miRNA binding sites to achieve cooperative regulation, the ability of a site to base pair nucleotides thirteen through sixteen, location of the site approximately fifteen nucleotides away from the stop codon, and location on either end of long 3' UTRs (Grimson et al., 2007). Taken together, these observations provide a reasonable set of rules for assessing a miRNA-mRNA target interaction. With similar rules in mind, along with the original requirement for site conservation among related species, miRNA target-prediction algorithms have been developed (Enright et al., 2003; Friedman et al., 2009; Grimson et al., 2007; John et al., 2004; Kiriakidou et al., 2004; Lewis et al., 2005; Lewis et al., 2003; Rajewsky, 2006; Stark et al., 2003). Such approaches provide a powerful set of resources for predicting germane miRNA-mRNA interactions. However, experimental validation of any individual miRNA-mRNA target interaction is still necessary to establish a functional relationship, as comprehensive functional assessment of the various approaches is still lacking.

To perform such functional validation of miRNA-mRNA interactions, several technologies have been developed for the purpose of inhibiting miRNA function. An initial approach was the development of modified oligonucleotides that could bind to miRNAs in order to block miRNA-mRNA interactions in tissue culture (Meister et al., 2004). More recently,

further modifications have been generated to create "antagomiRs," which act both to inhibit and turn over target miRNAs (Krutzfeldt et al., 2005). While the original modified oligonucleotides could cause transient inhibition of miRNAs in cell culture, antagomiRs can be delivered to whole animals and appear to be stable in tissues for several weeks. It is not, however, clear how uniform antagomiR delivery is to individual cells and subsets of tissues in an animal. Furthermore, as antagomiRs are broadly delivered throughout the animal, it is neither possible to induce miRNA inhibition in different cell types, nor to track inhibition of a miRNA by the antagomiR. Moreover, longer-term studies of miRNA inhibition would require repeated delivery of the antagomiR, which is both costly and time-consuming.

As an alternative, a genetic approach to miRNA inhibition has been developed, termed miRNA sponges (Ebert et al., 2007). These sponges work through expression from a plasmid of a synthetic transcript containing many copies of an idealized miRNA binding site. With sufficient expression of the sponge, the miRNA is "sopped up" by the synthetic target, leading to subsequent derepression of endogenous targets. While this allows for both regulatable and stable inhibition of an individual or family of miRNAs, it is still limited by not knowing the completeness of miRNA inhibition in the animal. Considering that miRNAs, which require stable association with a given transcript for repression, are concentration-dependent regulators, complete inhibition of a miRNA may have distinct effects from incomplete inhibition. Thus, traditional genetic strategies have provided crucial insights to the role of miRNAs in development and disease.

MiRNA mammalian genetics—global consequences

To initially examine the global role of miRNAs in mammalian development, a germline knockout of *Dicer1* was generated (Bernstein et al., 2003). These animals are embryonic lethal, with failure to collect *Dicer1*-null progeny after embryonic day 6.5 (E6.5) because of a depletion of stem cells. This stem cell failure was bolstered by the inability to generated *Dicer1*-null embryonic stem (ES) cells. In fact, once *Dicer1*-null ES cells were generated by forced deletion of a conditional allele of *Dicer1*, they underwent a sharp cell cycle arrest (Kanellopoulou et al., 2005; Murchison et al., 2005). In cells that eventually bypassed this arrest, both self-renewal and differentiation were severely compromised. This suggested that *Dicer1* was essential for ES cell identity and function.

While these findings demonstrated that Dicer was required for early mammalian development, it was unclear whether this requirement was due to Dicer's role in miRNA biogenesis or its function in either alternative small RNA processing events or functions unrelated to small RNA maturation. To assess further the contribution of miRNA biogenesis to mammalian development, a similar knockout allele was generated for *Dgcr8* (Wang et al., 2007). While *Dgcr8*-null progeny are also inviable, they survive longer than *Dicer1*-null embryos. Moreover, it was possible to generate *Dgcr8*-null ES cells; however, these cells also experience a cell cycle block at the G1 phase of the cell cycle that is triggered by the embryonic miRNA family miR-290 (Wang et al., 2008b). These *Dgcr8*-null ES cells, though experiencing impaired self-renewal, were able to express both pluripotency and differentiation markers. Overall, these observations suggested that there were important differences between mutations in different components of the miRNA processing pathway, with the particular potential for miRNA-independent functions for *Dicer1*. This idea has been further supported by small RNA cloning and sequencing from *Dicer1* and *Dgcr8*-null ES cells, which has revealed several small RNA

species that are produced in a *Dicer1*-dependent, *Dgcr8*-independent manner (Babiarz et al., 2008).

In addition to ES cells, mouse embryonic fibroblasts (MEFs) have been analyzed in the context of depleted miRNA processing. In these studies, it was initially noted that *Dicer1* loss triggered cell cycle defects similar to those in ES cells, with a particular increase in aneuploidy due to extended chromosome bridges, which was in line with an earlier analysis of Dicer-depleted chicken cells, which noted increases in chromosome segregation defects and changes in centromeric heterochromatin structure (Fukagawa et al., 2004; Harfe et al., 2005). These findings were followed by a more temporally regulated deletion of *Dicer1* in MEFs, which found its loss induced cellular senescence via the p19Arf-p53 pathway (Mudhasani et al., 2008). While the trigger for p19Arf was not determined in this study, it further highlighted the cell cycle consequences of miRNA processing ablation.

While these cellular studies have provided an important groundwork for examining the loss of miRNA processing in development, more precise genetic studies have allowed for the characterization of miRNAs in general on tissue-specific developmental pathways. Some of the earliest examinations of *Dicer1* loss occurred in the lymphoid system, with deletion of *Dicer1* in T cells. It was initially observed that loss of *Dicer1* caused aberrant lineage choice and differentiation in tandem with significantly reduced survival (Cobb et al., 2005; Muljo et al., 2005). Moreover, mutation of either *Dicer1* or *Drosha* in various subsets of T cells was shown to cause specific depletion of regulatory T cells (T-regs) (Chong et al., 2008; Cobb et al., 2006; Liston et al., 2008; Zhou et al., 2008). This loss of T-regs led to a robust autoimmune disorder in the *Dicer1*-null animals, and this was a cell autonomous consequence of loss of miRNA processing within the T-cell lineage. In sum, these data highlight a role for miRNA biogenesis in

T-cell lineage choice and a potential role for mutated miRNA processing factors in autoimmune diseases.

Further examination of miRNAs in the lymphoid compartment occurred with mutation of *Dicer1* in the B-cell lineage. In these animals, loss of miRNAs caused a potent block of differentiation at the pro- to pre-B cell transition (Koralov et al., 2008). This was coupled to a survival defect largely related to a specific miRNA cluster, miR-17~92. When survival was sustained by expression of anti-apoptotic factors, *Dicer1* loss further altered V(D)J recombination at stages downstream of gene rearrangements. These results further support the idea of miRNAs regulating developmental choice, frequently through changes in cell survival.

Another tissue type exhibiting striking changes in response to abrogated small RNA processing is the reproductive tract. A variety of studies have examined *Dicer1* loss in both the male and female reproductive systems, along with loss in the gametes proper (Gonzalez and Behringer, 2009; Hayashi et al., 2008; Hong et al., 2008; Maatouk et al., 2008; Murchison et al., 2007; Nagaraja et al., 2008; Otsuka et al., 2008; Papaioannou et al., 2009; Tang et al., 2007). In all cases, loss of *Dicer1* severely restricted both reproductive system development and gamete maturation, causing near to complete sterility. Interestingly, small RNA cloning from mouse oocytes revealed a novel class of endogenous siRNAs (endo-siRNAs) that, along with the piRNAs mentioned above, appear to target transposons and related mobile genetic elements for suppression (Tam et al., 2008; Watanabe et al., 2008). Notably, these endo-siRNAs are depleted in the absence of Dicer1, suggesting their processing through at least the Dicer1 step of small RNA maturation. Overall, these studies demonstrate a required function for small RNA processing in mammalian reproduction.

MiRNA processing has further been examined in the differentiation program of the skin, where bulge stem cells regulate development of both skin cells and the hair follicle (Andl et al., 2006; Yi et al., 2006; Yi et al., 2009; Yi et al., 2008). With loss of both *Dicer1* and *Dgcr8*, embryonic skin morphogenesis is strikingly impaired. This impairment is largely due to failure to produce the miRNA miR-203, whose repression of the stem-cell maintenance factor p63 is vital for differentiation of both the skin and hair follicle compartments. Similar defects in proper development in the absence of *Dicer1* have been seen in the kidney, where loss of *Dicer1* causes glomerular disease related to tubular injury (Harvey et al., 2008; Shi et al., 2008). Moreover, loss of *Dicer1* in the developing lung causes a similar morphogenetic defect in the lung epithelium, arguably through altered FGF signaling between lung epithelial cells and the mesenchyme (Harris et al., 2006). Similar failures in morphogenesis are further seen in the developing limb bud and postnatal endothelium upon *Dicer1* deletion (Harfe et al., 2005; Suarez et al., 2008). Overall, a role for the miRNA processing pathway in developmental morphogenesis and stem-cell maintenance, particularly in epithelial tissues, has been firmly established.

Examination of *Dicer1* loss in a variety of mesenchymal tissues has further demonstrated a role for miRNA processing in tissue remodeling (da Costa Martins et al., 2008; O'Rourke et al., 2007). In developing skeletal muscle, loss of *Dicer1* caused a potent cell death response in myoblasts, leading to significantly reduced skeletal muscle mass and abnormal myofiber structure. Moreover, loss of *Dicer1* in adult cardiac muscle triggered a potent remodeling of the cardiac myocardium, leading to atrial enlargement and eventual hypertrophy. Similar effects were seen in *Dicer1*-null chondrocytes, where there was a concomitant reduction in proliferation and enhancement of differentiation upon *Dicer1* mutation (da Costa Martins et al., 2008). In

total, these findings demonstrate that *Dicer1* regulates both differentiation and remodeling in mesenchymal tissues.

Additional evidence for miRNA-mediated control of cell survival is seen in the developing nervous system. In a variety of regions within the brain, including the cortex, hippocampus, striatal neurons, and cerebellum, deletion of *Dicer1* causes defective morphogenesis at both the cell and tissue level (Cuellar et al., 2008; Davis et al., 2008; De Pietri Tonelli et al., 2008; Schaefer et al., 2007). Frequently, this morphogenetic failure induces neurodegeneration; however, *Dicer1* loss can trigger neurological changes without neurodegeneration. In addition, *Dicer1* mutation in the mouse retina causes a progressive neurodegeneration, with animals unable to respond to light within weeks of *Dicer1* loss (Damiani et al., 2008). Overall, these results suggest a broad role for miRNA biogenesis in the nervous system.

A final tissue for which impaired miRNA maturation has been examined is the liver. Two studies examined loss of *Dicer1* in the late embryo and adult liver (Hand et al., 2009; Sekine et al., 2009). In both cases, loss of *Dicer1* led to a concomitant increase in both proliferation and apoptosis in hepatocytes that frequently induced replacement of the organ with wild type cells. Importantly, these changes were coupled to increases in inflammation and broad up-regulation of genes expressed in the fetal liver. In one study, aged mice, though largely repopulated with *Dicer1* wild type hepatocytes, developed hepatocellular carcinomas that exhibited some level of *Dicer1* recombination. Overall, these studies provide important evidence for the loss of miRNA processing altering not only tissue regeneration, but also proliferative and apoptotic programs that can promote oncogenic transformation.

MiRNA mammalian genetics—miRNAs and miRNA clusters

While the above methods of globally inhibiting miRNA biogenesis have provided valuable insights into the broad role of small RNAs in a variety of tissues, they face two important limitations. First, due to the clear necessity of some small RNAs for cellular viability, both germline and conditional loss of miRNA processing components causes early lethality. Second, while these studies demonstrate a role for small RNAs in general within a given tissue, they do not allow for simple examination of the particular miRNAs important within a given cell or tissue type. In order to examine particular small RNAs, targeted deletion of individual miRNAs, miRNA families, and miRNA clusters has been performed.

Some of the earliest examinations of particular miRNAs in mammalian development have occurred in the muscle, where highly expressed miRNAs have been identified. An initial study was performed on the muscle-specific miRNA miR-1-2 (Zhao et al., 2007). Comparable to *Dicer1* deletion in the heart, mutation of miR-1-2 caused defects in morphogenesis and function of cardiomyocytes, particularly through difficulties in electrical conduction. In addition to miR-1, a loss-of-function allele has been generated for miR-208, an intronic miRNA within the alpha-myosin heavy chain (α-MHC) gene (van Rooij et al., 2007). Its deletion, without changing α-MHC expression, caused pronounced defects in cardiac hypertrophy and fibrosis, along with effects on expression of other MHC members. Beyond the muscle, deletion of an endothelial-specific miRNA, miR-126, caused specific defects in vascular development and angiogenesis, which was more pronounced during vascular remodeling after myocardial infarction (Wang et al., 2008a). Overall, these early knockout studies provided valuable evidence for individual miRNAs regulating cardiac muscle and endothelial cell function.

While these studies develop a role for individual miRNAs in muscle and endothelial cell development, the majority of analyses of loss-of-function for miRNAs and miRNA families have occurred in the hematopoeitic system. In the myeloid compartment, the clearest example of miRNA regulation has been shown in the granulocyte lineage by mutation of miR-223 (Johnnidis et al., 2008). In miR-223 mutant mice, there is a specific increase in the number and proliferation of granulocyte progenitors. Strikingly, these consequences on differentiation are largely regulated by a single target, the transcription factor Mef2c, whose additional mutation reversed the effects of miR-223 loss. In the lymphoid lineage, a set of diverse phenotypes have been explored by loss-of-function mutation. The earliest example of lymphoid consequences for loss of a miRNA was miR-155, whose mutation led to defects in T helper cell differentiation and function in antibody production, particularly through alterations in cytokine production (Rodriguez et al., 2007; Xiao et al., 2007). Moreover, specific depletion of miR-155 in T-regs, previously shown to be impaired by loss of *Dicer1*, caused defects in proliferation and T-reg number comparable to complete loss of miRNA processing (Lu et al., 2009). Overall, these studies provide striking evidence for individual miRNAs in both myeloid and T cell differentiation.

In the B lymphocyte lineage, several groups have reported mutations of miRNAs or miRNA clusters that contribute to differentiation. For miR-150, mutation impacted the later stages of B cell development, as it is most strongly expressed in mature B lymphocytes (Xiao et al., 2007). As with miR-223, these effects appear to be largely controlled by a single miRNA target, the transcription factor c-Myb, as deletion of c-Myb causes the opposite effect of miR-150 loss. Of note, the miR-150/c-Myb pathway has also been shown to be important in myeloid

differentiation, where altering miR-150 regulates the megakaryocyte versus erythroid transition via c-Myb (Lu et al., 2008).

While the above studies have examined the role of individual miRNAs in developmental decisions, as of now there is a single report of mutation of a miRNA cluster in the mouse (Ventura et al., 2008). In this study, the miR-17~92 cluster (discussed in detail below) and its paralogs were mutated. While the germline nulls of miR-17~92 die perinatally due to defects in lung and vascular development, inactivation of the cluster in the adult caused a striking developmental phenotype in B cells. In particular, miR-17~92-null B cells had a block in differentiation specifically at the pro- to pre-B cell transition, likely through up-regulation of the pro-apoptotic factor Bim due to loss of repression by several members of the cluster. This work was complemented by work showing that the effects of *Dicer1* loss on B cell differentiation were largely reversed via re-expression of mature miRNAs from the miR-17~92 cluster, again through altered expression of Bim and its effects on apoptosis (Koralov et al., 2008). Taken together, this collection of genetic analyses provides strong evidence for both individual miRNAs and miRNA clusters in regulating important developmental transitions in mammals.

MiRNAs and cancer

Overview

Cancer is a multifactorial disease in which acquisition of either an individual change or a series of changes promotes uncontrolled growth of a subset of cells within a tissue; in many cases, these changes are DNA mutations. In oncogenes, these changes function by promoting the

activity of a target gene; in tumor suppressor genes, these changes function by inhibiting or eliminating the activity of a target gene. Through these changes, tumor cells broadly acquire several features requisite for development of cancer: self-sufficiency in growth signals, insensitivity to anti-growth signals, escape from apoptosis, replicative immortality, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000).

Based upon the known regulatory function of miRNAs, Ron Plasterk posited four ways that miRNAs and their targets could be regulated in human disease: there could be a gain of function mutation of a miRNA, there could be a loss-of-function mutation of a miRNA, there could be a loss-of-function mutation in an target mRNA so that it avoids miRNA-mediated repression, or there could be a gain of function mutation in a target mRNA so that it gains novel-miRNA mediated repression (Plasterk, 2006). While the fourth possibility has not yet been observed, there are several lines of evidence suggesting the first three types of changes occur in many, if not all, cancers. Here I will describe the most important observations for miRNAs and mRNA targets undergoing these classes of alterations in human cancers, and what insights they can provide on the role of miRNAs in tumorigenesis.

Gain of function mutation of miRNAs

MiR-17~92

The earliest studies of miRNAs in tumorigenesis focused on the role of miRNAs as oncogenes. These initial studies were largely led by examination of the miR-17~92 cluster. MiR-17~92 are particularly important in the study of oncogenic miRNAs, as the cluster was the first

polycistronic miRNA shown to be altered in the disease. This cluster is position in 13q31.2, which is frequently amplified in a wide collection of of B-cell lymphomas, including Burkitt's lymphomas, Diffuse Large B-Cell Lymphomas, and Mantle Cell Lymphomas (He et al., 2005; Lenz et al., 2008; Ota et al., 2004; Rinaldi et al., 2007; Tagawa et al., 2007; Tagawa and Seto, 2005). The cluster is also found overexpressed, and occasionally amplified, in a number of other cancer types, including lung cancers, hepatocarcinomas, neuroblastomas, medulloblastomas, and colorectal cancers (Connolly et al., 2008; Hayashita et al., 2005; He et al., 2005; Uziel et al., 2009).

The argument that *miR-17~92* is a *bona fide* oncogene is strengthened by the finding that a truncated version of this cluster (miR-17~19b, lacking miR-92) greatly accelerates tumorigenesis in a mouse model of B cell lymphoma in which the initiating oncogene is an Eμ-Myc transgene (Figure 1.2) (He et al., 2005). Interestingly, *miR-17~92* not only cooperates with the Myc oncogene but it is also one of its transcriptional targets (O'Donnell et al., 2005). A molecular circuit has emerged, linking Myc, *miR-17~92*, and members of the E2F family of transcription factors. According to this model, c-Myc can directly induce transcription of *miR-17~92,* and two members of this cluster (*miR-17* and *miR-20*), can in turn repress the translation of E2F proteins (E2F1, E2F2 and E2F3). The circuitry is closed by E2F1-3 that can also induce the transcription of *miR-17~92* (O'Donnell et al., 2005; Sylvestre et al., 2007; Woods et al., 2007).

The significance of these findings with respect to the oncogenic properties of *miR-17~92* is unclear. E2F proteins are key regulators of cell-cycle progression, but are also inducers of

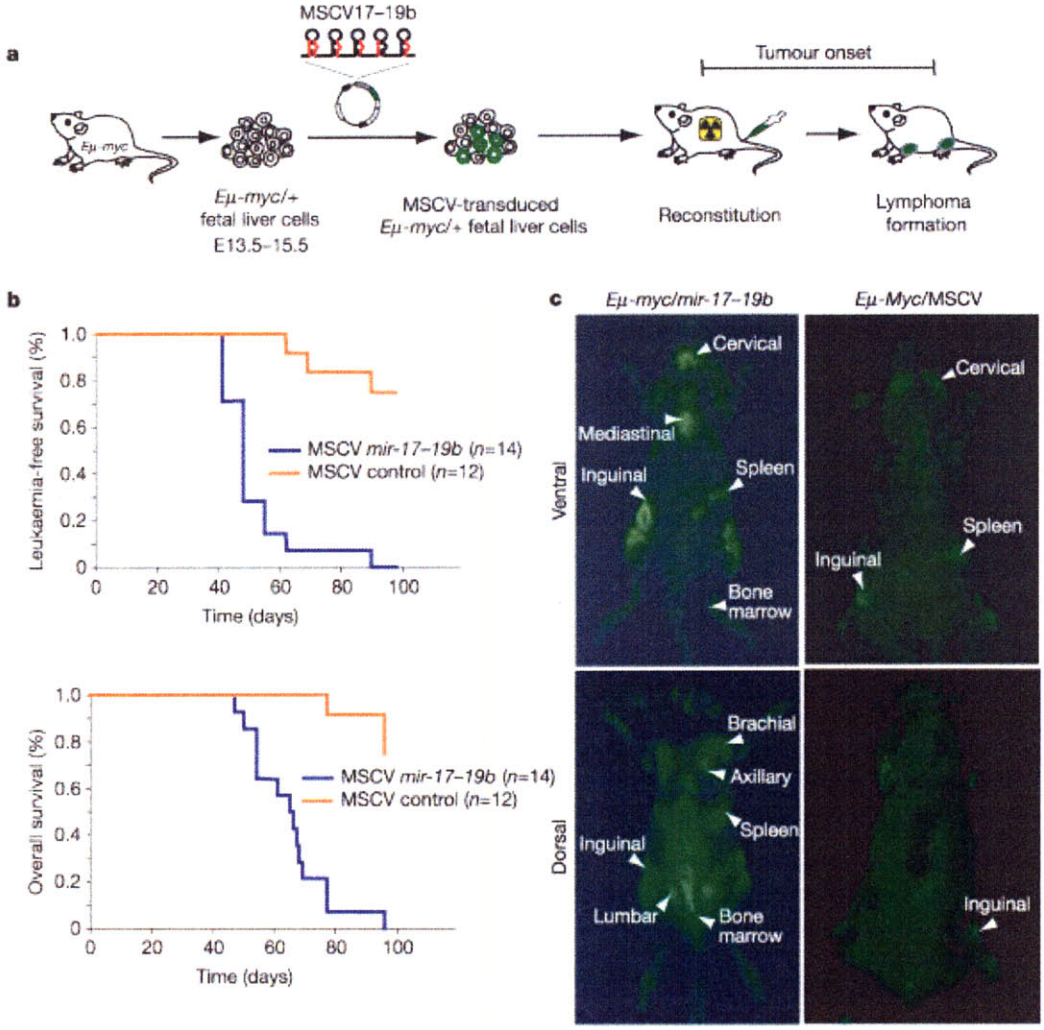


Figure 1.2 Overexpression of the *mir-17–19b* cluster accelerates *c-myc*-induced lymphomagenesis in mice. a, Schematic representation of the adoptive transfer protocol using *Eμ-myc* HSCs. **b**, Mice reconstituted with HSCs expressing *mir-17–19b* (MSCV *mir-17–19b*) or infected with a control MSCV virus were monitored by blood smear analysis. The Kaplan-Meier curves represent the percentage of leukemia-free survival or overall survival. **c**, External GFP imaging of tumor-bearing mice shows the overall distribution of tumor cells. Adapted from (He et al., 2005).

apoptosis (Iaquinta and Lees, 2007). This latter function is likely mediated via activation of the p19-Arf/p53 pathway. It is tempting to speculate that the modulation of E2F1-3 expression by *miR-17~92* may contribute to Myc-induced transformation by suppressing cell death. This would be consistent with the observation, by He and colleagues, that in the Eμ-Myc model B cell lymphomas over-expressing *miR-17~19b* show greatly reduced spontaneous apoptosis compared to controls (He et al., 2005). While repression of E2F1-3 is likely to contribute to the oncogenic activity of *miR-17~92,* it does not explain it fully. Indeed, not only does deletion of *E2f1* not accelerate Eμ-Myc-induced lymphomagenesis, it actually suppresses it (Baudino et al., 2003). Indeed a number of other targets of *miR-17~92* have also been proposed to mediated its tumor promoting activities, among them TGF-β signaling pathway factors, PTEN, p21, AML1, Bim, p107, TSP1, and CTGF (Dews et al., 2006; Fontana et al., 2008; Fontana et al., 2007; Lu et al., 2007; Petrocca et al., 2008b). In particular, the finding of regulation by miR-17~92 of TSP1 and CTGF, both regulators of angiogenesis, provide particular evidence for this miRNA cluster in tumor progression (Dews et al., 2006).

The recent generation of loss-of-function and gain-of-function alleles of *miR-17~92* in the mouse has provided important insights into its role in tumorigenesis (Lu et al., 2007; Uziel et al., 2009; Xiao et al., 2008). Transgenic mice overexpressing miR-17~92 in lymphocyte progenitors develop a lymphoproliferative disorder affecting both B and T cells that eventually results in autoimmunity (Xiao et al., 2008). Moreover, transgenic mice overexpressing miR-17~92 in the lung epithelium undergo a potent increase in lung epithelial proliferation coupled to defective differentiation of the proximal lung, with an expansion of the neuroendocrine cell compartment; this effect could be at least partly mediated by inhibition of p107, a member of the Rb family of cell cycle regulators (Lu et al., 2007). This lung hyper-proliferation is of particular

interest, as miR-17~92 overexpression, frequently via amplification of the *miR-17~92* locus, has been reported in human lung cancers (Hayashita et al., 2005; Matsubara et al., 2007). Finally, expression of the miR-17~92 cluster in cerebellar granule neuron progenitors from *Ink4c*$^{-/-}$; *Ptch1*$^{+/-}$ but not *Ink4c*$^{-/-}$; *Trp53*$^{-/-}$ mice cooperated to promote development of medulloblastoma in a mouse transplant model (Uziel et al., 2009). Taken together, these reports provide direct evidence for this oncogenic miRNA cluster in the mouse.

These findings lend additional support to the hypothesis that one or more miRNAs expressed from the *miR-17~92* are critical regulators of cell survival in lymphocytes. While we are far from a full identification of the key set of genes controlled by *miR-17~92,* the works of Ventura and Xiao suggest that the effect on cell survival may be mediated, at least in part, by the BH3-only protein Bim. Both authors have reported that the Bim 3' UTR contains multiple binding sites for miRNAs expressed by *miR-17~92* and that the expression of this pro-apoptotic gene is suppressed by this cluster (Koralov et al., 2008; Xiao et al., 2008). Bim is a crucial regulator of cell survival in lymphocytes and a potent suppressor of Myc-induced B cell lymphomagenesis (Bouillet et al., 1999; Bouillet et al., 2002; Egle et al., 2004; Hemann et al., 2005).

Moreover, the tumor suppressor function of Bim is dosage-sensitive, as even deletion of a single *Bim* allele accelerates lymphomagenesis in the Eμ-Myc model without concomitant loss of the remaining wild type allele (Bouillet et al., 1999; Bouillet et al., 2002; Egle et al., 2004; Hemann et al., 2005). It is therefore likely that Bim suppression by *miR-17~92* contributes to both the tumor promoting activity of *miR-17~92* overexpression and to its physiologic function in regulating normal B cell development. In support of this hypothesis, Koralov and colleagues have found that deletion of the miRNA processing enzyme *Dicer1* in B cell progenitors leads to

a drastic defect in B cell proliferation that is reminiscent of what was observed in *miR-17~92*–deficient mice (Koralov et al., 2008). This defect is accompanied by elevated expression of Bim and, more importantly, can be partially rescued by *Bim* deletion.

A recurring theme with miRNAs is the frequent occurrence of multiple copies of the same, or closely related, miRNAs at different loci. This fact raises the possibility of functional overlap and compensation and significantly complicates the interpretation of loss-of-function studies where only one member of a miRNA family is deleted. The *miR-17~92* cluster is no exception to this observation. Both the human and the mouse genomes contain two closely related paralogs: *miR-106a~363* (on the X chromosome), and *miR-106b~25* (on chromosome 7 in humans and chromosome 5 in mice). These two paralogs express miRNAs that have the same seed sequences as the miRNAs expressed from *miR-17~92* and in some cases are absolutely identical. While more work has been done to characterize *miR-17~92*, emerging evidence suggest that *miR-106a~363* and *miR-106b~25* are also potential oncogenes. *MiR-106b~25* is overexpressed in gastric cancers, where it impairs signaling through the TGF-β pathway, and *miR-106a~363* has been reported to be frequently activated by insertional mutagenesis in mouse models of T cell leukemias and lymphomas (Landais et al., 2007; Lum et al., 2007; Petrocca et al., 2008a; Uren et al., 2008).

To determine the extent of functional cooperation between these three highly related miRNA clusters, Ventura and colleagues have generated mutant mice for each of these three clusters, as well as compound mutants in which two or all three clusters were simultaneously deleted. Their analysis shows that *miR-17~92* alone is essential for mouse development, as even double mutant mice simultaneously deficient for *miR-106a~363* and *miR-106b~25* develop normally and are fertile. Interestingly, however, while deletion of *miR-106b~25* does not result

in any obvious defect, simultaneous deletion of *miR-106b~25* and *miR-17~92* leads to embryonic lethality at mid-gestation, with embryos showing more severe developmental defects and increased apoptosis than for deletion of *miR-17~92* alone (Ventura et al., 2008).

At the moment it is unclear why *miR-106b~25* and *miR-106a~363* are not essential genes while *miR-17~92* is. It must be noted, however, that *miR-106a~363* is expressed at much lower levels compared to *miR-17~92* in most tissues and that the *miR-106b~25* cluster does not contain miRNAs of the miR-19 and miR-18 family (which are present in the *miR-17~92* cluster). It is likely that generation of more refined mutant mice, in which individual miRNAs belonging to the *miR-17~92* cluster are deleted or added back, will provide answers to these important questions.

MiR-155

Mir-155 is a well established oncogenic miRNA that is frequently over-expressed in human B cell malignancies, including Diffuse Large B Cell lymphomas, primary mediastinal B cell lymphomas, and Hodgkin's lymphomas (Eis et al., 2005; Kluiver et al., 2006; Kluiver et al., 2005; Kluiver et al., 2007; van den Berg et al., 2003). The history of miR-155 is particularly instructive. In 1989, well before the discovery of miRNAs, Clurman and Hayward identified a common proviral DNA insertion site in lymphomas induced by the avian leucosis virus and named the corresponding gene BIC (Clurman and Hayward, 1989). Although it was noted that the BIC RNA could form extensive secondary structures, and in particular a 145-nucleotide stem-loop, the absence of an obvious open reading frame remained a puzzling feature, even after

it was conclusively shown that BIC could cooperate with Myc in lymphomagenesis and erythroleukemogenesis (Tam, 2001; Tam et al., 1997; Tam et al., 2002).

The puzzle was solved when it became clear that the BIC gene hosts a microRNA—miR-155—and that miR-155 overexpression in B cells is sufficient to induce polyclonal proliferation of pre-B cells followed by full-blown B cell leukemia in a transgenic mouse model (Costinean et al., 2006; Eis et al., 2005; Kluiver et al., 2005; Lagos-Quintana et al., 2002). A different and complementary approach to investigate the function of miR-155 has been recently reported by two groups. Rather than deleting the miR-155 gene, these authors have generated mice carrying a mutation disrupting a miR-155 binding site in the 3' UTR of a predicted miR-155 target gene.

Both groups selected the Activation-Induced Cytidine Deaminase (AID) gene, which allows immunoglobulin diversification by promoting somatic hypermutation (SHM) and class-switch recombination (CSR) in B cells. The 3' UTR of this gene contains a single binding site for miR-155 that confers sensitivity to miR-155-mediated repression in a reporter assay (Teng et al., 2008). Interestingly, mutation of the miR-155 binding site on AID and deletion of miR-155 lead to a comparable increase in AID expression in activated B-cells, suggesting that AID is a bona fide target of miR-155 (Dorsett et al., 2008; Teng et al., 2008).

While both approaches observed comparable changes in expression, the functional consequences were significantly different in the two systems; while in miR-155 deficient B cells CSR was reduced, it was increased in B cells carrying the mutant AID (Dorsett et al., 2008; Teng et al., 2008; Thai et al., 2007; Vigorito et al., 2007). This contrast was observed with respect to SHM, which is normal in miR-155-deficient B cells, but appears increased in B cells with the mutant miR-155 binding site in the AID gene elegantly demonstrate how loss of miRNA-mediated regulation of a single target gene can have profound physiologic effects, they also

serve as a reminder that the phenotypic consequences of loss of a miRNA are likely due to the simultaneous deregulation of more than one gene.

MiR-372~373

MiR-373 was one of the earliest known miRNAs found as an oncogene. It was discovered in a reverse-genetic screen, in which a library of known miRNAs and miRNA clusters was expressed in fibroblasts and screened for their ability to bypass oncogene-induced senescence (Voorhoeve et al., 2006). In this screen, two members of a miRNA cluster (miR-372~373) were found to suppress p53-dependent cell cycle arrest in response to H-Ras^{G12V}. In this study, they observed that miR-373 was able to escape this cellular senescence via repression of the LATS2 tumor suppressor, which subsequently activates the p53 effector, p21. Importantly, miR-372 and miR-373 were observed to be frequently overexpressed in testicular cancer, a disease noted for the dearth of mutations in *TP53*. In total, these findings provided strong evidence for a role of this miRNA family in oncogenic transformation.

Additional data for the role of miR-373 orthologs has been found in examination of the proliferation of ES cells. As mentioned above, it is possible to generate *Dgcr8*-null ES cells, which, coincident with a block in miRNA biogenesis, undergo a potent block in the G1/S phase of the cell cycle (Wang et al., 2008b). Importantly, expression of a pre-miRNA library in *Dgcr8*-null ES cells revealed that numerous members of the miR-290 miRNA family (of which miR-373 is a member) were able to rescue proliferation via suppression of a variety of predicted

mRNA targets involved in cell cycle regulation. In sum, these findings bolster the notion of the greater miR-373 family regulating transformation through cell cycle control.

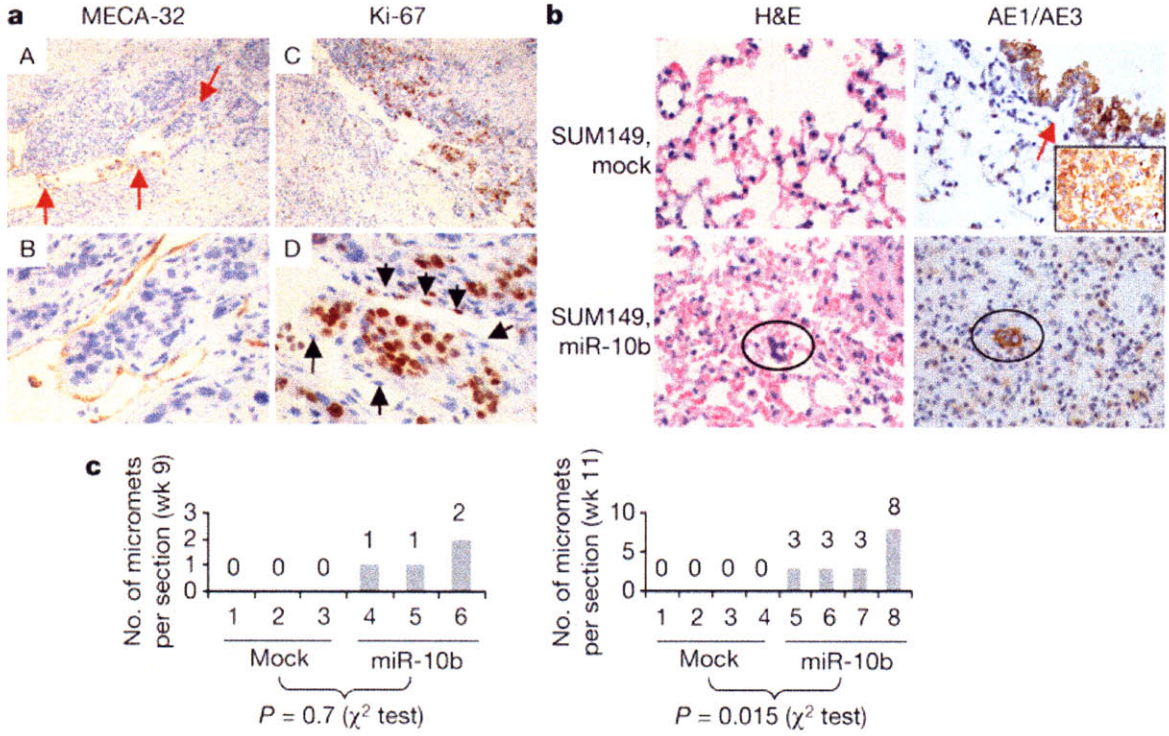
Beyond these effects on transformation, further data has suggested a role for miR-373 in several steps of tumor progression, including invasion and metastasis (Huang et al., 2008). In this study, they performed a reverse-genetic screen to identify miRNAs that promote cell migration *in vitro* and validated the hits *in vivo* using breast cancer cell lines. Three miRNAs sharing the same seed sequence (miR-373, miR-520c and miR-520e) scored positively in the assays. These three miRNAs are expressed from a common region on human chromosome 19. While miR-373 has homologs in the mouse, dog, and rat genomes, miR-520c and miR-520e belong to a very large family of miRNAs that have no homologs in non-primate genomes (Bentwich et al., 2005).

Importantly, the pro-metastatic activity of miR-373 appears to be independent from its ability to suppress the p53 pathway, as RNAi-mediated depletion of neither p53 nor LATS2 conferred increased cell migration and invasion *in vitro* to non-metastatic breast cancer cells. Rather, Huang and colleagues propose that miR-373 may promote metastasis by suppressing the cell surface receptor CD44, since CD44 is a direct target of miR-373 and injection of MCF-7 cells stably expressing shRNAs against CD44 promoted the formation of distant metastases (Huang et al., 2008). While they observed elevated expression of these miRNAs in metastatic breast cancer patients, additional work will be required to determine the widespread relevance of these results for human breast cancer patients and their treatment.

MiR-10b

By performing miRNA expression profiling in primary human breast cancers and breast cancer cell lines, Iorio and colleagues had identified a set of 29 miRNAs that were significantly deregulated compared to normal breast tissues (Iorio et al., 2005). To determine whether any of these miRNAs contribute to metastasis, Ma and colleagues compared their expression in a set of metastatic and non-metastatic human breast cancer cell lines. With this approach they identified miR-10b as a miRNA whose expression was increased only in metastatic cell lines (Ma et al., 2007). They also identified the mechanism underlying increased expression of miR-10b, by showing that it is a direct transcriptional target of TWIST1, a master regulator of embryogenesis and a powerful inducer of the Epithelial-Mesenchimal Transition (EMT) (Yang et al., 2004). Ma and colleagues went on to demonstrate that ectopic expression of miR-10b was sufficient to convert the non-metastatic cell lines into metastatic ones, as determined by *in vitro* invasion assays and a xenograft mouse model (Ma et al., 2007). Importantly, miR-10b overexpression caused increased motility, invasion, and angiogenesis, but not increased proliferation, indicating that miR-10b specifically affects the metastatic process and not primary tumor formation (Figure 1.3). Consistent with this hypothesis, Ma and colleagues also showed that while miR-10b expression is elevated in 50% of primary breast tumors from patients with metastasis (9 out of 18), it is low in primary tumors from metastasis-free women (5/5).

As for the mechanism by which miR-10b exerts its pro-metastatic influence, Ma and colleagues showed that it is at least in part mediated by the direct repression of HOXD10, a homeobox gene that is known to repress the transcription of several genes involved in cell



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	Lung metastasis	Peritoneal metastasis
Mock	0/10	0/10
miR-10b	8/10	3/10
P (χ^2 test)	0.01	0.1

Figure 1.3 MiR-10b induces distant metastasis. a, MECA-32- and Ki-67-stained sections of a primary mammary tumor formed by miR-10b-transduced cells, at week 6 after orthotopic transplantation. Arrows in panel **a** indicate tumor cells within a vessel, and arrows in panel **d** indicate endothelial cells.**b**, H&E- and AE1/AE3-stained sections of lungs isolated from mice that received miR-10b-transduced or mock-infected cells, at week 9 after transplantation. Circles indicate clusters of metastatic cells. The arrow indicates normal bronchial epithelium. Inset, AE1/AE3 staining of a primary tumor. **c**, Numbers of lung micrometastases per section in individual mice that received orthotopic injection of miR-10b-transduced or mock-infected SUM149 cells, at week 9 (left panel) and week 11 (right panel) after transplantation, respectively. **d**, Bright field, GFP imaging, and H&E staining of lungs isolated from mice that received orthotopic injection of miR-10b-transduced or mock-infected cells, at week 11 after transplantation. **e**, GFP imaging and H&E staining of a macroscopic peritoneal metastasis in a mouse that received orthotopic injection of miR-10b-transduced cells, at week 11 after transplantation. **f**, Incidence of lung metastasis and macroscopic peritoneal metastasis in mice that received orthotopic injection of miR-10b-transduced or mock-infected cells. Adapted from (Ma et al., 2007).

migration and invasion. One of the genes repressed by HOXD10 is the small GTPase RhoC, a well characterized player in the metastatic process (Clark et al., 2000). Accordingly, expression of a miR-10b-resistent HOXD10 (missing the 3' UTR) or knockdown of RHOC largely suppressed the miR-10b-induced cell migration and invasion phenotype. Thus, the work of Ma and colleagues has defined a novel pathway that contributes to the metastatic phenotype of a subset of human breast cancers and paves the way for additional studies aimed at determining the general relevance of miR-10b as a prognostic indicator and as a possible pharmacological target in human breast cancer patients.

Loss-of-function mutation of miRNAs

Global down-regulation of miRNAs

One of the earliest indications that miRNAs may participate in tumorigenesis was the finding that tumor cells display a global reduction in miRNA abundance compared to normal tissue (Lu et al., 2005). This global examination of miRNAs occurred via bead-based miRNA profiling, in which oligonucleotide-capture probes complementary to human and mouse miRNAs are coupled to polystyrene beads impregnated with various mixtures of fluorescent dyes so that each individual miRNA is represented by a distinct color. Following adaptor ligation, reverse-transcribed miRNAs are amplified using a common primer, hybridized to the capture beads and stained. Finally, the beads are analyzed on a high-speed flow cytometer capable of identifying bead color and intensity. These data can be used to determine the abundance of individual

miRNA species. In addition to being sensitive and economic, the bead-based hybridization approach described above appears to have a higher specificity compared to glass-hybridization methods. Indeed, in a spiking experiment involving 11 closely related sequences, the bead method was capable of efficiently discriminating between single base-pair mismatches.

This method was applied to profile the expression of 217 miRNAs in human tumors compared to normal tissues, leading to the striking observation that tumor cells display a global reduction in miRNA expression levels (Figure 1.4) (Lu et al., 2005). An analogous difference was observed when normal lungs and lung tumors from K-Ras mutant mice were compared. Although this finding does not prove that microRNAs play a functional role in tumorigenesis, the observation that more differentiated cells express higher levels of miRNAs than do undifferentiated or poorly differentiated cells suggests that miRNA might be involved in determining and maintaining the differentiated state.

This global loss was not found in other large-scale studies of miRNA profiling (Volinia et al., 2006). These differences were largely due to alternative means of normalization. While the Golub group normalized against synthetic RNA spikes introduced before and after hybridization, the Croce group normalized against the total small RNA content. Since the loss of miRNAs is so broad, such a change would not be observed by the latter normalization scheme. Additionally, the Golub group's miRNA profiles were shown to be an accurate predictor of both the differentiation state and developmental origin of the tumor. Moreover, classifiers based on these miRNA profiles were found to be more informative than mRNA profiles in classifying poorly differentiated tumors. In total, these reports demonstrate that miRNA profiling is a powerful method for cataloging the miRNA content in a given cell type, developmental stage, or disease state.

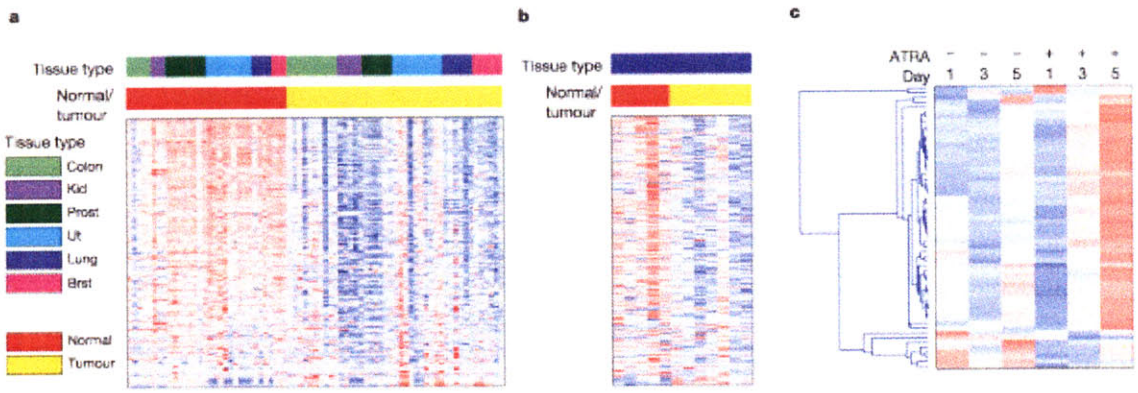

Figure 1.4. Comparison between normal and tumor samples reveals global changes in miRNA expression. a, Markers were selected to correlate with the normal versus tumor distinction. A heatmap of miRNA expression is shown. **b**, miRNA markers of normal versus tumor distinction in human tissues from **a**, applied to normal lungs and lung adenocarcinomas of $Kras^{LA1}$ mice. **c**, HL-60 cells were treated with *all-trans* retinoic acid (ATRA +) or vehicle (-) for the indicated number of days. A heatmap of miRNA expression from a representative experiment is shown. Adapted from (Lu et al., 2005).

At first, the mechanism by which miRNAs are globally lost in mammalian cancers was largely undefined. An initial observation, in which primary miRNA transcripts were analyzed in the same samples profiled at the miRNA level, revealed that many pri-miRNAs were unchanged in the same tumors that experienced down-regulation of the mature miRNA (Thomson et al., 2006). This data indicated that the global loss of miRNAs occurred at the level of processing or miRNA product stability. In contrast to this result, an analysis of Myc-regulated miRNAs suggested a widespread silencing of miRNAs at the transcriptional level by c-Myc (Chang et al., 2008). A series of recent findings has provided further evidence for miRNA down-regulation at the level of miRNA processing. First, an analysis of mRNA expression for a series of miRNA processing components in ovarian cancer revealed decreases in Dicer1 and Drosha transcript abundance in both ovarian cancer cell lines and in primary tumors, and that patients with reduced expression of these components have a significant worsening of prognosis (Merritt et al., 2008). Similar changes in Dicer1 expression, and a subsequent worsening of prognosis, have been observed in lung adenocarcinomas (Karube et al., 2005). Moreover, they discovered somatic point mutations in *DICER1 and RNASEN* (the gene which encodes Drosha) within both ovarian cancer cell lines and patient material.

Beyond this, a recent examination of mismatch-repair deficient cancers observed a striking rate of frameshift mutations in *TARBP2*, the gene encoding TRBP (Melo et al., 2009). These frameshifts led to premature stop codons in *TARBP2* and subsequent decreases in TRBP levels. This decrease in TRBP leads to reduced levels of Dicer1 and reductions in miRNA processing. Notably, overexpression of either wild type *TARBP2* or wild type *DICER1* in these mutant cells, which restored miRNA processing to wild type levels, was sufficient to slow tumor growth, suggesting that the defect in miRNA processing caused by these mutations altered the

tumor phenotype. This was in line with other work showing that impaired miRNA processing enhances the tumorigenic potential of cancer cells (discussed in detail in Chapters 2 and 3 of this thesis) (Kumar et al., 2007). In total, this collection of studies provides strong evidence for abrogation of the miRNA processing machinery within human cancer.

MiR-34

A series of recent reports has explored the regulation of miRNAs by tumor suppressor genes. These studies have focused on the miRNAs regulated by p53, a tumor suppressor gene that is frequently inactivated in human cancers. This approach has lead to the identification of the miR-34 family as being an important mediator of p53 activity (Bommer et al., 2007; Chang et al., 2007; Cole et al., 2008; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Tazawa et al., 2007; Welch et al., 2007). This family consists of three highly related miRNAs expressed from two separate loci: miR-34a from chromosome 1p36 and miR-34b/miR-34c as a cluster from 11q23. The transcription of both loci appears to be directly regulated by p53 via binding to conserved sites in the respective promoters.

TP53 is a very well characterized transcription factor and a potent tumor suppressor whose activity is induced in response to a variety of stimuli, including DNA damage, oncogene activation, hypoxia, and oxidative stress (Vogelstein and Kinzler, 1992). *TP53* exerts its tumor suppressive function by activating the promoters of a large number of genes whose function is to act as cell-cycle inhibitors or pro-apoptotic factors. The result is, depending on the cellular context, either cell death or growth arrest (in the form of cell cycle arrest or senescence).

Intriguingly, cellular senescence appears to function as a longer-term effector of p53 activation. As such, regulation of the transcriptome by miRNAs would be an effective means of establishing the p53 senescence response. Consistent with a role in the p53 pathway, ectopic miR-34 expression in cell culture systems leads to a partial arrest in the G1 phase of the cell cycle or to increased cell death. From the studies published so far, it appears that the three members of the miR-34 family are functionally equivalent. It must be noted, however, that while miR-34a is predominantly expressed in the brain, miR-34b/c are reported to be present at high levels in the lungs and are virtually undetectable in other tissues. It will be important to determine whether stimuli that induce p53 activation also induce broad expression of miR-34 members *in vivo* and whether miR-34 exerts p53-independent functions.

While there is consensus that miR-34 can mediate, at least in part, the p53 response, the exact mechanism is still unclear. Microarray experiments reveal that ectopic miR-34 expression results in a broad change in gene expression that resembles that induced by p53 activation, with an enrichment for genes involved in controlling apoptosis, cell cycle progression, angiogenesis, and DNA repair. Different groups have focused on different targets, including members of the E2F family of transcription factors, the antiapoptotic protein BCL2, the NAD-dependent deacetylase SIRT1, cell cycle regulators CDK4, CDK6, Cyclin E2, and even a membrane receptor (c-MET) (Bommer et al., 2007; He et al., 2007; Tazawa et al., 2007; Welch et al., 2007; Yamakuchi et al., 2008). Although it is formally possible that further studies will show that one of these genes is the critical target of miR-34, it is more likely that miR-34 acts by fine-tuning the response to p53 activation via the modulation of a large array of genes.

Finally, it must be noted that while most experiments indicate that miR-34 is downstream of p53, some data also suggest that miR-34 can act, at least in part, upstream of p53. In

particular, ectopic expression of miR-34a leads to increased p53 expression in colon carcinoma cell lines and induces apoptosis less efficiently in p53-/- cells than in cells with functional p53. The studies described above strongly suggest that members of the miR-34 family may act as tumor suppressor genes. Consistent with this hypothesis, hemizygous deletion of the miR-34a locus is a recurrent event in several human cancers (Bagchi and Mills, 2008). Furthermore, deletion of miR-34a and low or undetectable expression of this miRNA has been reported in neuroblastoma cell lines, where its reintroduction leads to apoptosis (Welch et al., 2007). Loss of miR-34b and miR-34c has also been observed in breast and in non-small cell lung cancers, and expression of these miRNAs is frequently reduced in non-small cell lung cancer cell lines (Bommer et al., 2007; Calin et al., 2004).

While all these observations seem to indicate an important role of the miR-34 family in tumorigenesis, determining whether loss of miR-34 expression can initiate or accelerate tumorigenesis will require the generation of miR-34 knockout mice. Moreover, the possible role of miR-34 in regulating cellular senescence in response to p53 activation provides an interesting basis for understanding the effects of p53 on aging, a process which functions largely through cellular senescence in tissues; these ideas are supported by the regulation of SIRT1, an important regulator of eukaryotic aging, by miR-34 (Serrano and Blasco, 2007; Yamakuchi et al., 2008). Such investigations will almost certainly require the use of miR-34 deficient animals.

MiR-206 and miR-335

If the work on miR-10b shows miRNAs can act by promoting metastasis, the work on miR-335 and miR-206 shows that other miRNAs have the opposite effect, acting as powerful suppressors of the metastatic phenotype. To identify anti-metastatic miRNAs, Tavazoie and colleagues also used breast cancer cell lines (Tavazoie et al., 2008). For their analysis, they subjected the poorly metastatic human breast cancer cell line MDA-MB-231 and a set of highly metastatic derivatives that preferentially colonize the bones or the lungs to miRNA expression profiling. Of the six miRNAs whose expression was most consistently decreased in the metastatic lines, three (miR-335, miR-126, and miR-206) were shown to actually suppress metastasis formation when their expression was restored in the metastatic lines.

While miR-126 also negatively affected tumor cell proliferation, expression of miR-335 and miR-206 did not result in significant changes in either proliferation or apoptosis of the tumor cells, indicating that these two miRNAs specifically affect the metastatic process. Indeed, by performing *in vitro* assays, Tavazoie and colleagues demonstrated that these two miRNAs have a profound effect on cell migration. As for the molecular mechanism, the authors provide an interesting list of pro-metastasis genes whose expression is regulated by miR-335, and show that knock down of two of them (SOX4 and TNC) can suppress the metastatic phenotype. Clearly, additional studies are warranted to fully dissect the critical pathways that are physiologically regulated by miR-335, miR-126, and miR-206, and to determine whether they can become useful anticancer targets, but at a minimum these three miRNA are promising novel prognostic indicators in breast cancer patients. Indeed, by analyzing a cohort of 20 primary breast cancers, Tavazoie and colleague have found that low expression of miR-335 and miR-126 (and to a lesser extent miR-206) correlates with very poor metastasis-free survival. In sum, this study provides

powerful evidence that this family of miRNAs suppresses breast cancer metastasis, and that these miRNAs are relevant in clinical progression.

Let-7

Let-7, one of the first miRNAs to be discovered, was initially identified in *C. elegans*, where it controls the timing of developmental events. In the nematode, loss of *let-7* prevents cell cycle exit and terminal differentiation of a particular type of cells (seam cells) at the transition from larval to adult life (Reinhart et al., 2000). Homologs of *let-7* have been identified in mammals, and the human genome contains at least 12 members of the let-7 family, organized in eight different loci (http://microrna.sanger.ac.uk).

Consistent with studies that show let-7's central role in controlling cell proliferation and differentiation in vertebrates, a number of studies have provided evidence supporting a role for let-7 in human cancer. First, various *let-7* members are located at chromosomal sites frequently deleted in a variety of human tumor types (Calin et al., 2004). In addition, reduced expression of let-7 family members has been reported in a subset of non-small lung cancers, where it correlates with poor prognosis and advanced staging of the disease (Takamizawa et al., 2004). Moreover, expression of let-7 family members was part of a miRNA expression set which predicted for poor prognosis in lung adenocarcinomas (Yanaihara et al., 2006).

Gene deletion is not the only mechanism through which expression of let-7 family members is deregulated in human cancers. As discussed above, the c-Myc oncogene can negatively regulate let-7, an ability that may very well contribute to its oncogenic potential

(Chang et al., 2008). Furthermore, recent work has shown that the Lin-28 protein binds to the pre-miRNAs of let-7 family members preventing the processing of the mature miRNAs, a finding that explains why some cell types and cancer cell lines have high levels of let-7 precursors, but undetectable mature let-7 (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). Interestingly, recent data suggests that c-Myc's ability to reduce let-7 levels is not due to transcriptional silencing, but to up-regulation of Lin-28, which subsequently inhibits processing of let-7 family members (Chang et al., 2009).

An exciting initial explanation for the role of let-7 in tumor suppression was provided by the Slack group, who showed that, in both the nematode and human cells, let-7 family members can suppress expression of the Ras family of proto-oncogenes via their 3' UTRs (Johnson et al., 2005). Beyond the Ras family, several other oncogenes have been described as targets of the let-7 miRNA family. For example, the HMG-domain containing protein HMGA2 is strongly repressed by let-7 (Lee and Dutta, 2007; Mayr et al., 2007; Park et al., 2007). The c-Myc oncogene itself appears to be targeted by let-7 family members in Burkitt's lymphoma (Sampson et al., 2007). Finally, a recent examination of gene expression in response to let-7 expression observed direct and widespread regulation of several positive regulators of the cell cycle, including CDC25A, CDK6, and Cyclin D2, by the let-7 family (Johnson et al., 2007). Overall, these studies posit that loss of let-7 expression may promote tumorigenesis by simultaneously leading to up-regulation of a wide network of oncogenes.

While a conclusive demonstration that loss of let-7 expression can initiate or promote tumorigenesis *in vivo* will likely have to wait for generation of mice carrying loss-of-function alleles of let-7 (a feat complicated by the presence of multiple let-7 members in mice), three recent reports have shown that forced let-7 expression can suppress tumorigenesis. Yu and

colleagues found that the expression of let-7 family members is markedly lower in breast tumor-initiating cells (BT-ICs) compared to non-BT-ICs derived from primary breast cancers (Yu et al., 2007). Consistent with a role in regulating self renewal of breast cancer cells, overexpression of let-7 in BT-ICs leads to reduced proliferation, increased differentiation, and suppression of tumor formation in immuno-compromised mice. The authors also propose that the effects of let-7 on self-renewal and differentiation are likely mediated by different targets; indeed, suppression of HMGA2 promoted differentiation with little effect on self-renewal, while suppression of H-Ras had a profound effect on self-renewal without promoting differentiation.

Beyond cancer stem cells, several reports have suggested a role for let-7 in normal stem cell function. An early finding was provided by the Hannon group, in which let-7 expression was strongly altered during differentiation *in vitro* in a mammary cell differentiation system (Ibarra et al., 2007). Moreover, a study by the Morrison laboratory provided specific evidence for let-7 in mediating stem cell self-renewal in young mice (Nishino et al., 2008). In both hematopoeitic and neural stem cells, let-7 expression increased with age, leading to suppression of HMGA2 and subsequent up-regulation of the Ink4a-Arf transcripts, which have been shown to regulate self-renewal of stem cells. While this data conflicts with the work of Yu and colleagues suggesting that HMGA2 suppression by let-7 changed differentiation but not self-renewal, it is possible that these differences are due to distinctions in stem-cell function in normal and cancer stem cells.

While these studies provide significant evidence for let-7 in tumor development and stem cell maintenance in the mammary, blood, and neural cell lineages, let-7 loss was initially observed to be important particularly in non-small cell lung cancer. However, the functional evidence for let-7 suppressing lung tumorigenesis has been relatively limited until two recent studies. In one, let-7 was induced in lung cancer cells and ectopically expressed in autochthonous

lung tumors via lentiviral expression (discussed in detail in Chapter 4 of this thesis) (Kumar et al., 2008). In another report, let-7 family members were delivered to lung cancer cells *in vitro* as siRNAs and delivered in an autochthonous model of non-small cell lung cancer by adenovirus (Esquela-Kerscher et al., 2008). Taken together, these studies provided an important demonstration for let-7 suppressing the initiation of non-small cell lung cancer.

Loss-of-function mutation of miRNA binding sites

While the altered expression of miRNAs has been shown to control several processes important to tumorigenesis, a small set of examples is being found in which target mRNAs undergo changes to avoid miRNA-mediated regulation. The clearest example has been seen for the HMG-domain containing protein HMGA2 (Mayr et al., 2007). As mentioned above, the HMGA2 mRNA contains multiple sites for the let-7 miRNA within its 3' UTR. Moreover, it has been known that HMGA2 translocations that occur and are important for the development of human lipomas, though removing only a small fragment of the coding sequence of the mRNA, cause a potent increase in its expression. Mayr and colleagues thus postulated that the rearrangements seen in human lipomas were not selecting for loss of the coding sequence, but instead loss of the 3' UTR and consequent miRNA regulation. In fact, point mutation of all of the let-7 binding sites in the HMGA2 3' UTR recapitulated the translocation mRNA's ability to transform cells (Figure 1.5). Overall, this study provided powerful evidence for genetic selection of an oncogene to lose its miRNA regulation.

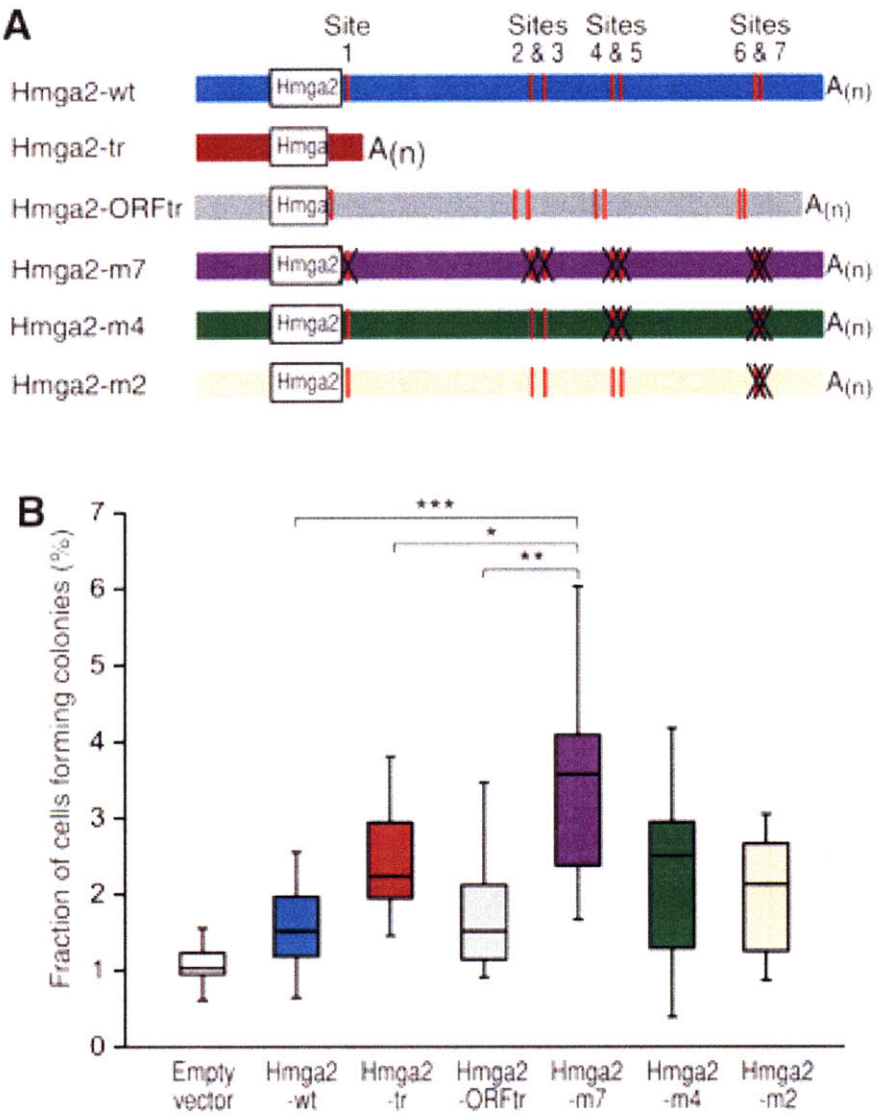

Figure 1.5 Soft-agar assay for anchorage-independent growth. a, Hmga2 constructs used for stable transfection **b,** Colony formation. For cells stably transfected with the indicated vector, the percentage that yielded colonies is plotted. When compared with Hmga2-wt, a significantly higher number of colonies was observed for Hmga2-tr. Hmga2-m7 showed significantly more colonies than any of the other constructs tested. Adapted from (Mayr et al., 2007).

While the above study suggested that 3' UTRs undergo mutagenic pressure in human cancer, it was unclear whether there were global changes comparable to that of HMGA2. A recent report has provided significant evidence for an epigenetic change of this kind (Sandberg et al., 2008). In this study, it was noted that active proliferating cells have shorter 3' UTRs due to upstream polyadenylation site usage. Notably, this alternative polyadenylation causes loss of many miRNA binding sites. This has led to the idea that cancer cells, which undergo increased proliferation relative to normal cells, might utilize such alternative polyadenylation to eliminate miRNA-mediated repression of oncogenic targets. While mechanisms of both alternative polyadenylation and oncogenic de-repression have yet to be determined, this observation has provided a novel means by which cancers can escape miRNA-mediated regulation.

Conclusions

MiRNAs are a novel small RNA species whose regulation of target mRNAs alters many processes germane to transformation and tumorigenesis. Taken together, the evidence for changes in miRNAs broadly and specifically is robust. However, the functional validation of these changes in models of cancer has been severely lacking. To better understand the role of miRNAs in cancer, we have performed a series of studies comparing loss and gain of function of miRNAs in mammalian cancer models. These studies provide important demonstrations of the effects of miRNAs on cancer and suggest novel therapeutic targets for the disease.

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CHAPTER 2: Impaired microRNA processing enhances cellular transformation and tumorigenesis

The work presented in this chapter is largely taken from a paper published in *Nature Genetics* (Kumar et al., 2007). I contributed all figures presented in this chapter. Jun Lu helped to design the original pSuper oligonucleotides and performed miRNA profiling on samples. Kim L. Mercer, independent of me, performed tumor grading analysis presented in Figure 2.9D.

MicroRNAs (miRNAs) are a novel class of small non-coding RNAs that post-transcriptionally regulate the expression of target mRNA transcripts. Many of these target mRNA transcripts are involved in proliferation, differentiation, and apoptosis (Bartel, 2004; Gregory and Shiekhattar, 2005), processes commonly altered during tumorigenesis. Recent work has shown a global decrease of mature miRNA expression in human cancers (Lu et al., 2005). However, it is unclear whether this global repression of miRNAs is reflective of the undifferentiated state of tumors or causally contributes to the transformed phenotype. Here we show that global repression of miRNA maturation promotes cellular transformation and tumorigenesis. Cancer cells expressing short hairpin RNAs (shRNAs) targeting three different components of the miRNA processing machinery exhibited a significant decrease in steady-state miRNA levels and displayed a more pronounced transformed phenotype. In animals, miRNA processing-impaired cells (hereafter referred to as miR KD cells) formed tumors with accelerated kinetics. These tumors were more invasive than controls, suggesting that global miRNA loss enhances tumorigenesis. Furthermore, conditional deletion of *Dicer1* enhanced tumor development in a K-Ras—induced mouse model of lung cancer. Overall, these studies indicate that abrogation of global miRNA processing promotes tumorigenesis.

In order to examine the role of miRNA processing in tumorigenesis, we designed shRNAs targeting the three established regulators of miRNA processing: Drosha, DGCR8, and Dicer1. In murine lung adenocarcinoma (LKR13) cells (Johnson et al., 2001; Wislez et al., 2006), these hairpins achieved robust knockdown of target mRNA levels (Figure 2.1A) and reduced steady-state miRNA levels (Figure 2.1B). Of note, these shRNAs did not completely eliminate mature miRNAs. For Dicer1 in particular, we would expect incomplete knockdown, as

Figure 2.1

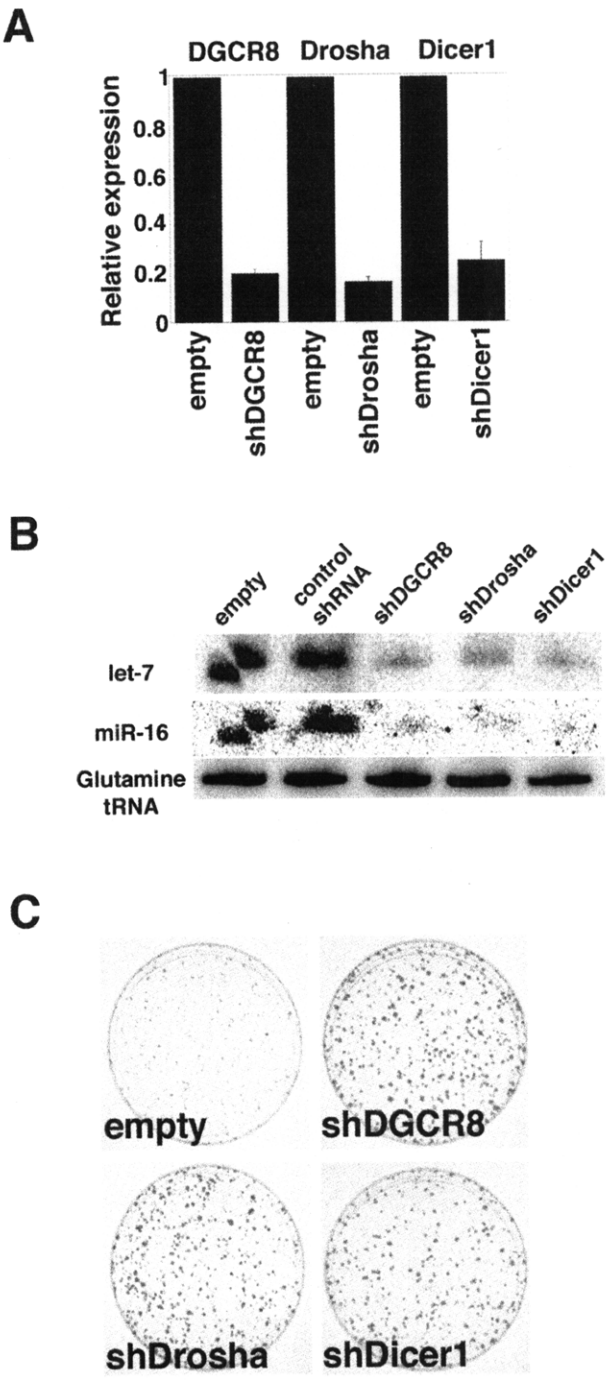

A
DGCR8
Drosha
Dicer1
Relative expression
1
0.8
0.6
0.4
0.2
0
empty
shDGCR8
empty
shDrosha
empty
shDicer1
B
empty
control shRNA
shDGCR8
shDrosha
shDicer1
let-7
miR-16
Glutamine tRNA
C
empty
shDGCR8
shDrosha
shDicer1

Figure 2.1 continued

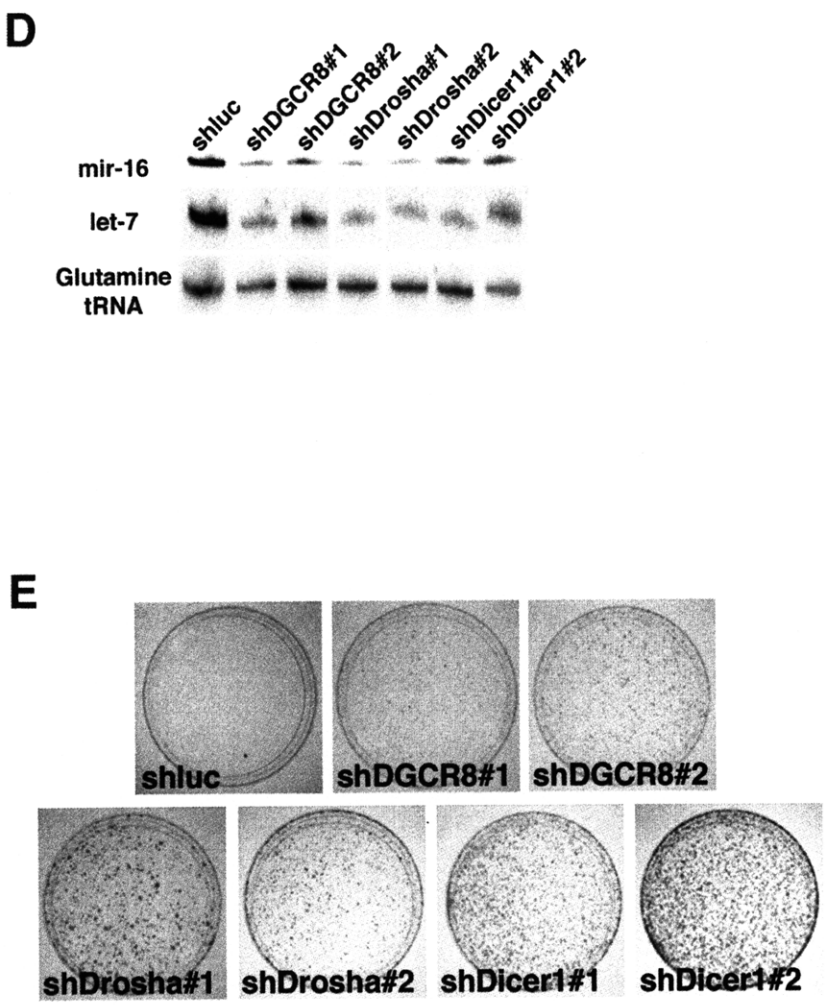


Figure 2.1 Impaired miRNA processing promotes cellular transformation. (A) Quantitative real-time polymerase chain reaction (Q-RT PCR) was performed with probes for DGCR8, Drosha, or Dicer1 in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 (see Supplementary Methods for details). Expression levels were initially normalized to TBP and subsequently normalized relative to DGCR8, Drosha, or Dicer1 levels in LKR13 cells infected with empty vector. Values are mean +/- s.d. (n=3) with propagated error. (B) Small RNA Northern blotting was performed against let-7 and glutamine tRNA in LKR13 cells infected with empty vector, a control shRNA (see Supplementary Methods for details), or shRNAs against DGCR8, Drosha, or Dicer1. (C) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at low density (2500 cells per 10 cm plate), grown for five days, and fixed and stained with crystal violet. (D) Small RNA Northern blotting was performed as above in U2OS cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 (see Supplementary Methods for details). (E) U2OS cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 were plated at low density as described above.

Dicer1 is required for the generation of its target small interfering RNA. We then tested the effect of impaired miRNA processing on the transformation properties of these cells. MiR KD LKR13 cells formed significantly larger colonies than controls when plated at low density (Figure 2.1C) and had improved colony formation in soft agar (Figure 2.2A). Additionally, miR KD cells formed more foci than control cells (Figure 2.2B). These enhanced transformation properties appear to be a consequence of increased proliferation, as miR KD cells grew faster than controls, exhibited a higher percentage of cells entering S phase, and reduced numbers of G_0/G_1 cells (Figure 2.2C-E). Thus, defective miRNA maturation (or some other unknown function of these genes) alters the transformed state of mouse tumor cells, accompanied by enhanced proliferative capacity.

To determine if impaired miRNA processing similarly affected murine and human cancer cells, we designed additional short hairpin RNAs against the human miRNA processing machinery. These hairpins also achieved knockdown of steady-state miRNA levels in three independent human cancer cell lines as assessed by Northern blotting (Figure 2.1D) and miRNA profiling (data not shown). Similar to the results from LKR13 cells, impaired miRNA processing enhanced both colony formation and growth in soft agar of human cancer cells (Figure 2.1E and Figure 2.2F). Importantly, this effect was independent of the tissue of origin; miR KD cells derived from osteosarcomas (U2OS), colorectal carcinomas (HCA7), and breast carcinomas (MCF7) all exhibited enhanced transformation (Figure 2.1E and Figure 2.2F-J). Overall, these results suggest that global repression of miRNAs enhances transformation of cancer cells.

To determine if global repression of miRNAs enhances the tumorigenic potential of cancer cells, we injected miR KD LKR13 cells subcutaneously into immune-compromised mice. MiR KD cells formed tumors with accelerated kinetics, with tumor volumes that were at least an

Figure 2.2

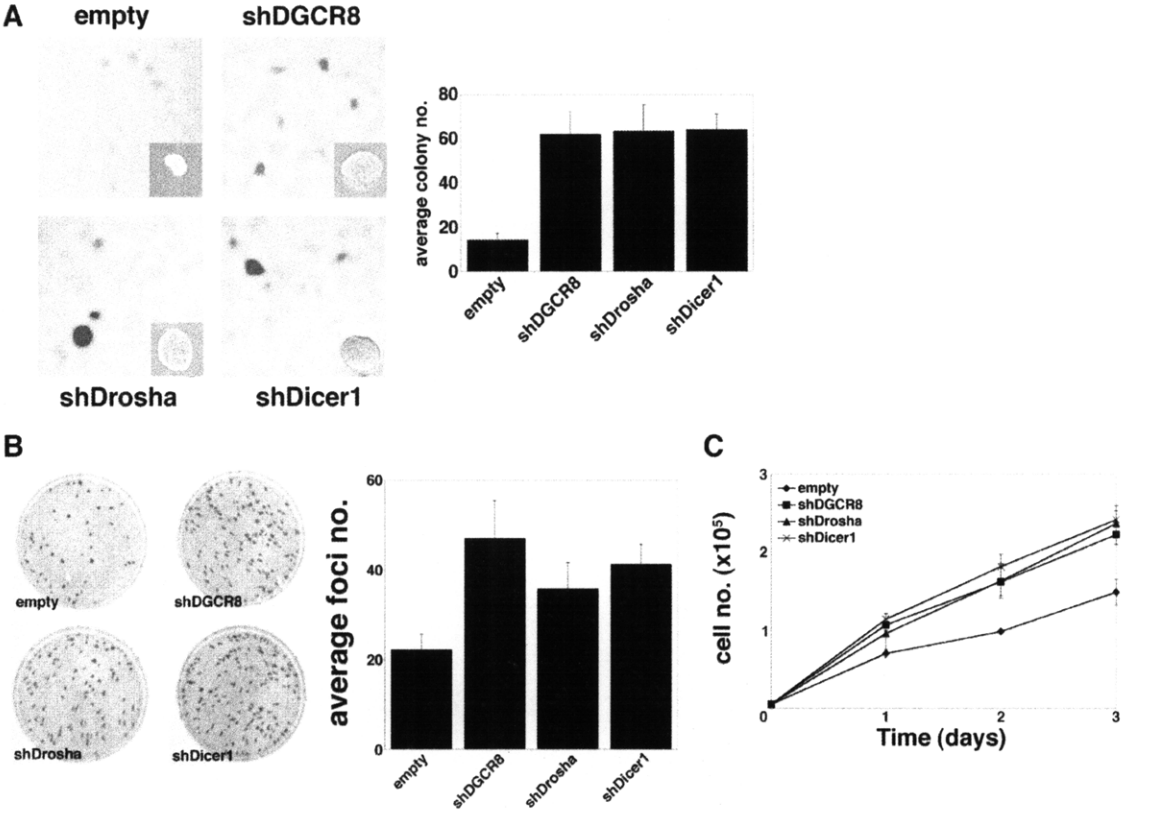

A
empty
shDGCR8
shDrosha
shDicer1
average colony no.
80
60
40
20
0
empty
shDGCR8
shDrosha
shDicer1
B
empty
shDGCR8
shDrosha
shDicer1
average foci no.
60
40
20
0
empty
shDGCR8
shDrosha
shDicer1
C
empty
shDGCR8
shDrosha
shDicer1
cell no. (x10^5)
3
2
1
0
1
2
3
Time (days)

Figure 2.2 continued

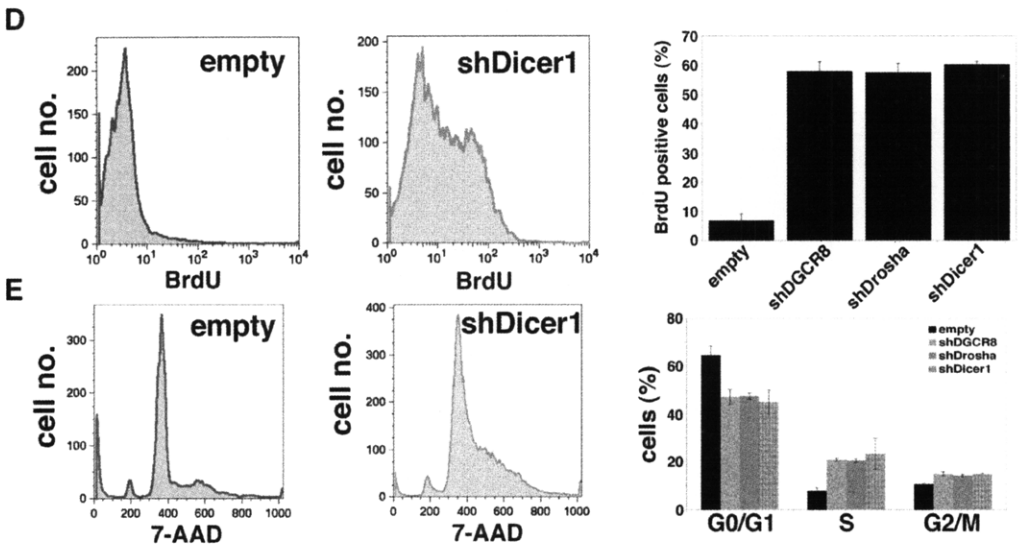


Figure 2.2 continued

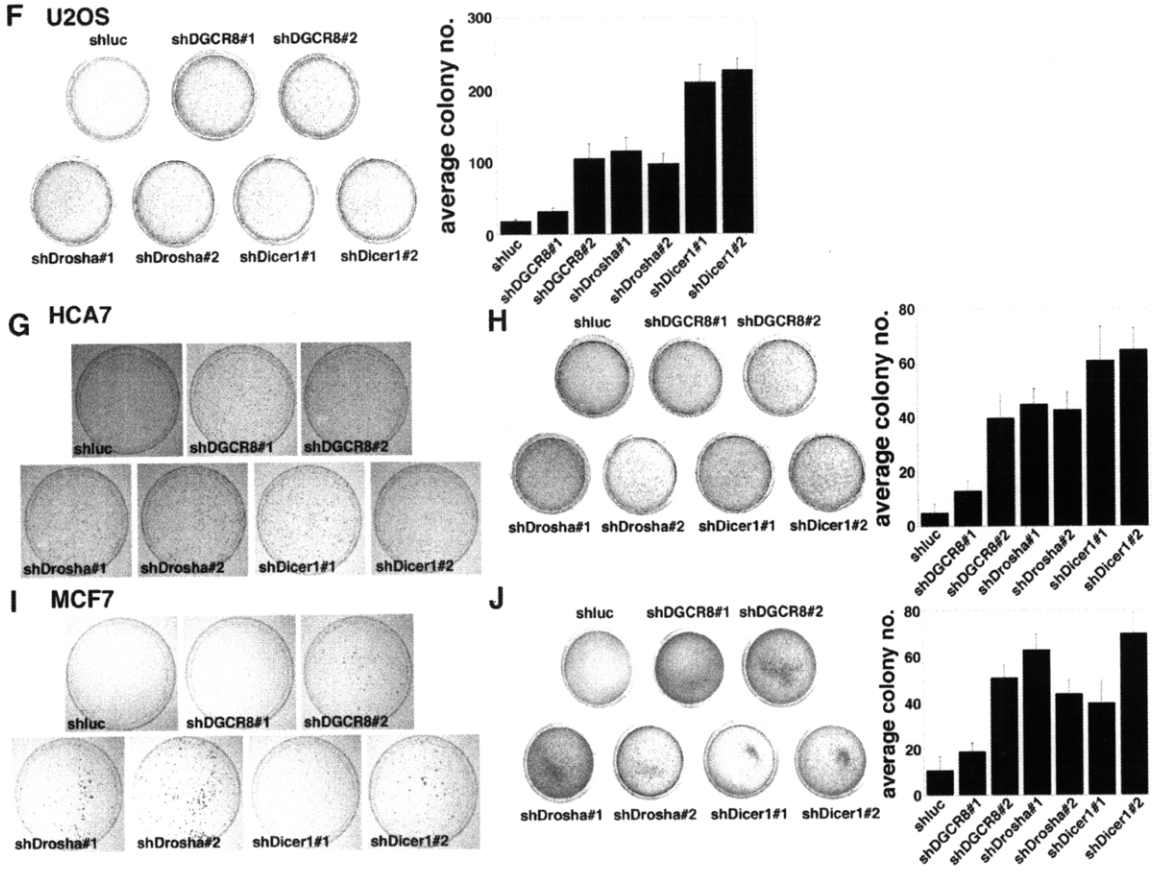


Figure 2.2. (A) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at 50,000 cells per plate in semisolid media, grown for two weeks, and fixed and stained with crystal violet. Colonies were counted from independent platings, with five random fields per condition. Values are mean +/- s.d. Plate magnification X4. Inset magnification, X20. (B) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at low density (1000 cells per plate) along with wild type mouse embryonic fibroblasts ($3X10^5$ cells per plate), grown for two weeks, and fixed and stained with crystal violet. Colonies were counted from independent platings. Values are mean +/- s.d. (n=3). (C) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at 5000 cells per well. Cells were counted over time in triplicate. Values are mean +/- s.e.m. (n=3). (D) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at 10^5 cells per well in media with reduced serum. Cells were subsequently pulse treated with BrdU, stained, and analyzed by flow cytometry (see Methods for details). Representative histograms of LKR13 cells infected with empty vector or a shRNA against Dicer1 are shown. Graph values are mean +/- s.d. (n=3). (E) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha or Dicer1 were plated at 10^5 cells per well in media with reduced serum. Cells were subsequently stained with 7-AAD and analyzed by flow cytometry (see Methods for details). Representative histograms of LKR13 cells infected with empty vector or a shRNA against Dicer1 are shown. Graph values are mean +/- s.d. (n=3). (F) U2OS cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 were plated in semisolid media and colonies counted as described above. (G) HCA7 cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 were plated at low density as described above. (H) HCA7 cells infected with shRNAs against *Renilla* luciferase, DGCR8,

Drosha, or Dicer1 were plated in semisolid media and colonies counted as described above. (I) MCF7 cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 were plated at low density as described above. (J) MCF7 cells infected with shRNAs against *Renilla* luciferase, DGCR8, Drosha, or Dicer1 were plated in semisolid media and colonies counted as described above.

order of magnitude greater than controls (Figure 2.3A). Steady-state miRNA levels in miR KD tumors were decreased compared to controls (Figure 2.4A). Earlier work suggested that the global loss of miRNAs in human cancers corresponds to altered differentiation in tumors relative to normal tissue (Lu et al., 2005). Thus, it was possible that miR KD tumors might be histologically distinct from controls. Surprisingly, miR KD tumors were similar to controls by histological analysis, all appearing moderately differentiated with the clear presence of glandular structures (Figure 2.4B). Importantly, however, miR KD tumors commonly invaded the surrounding normal tissue of the host, including skeletal muscle, adipose tissue, and nerve sheaths (Figure 2.3B). Careful histological analysis of control tumors revealed no such invasion (data not shown). Consistent with the invasive phenotype in miR KD tumors, miR KD cells demonstrated enhanced migration through a collagen matrix *in vitro*, suggesting that repression of miRNAs in cancer cells results in increased motility (Figure 2.3C).

Recent work with loss-of-function alleles of *Dicer1* in mammals has described defects in processes potentially unrelated to miRNAs, such as silencing of heterochromatic regions and chromosome segregation (Harfe et al., 2005; Kanellopoulou et al., 2005; Murchison et al., 2005). We observed no changes in transcript levels from major satellite repeats (Figure 2.5A); in addition, we noted similar levels of phosphorylated ribosomal S6 and eIF2α in miR KD and control cells, suggesting that global translation is not altered (Figure 2.5B). However, it remained possible that miR KD cells had undergone irreversible changes, independent of miRNAs, which enhance transformation. To investigate this possibility, we generated a shRNA against Dicer1 flanked by loxP sites (pSico^{R}-shDicer1), allowing subsequent removal of the hairpin via Cre-mediated recombination (Ventura et al., 2004). Upon delivery of adenoviral Cre to pSico^{R}-shDicer1 cells, we observed a concomitant restoration of miRNA processing (Supplementary

Figure 2.3

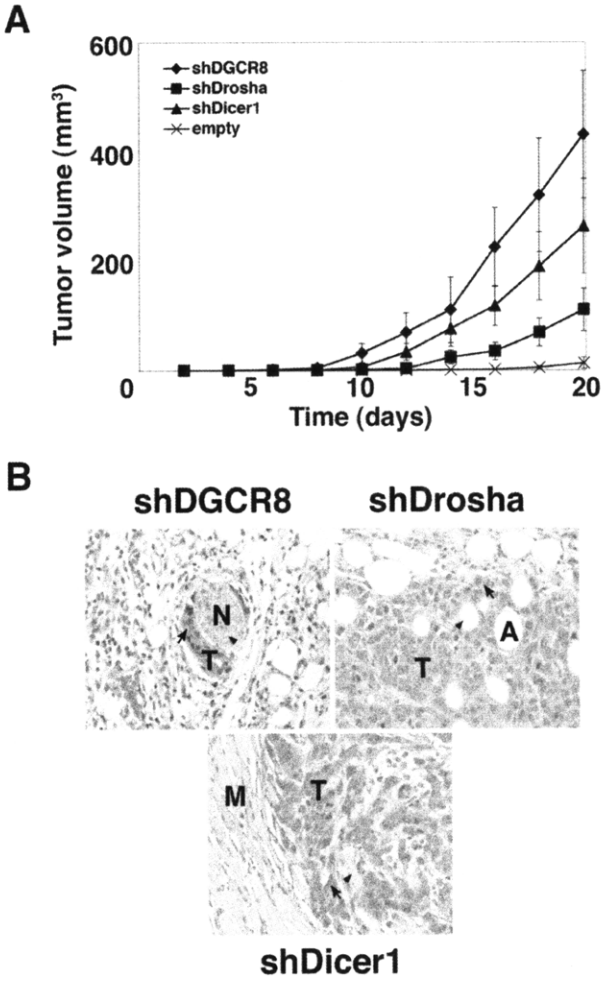

A
600
400
200
0
Tumor volume (mm³)
shDGCR8
shDrosha
shDicer1
empty
5
10
15
20
Time (days)
B
shDGCR8
shDrosha
N
T
A
T
M
T
shDicer1
C
9000
6000
3000
0
Rel. fluorescence
empty
shDGCR8
shDrosha
shDicer1

Figure 2.3 Impaired miRNA processing promotes tumorigenesis *in vivo*. (A) LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were injected subcutaneously into immunocompromised mice (10^5 cells/injection) and tumor growth was measured over time. Values are mean +/- s.e.m. (n=6). (B) Hematoxylin/eosin staining of tumors. Examples of tumor cells surrounding nerve sheaths and infiltrating into host adipose tissue and skeletal muscle of the host are shown. Original magnification, X40. N, nerve sheath. A, adipose tissue. M, skeletal muscle. T, tumor cells. Arrows indicate infiltrating tumor cells. Arrowheads indicate host tissue infiltrated by tumor cells. (C) *In vitro* migration assays using LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were performed (see Methods for details). Results are representative of three experimental repeats. Values are mean +/- s.d. (n=3).

Figure 2.4

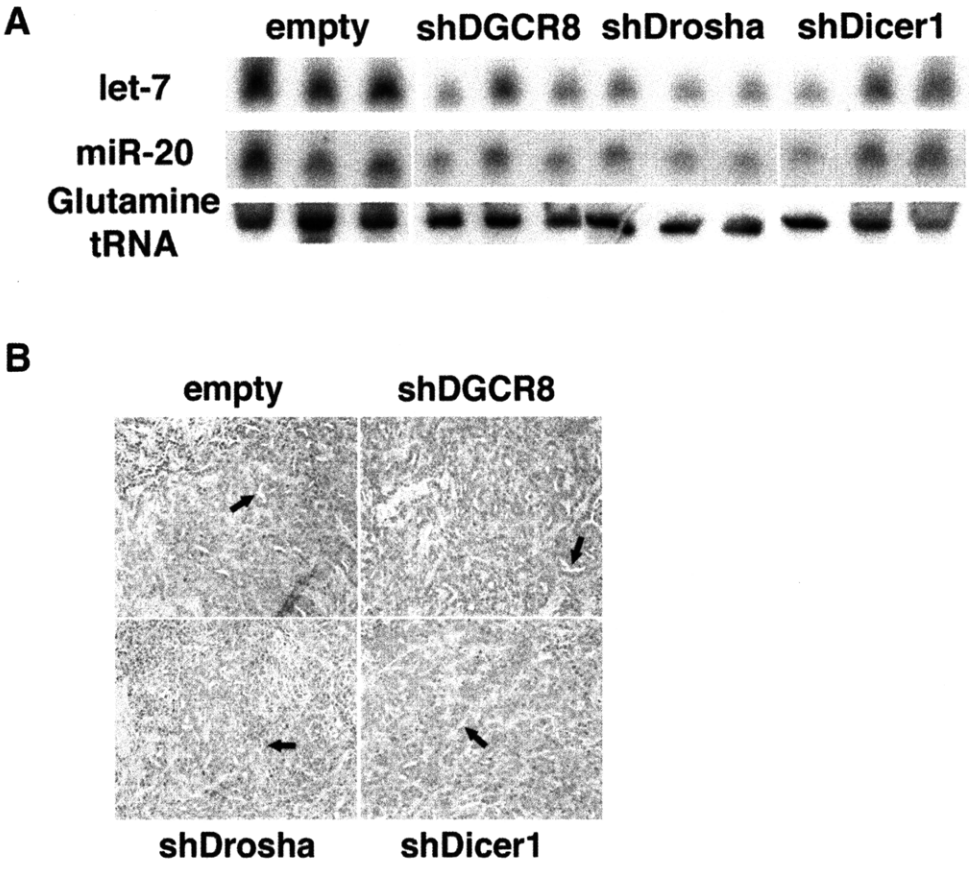
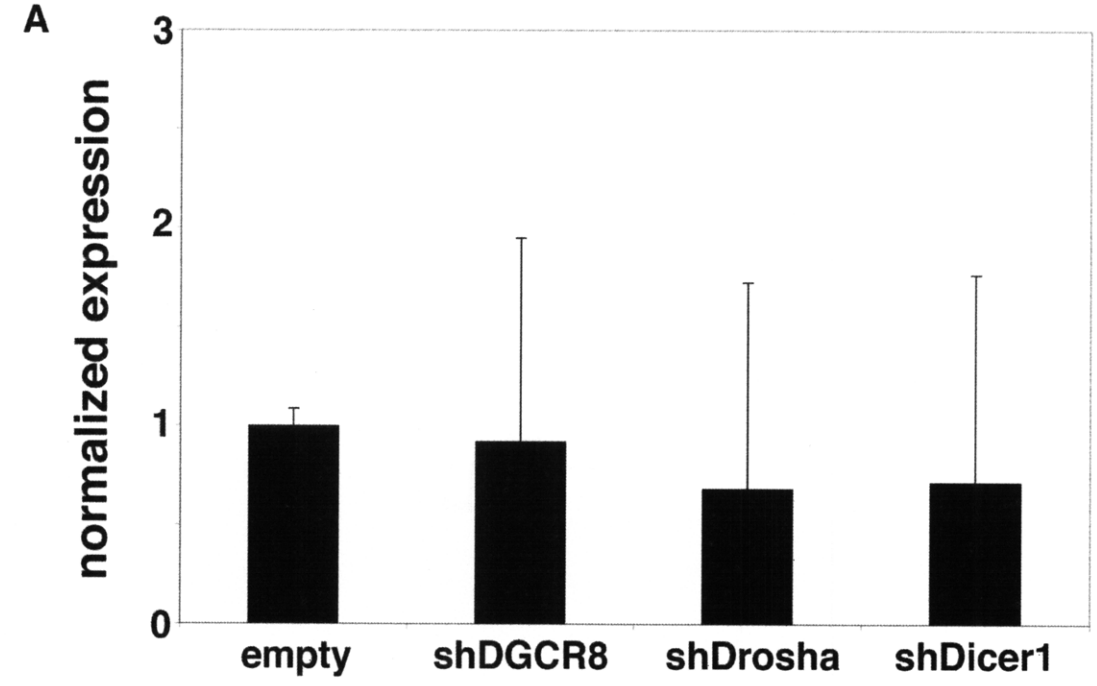

A
empty
shDGCR8
shDrosha
shDicer1
let-7
miR-20
Glutamine tRNA
B
empty
shDGCR8
shDrosha
shDicer1

Figure 2.4 (A) Small RNA Northern blotting was performed against miR-20, let-7, and glutamine tRNA on tumor RNA from three separate tumors of LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1. (B) Hematoxylin/eosin staining of tumors. Note the presence of glandular structures (arrows) representative of moderate differentiation in all of the different tumors. Original magnification, X20.

Figure 2.5


A
normalized expression
3
2
1
0
empty
shDGCR8
shDrosha
shDicer1

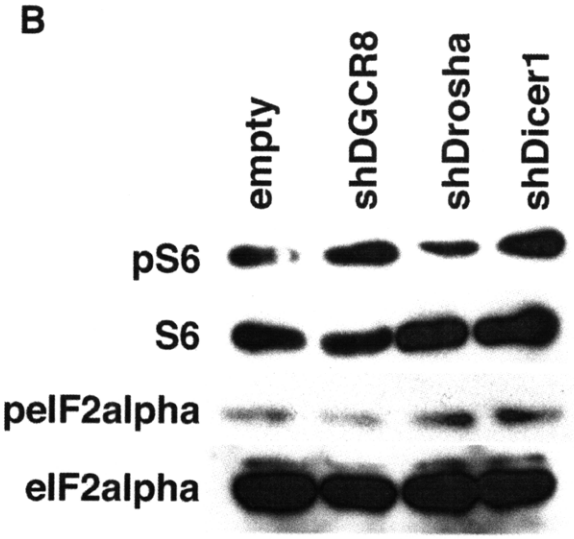

B
empty
shDGCR8
shDrosha
shDicer1
pS6
S6
peIF2alpha
eIF2alpha

Figure 2.5 (A) Quantitative real-time polymerase chain reaction (Q-RT PCR) was performed with probes for the major satellite repeat transcript in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 (see Supplementary Methods for details). Expression levels were initially normalized to TBP and subsequently normalized relative to the major satellite repeat transcript in LKR13 cells infected with empty vector. Values are mean +/- s.d. (n=4) with propagated error. (B) Immunoblotting for the expression of total and phosphorylated S6 and eIF2α in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1.

Figure 2.9a). MiRNA processing continued to be impaired in pSico^{R}-shDicer1 cells infected with a control adenovirus. Notably, adenoviral Cre-mediated removal of the Dicer1 hairpin resulted in reduced soft agar growth relative to control adenovirus-treated cells. This reversal was not observed in pSico^{R}-shluc cells (Supplementary Figure 2.9b). Thus, the enhanced transformation associated with impaired miRNA processing was reversible.

To determine if this enhanced transformation was specific to depletion of the miRNA processing machinery, we attempted to overcome the effect through expression of human cDNAs in miR KD LKR13 cells. As murine shRNAs do not demonstrate full sequence complementarity with their human cDNA counterparts, the human cDNAs were expected to retain expression. In this context, miRNA processing would be maintained while the shRNA was expressed and functional. We observed a near complete restoration of miRNA expression in cells containing the human cDNA in tandem with its target shRNA (Supplementary Figure 2.9c). Of note, shRNA-expressing cells lacking their corresponding cDNA or expressing a cDNA against a different miRNA processing component failed to restore mature miRNA levels, validating the specificity of the effect. MiRNA processing-rescued cells showed reductions in both low density colony formation and growth in soft agar compared to miR KD cells (Supplementary Figure 2.9d, e), demonstrating that protection against impaired miRNA processing was sufficient to prevent the phenotype triggered by the shRNAs. Therefore, the enhanced transformation corresponding to defective miRNA maturation was specific to the miRNA processing machinery and not a non-specific effect of shRNA expression.

In order to test if impaired miRNA processing was sufficient to transform non-cancerous cells, we expressed shRNAs against the miRNA processing machinery in K-Ras^{G12D}-expressing murine embryonic fibroblasts (MEFs), which have been shown to be incompletely transformed

Figure 2.6

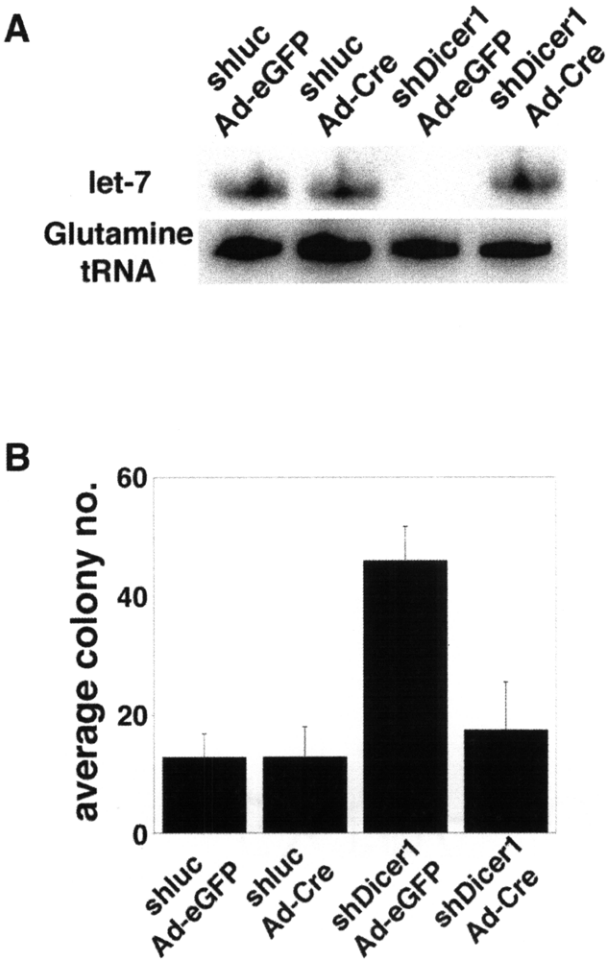
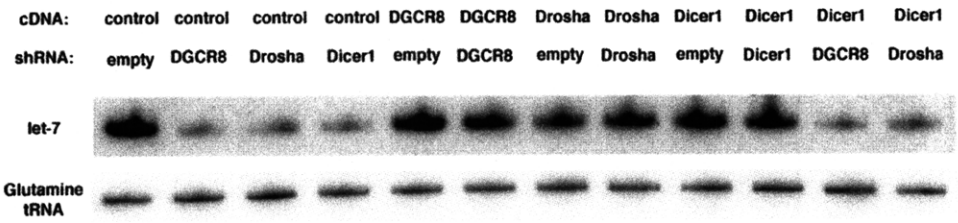

A
shluc Ad-eGFP
shluc Ad-Cre
shDicer1 Ad-eGFP
shDicer1 Ad-Cre
let-7
Glutamine tRNA
B
average colony no.
60
40
20
0
shluc Ad-eGFP
shluc Ad-Cre
shDicer1 Ad-eGFP
shDicer1 Ad-Cre

Figure 2.6 continued

C



D

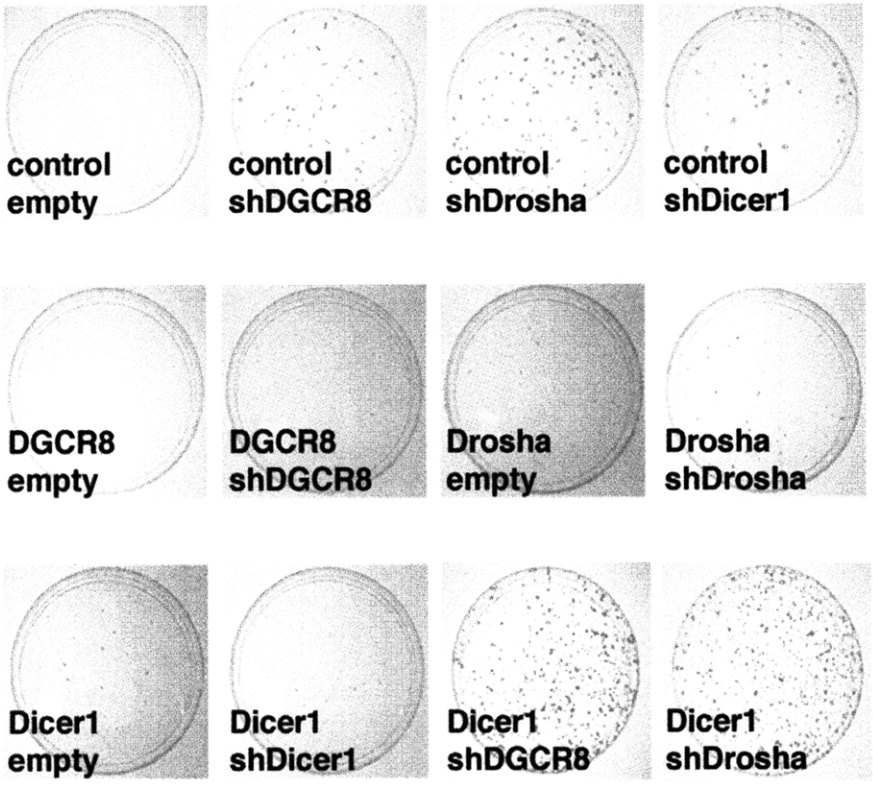


Figure 2.6 continued

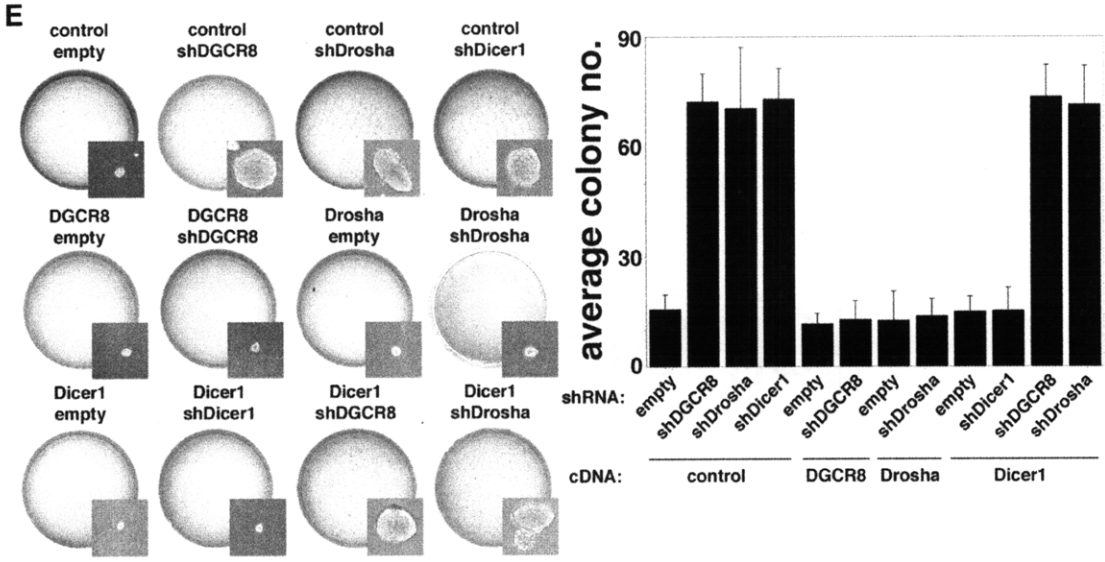


Figure 2.6 (A) LKR13 cells were infected with shRNAs against Dicer1 or *Renilla* luciferase flanked by loxP sites. Cells were subsequently infected with adenovirus expressing eGFP or Cre, resulting in removal of the shRNA. Small RNA Northern blotting was performed as above on LKR13 cells infected with shRNAs against Dicer1 or *Renilla* luciferase and subsequently infected with adenovirus expressing eGFP or Cre. (B) LKR13 cells infected with shRNAs against Dicer1 or *Renilla* luciferase and subsequently infected with adenovirus expressing eGFP or Cre were plated at 50,000 cells per plate in semisolid media, grown for two weeks, and fixed and stained with crystal violet. Colonies were counted from independent platings, with five random fields per condition. Values are mean +/- s.d. (C) LKR13 cells were stably transfected with control vector or Flag-tagged human DGCR8, Drosha, or Dicer1 cDNA. These cells were subsequently infected with shRNAs against DGCR8, Drosha, or Dicer1. Small RNA Northern blotting was performed as above in LKR13 cells containing the constructs described. (D) LKR13 cells containing the constructs described in **c** were plated at low density as described above. (E) LKR13 cells containing the constructs described in **c** were plated in semisolid media and colonies counted as described above. Inset magnification, X20.

(Tuveson et al., 2004). Compared to control *K-Ras*^{G12D} MEFs, miR KD *K-Ras*^{G12D} MEFs had an elevated growth rate (Figure 2.7A). However, impaired miRNA processing was not sufficient to fully transform *K-Ras*^{G12D} MEFs, as evidenced by the inability of the cells to form colonies in soft agar (data not shown). To further address the role of miRNA processing on proliferation, we infected shRNAs against the miRNA processing machinery into wild-type MEFs and examined long-term population growth. Interestingly, wild-type cells had a markedly reduced long-term proliferative capacity compared to controls (Figure 2.7B), consistent with observations from *Dicer1*-null MEFs and embryonic stem cells (Harfe et al., 2005; Kanellopoulou et al., 2005; Murchison et al., 2005). Therefore, while defective miRNA biogenesis improves the transformation of cancer cells, it is not sufficient to promote *de novo* transformation.

To begin to investigate the basis for the enhanced transformation associated with repression of miRNAs, we measured the expression of several oncogenes in miR KD LKR13 cells. As shown in Figure 2.8A, significantly higher protein levels of the oncogenes c-Myc and K-Ras were detected without changes in other known oncogenes like N-Ras, H-Ras, and E2F-1. K-Ras mRNA levels were slightly elevated in miR KD cells, while c-Myc mRNA levels were unchanged compared to controls (Figure 2.7C). As Ras family members have been shown to stabilize the c-Myc protein post-translationally through activation of the MEK/ERK and Akt signaling pathways (Adhikary and Eilers, 2005), we investigated whether these pathways were altered in miR KD cells. Surprisingly, no consistent change in MEK1/2, ERK1/2, or Akt phosphorylation was noted in response to impaired miRNA processing (Figure 2.7D). These data suggest that miRNAs directly regulate c-Myc expression post-transcriptionally.

To examine potential miRNA-mediated regulation, we used the miRNA target prediction program miRanda (John et al., 2004) and observed that the 3' UTRs of murine K-Ras and c-Myc

Figure 2.7

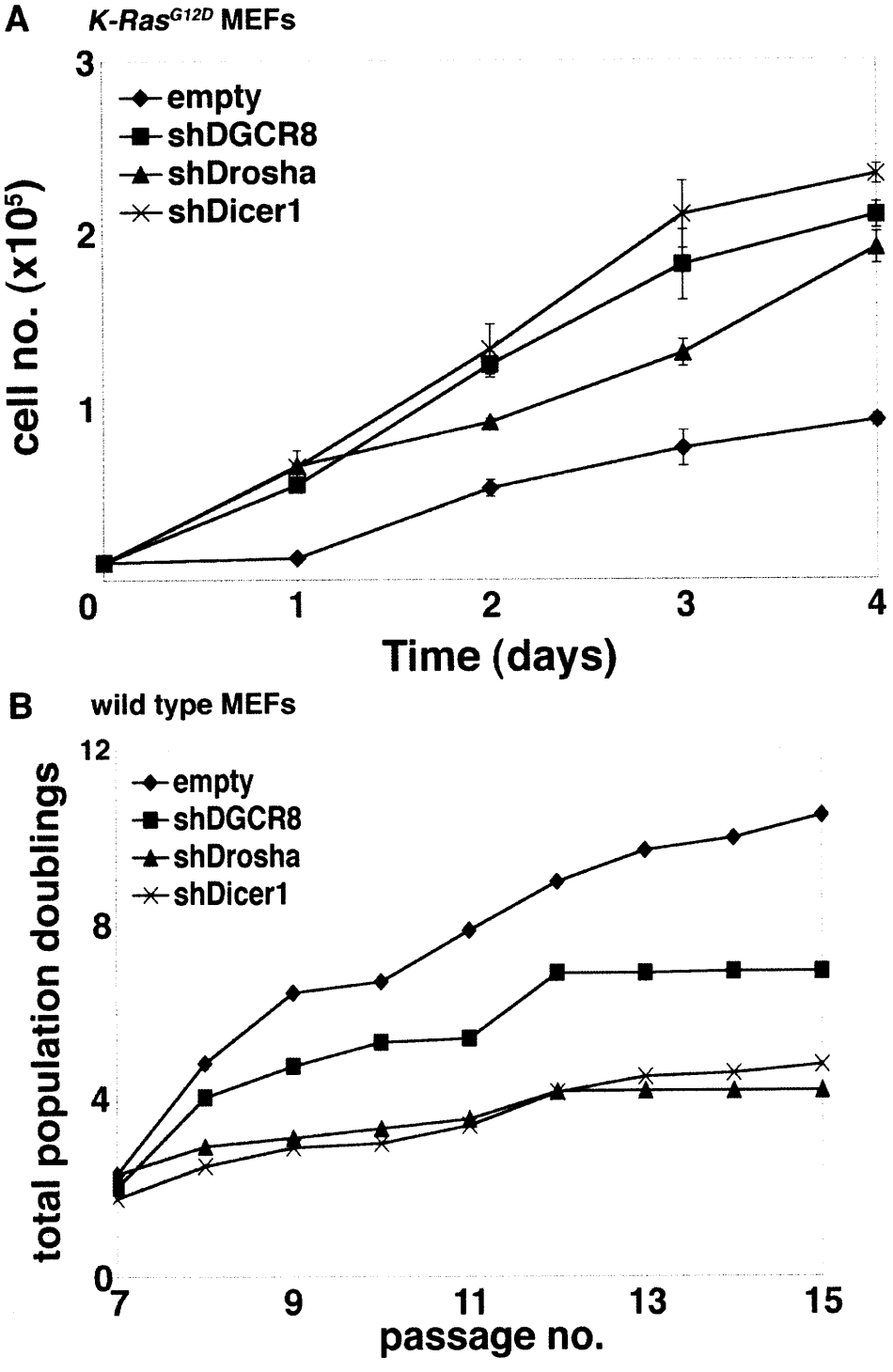

A
K-Ras^G12D MEFs
empty
shDGCR8
shDrosha
shDicer1
cell no. (x10^5)
Time (days)
B
wild type MEFs
total population doublings
passage no.

Figure 2.7 continued

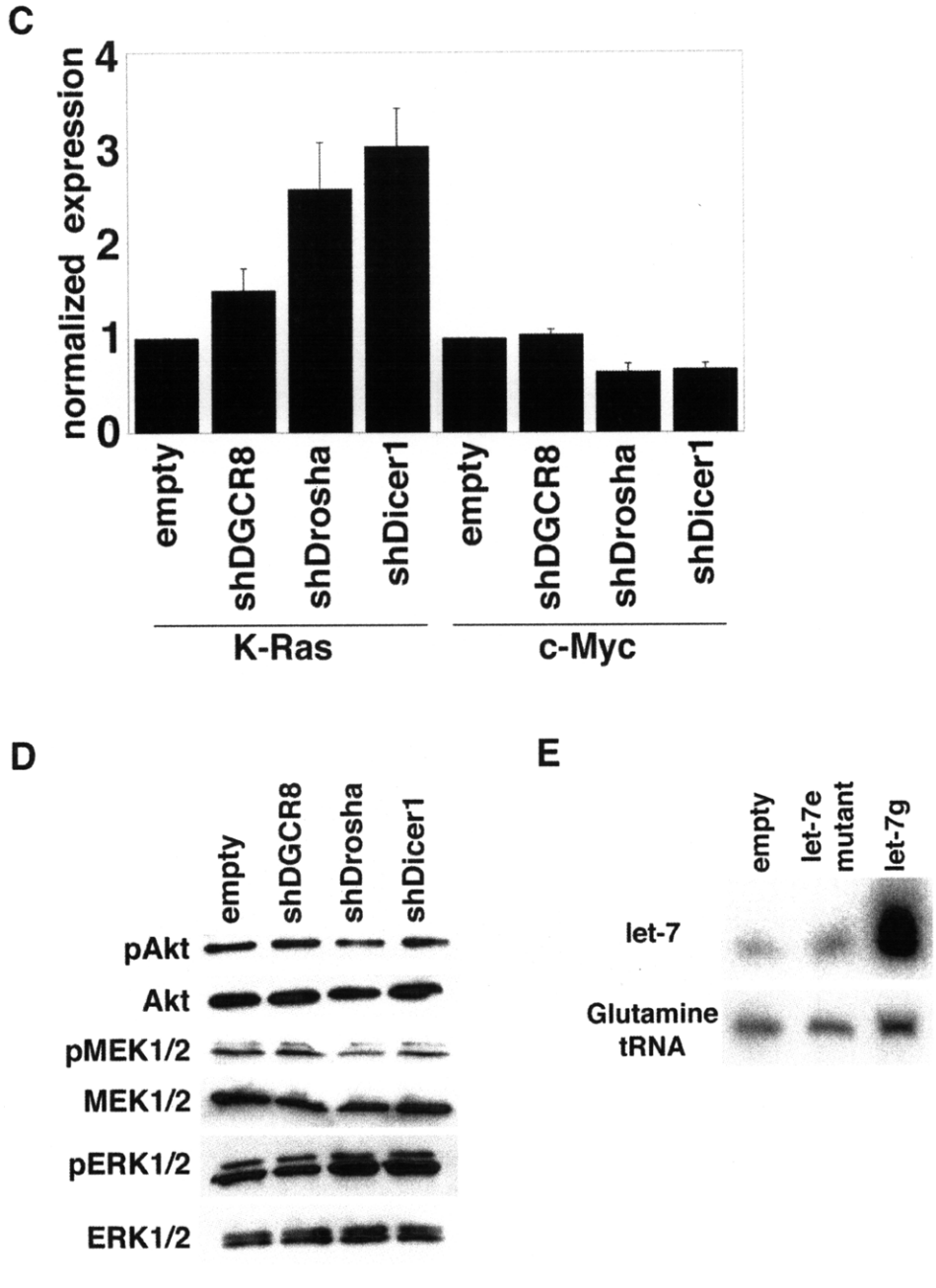


Figure 2.7 continued

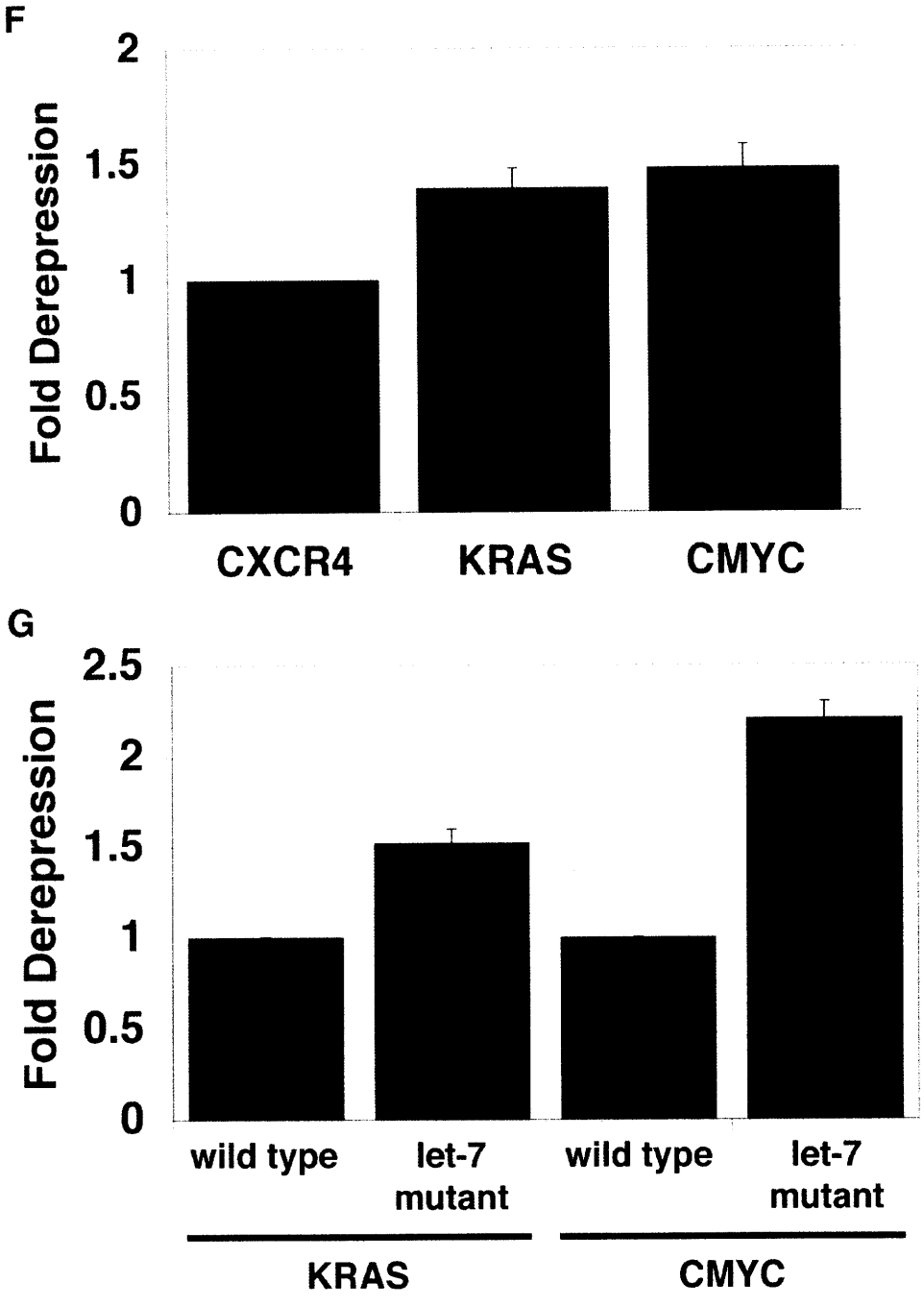


Figure 2.7 continued

H

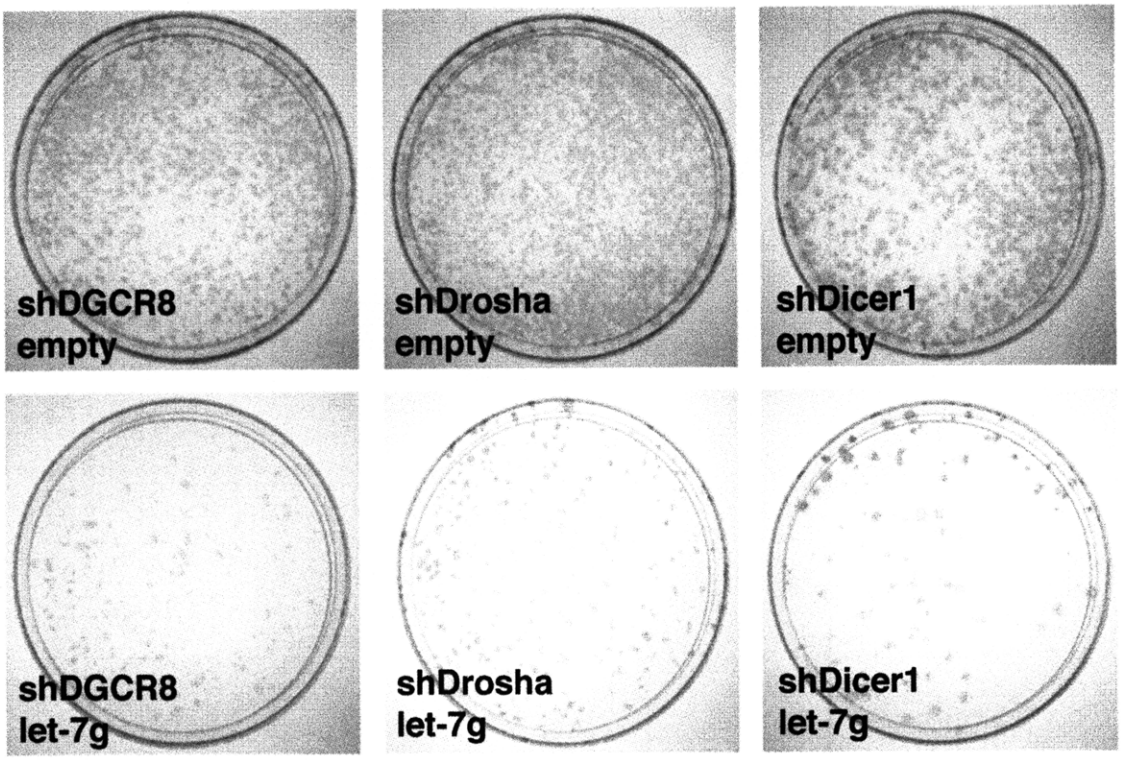


Figure 2.7 (A) *K-Ras*^{G12D} murine embryonic fibroblasts (MEFs) infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at 5000 cells per well. Cells were counted over time in triplicate. Values are mean +/- s.e.m. (n=3). (B) Wild type MEFs infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1 were plated at $3X10^5$ cells and serially passaged and counted, with total population doublings measured over time. (C) Q-RT PCR was performed with probes for K-Ras and c-Myc in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1. Normalization was performed as described. (D) Immunoblotting for the expression of total and phosphorylated Akt, MEK1/2, and ERK1/2 in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1. (E) small RNA Northern blotting was performed as above in 293T cells transfected with empty vectors or vectors expressing a seed mutant let-7e (control miRNA) or wild type let-7g. (F) Luciferase assays were performed in 293T cells co-transfected with 2'-O-methyl oligonucleotides complementary to either miR-181a or let-7g and pRL-TK containing bulged siCXCR4 binding sites, or murine K-Ras and c-Myc 3' UTRs. Firefly luciferase (pGL3) was used as a transfection control. Expression was normalized to pGL3 levels and subsequently normalized relative to pRL-CXCR4 expression. Values are mean +/- s.d. (n=6) with propagated error. (G) Luciferase assays were performed in 293T cells transfected with pRL-TK containing either wild type murine K-Ras and c-Myc 3' UTRs or murine K-Ras and c-Myc 3' UTRs containing point mutations in their let-7 miRNA binding sites. K-Ras (EMBL accession no. BC004642) mutations correspond to a series of point mutations ("ACAGTGGAAACC" to "TGAGCGGCCAGG") from positions 157 to 168 of the 3' UTR. C-Myc (EMBL accession no. X01023) mutations correspond to a G to A point mutation at position 165 of the 3' UTR. Expression was normalized to pGL3 levels and subsequently normalized relative to

corresponding wild type 3' UTR expression. Values are mean +/- s.d. (n=6) with propagated error. (H) LKR13 cells infected with shRNAs against DGCR8, Drosha, or Dicer1 were transfected with empty vector or with a vector overexpressing let-7g. Cells were then plated at low density as described in the text.

Figure 2.8

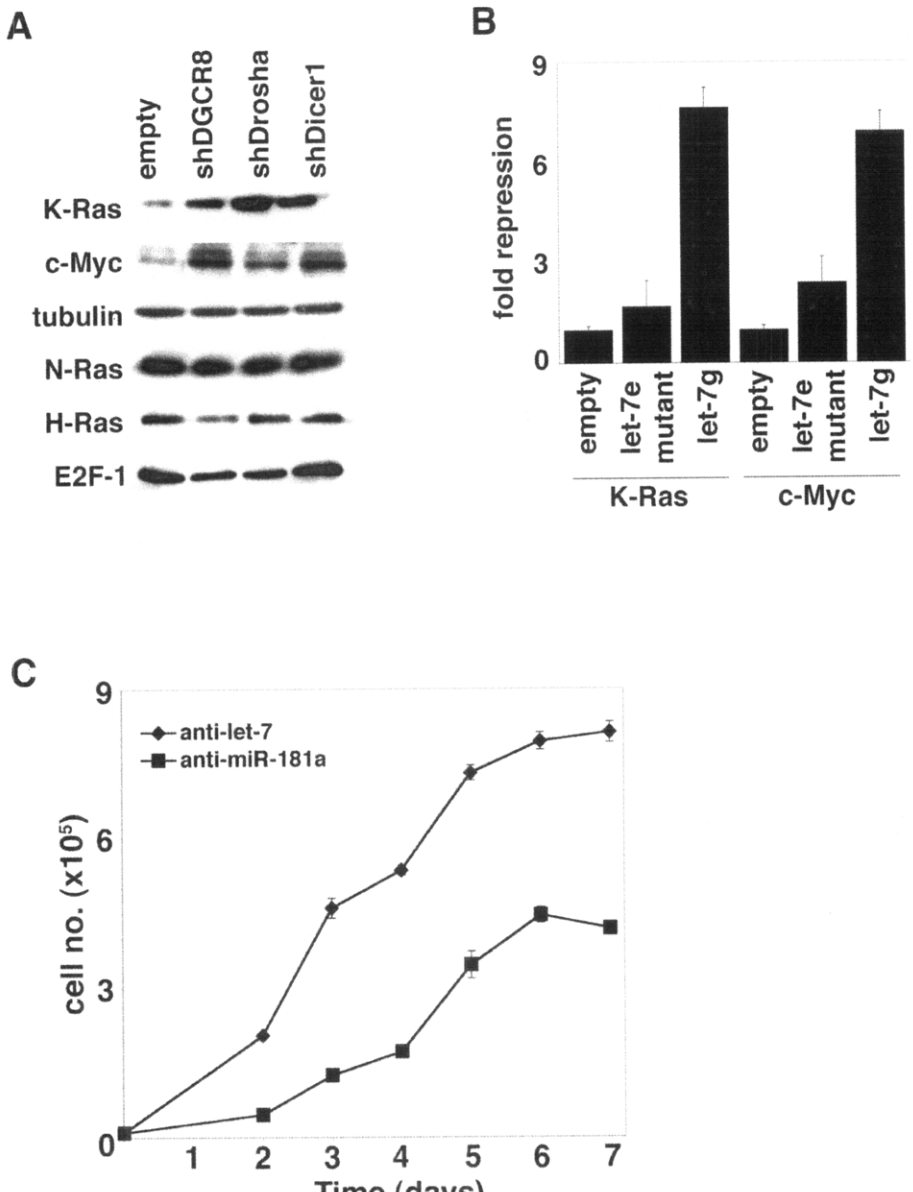

A
empty
shDGCR8
shDrosha
shDicer1
K-Ras
c-Myc
tubulin
N-Ras
H-Ras
E2F-1
B
fold repression
9
6
3
0
empty
let-7e mutant
let-7g
empty
let-7e mutant
let-7g
K-Ras
c-Myc
C
cell no. (x10^5)
9
6
3
0
anti-let-7
anti-miR-181a
1
2
3
4
5
6
7
Time (days)

Figure 2.8 Impaired miRNA processing increases expression of specific oncogenes. (A) Immunoblotting for K-Ras, c-Myc, α-tubulin, N-Ras, H-Ras, and E2F-1 in LKR13 cells infected with empty vector or shRNAs against DGCR8, Drosha, or Dicer1. (B) Luciferase assays were performed in 293T cells transfected with pRL-TK containing bulged siCXCR4 binding sites (Doench et al., 2003) or murine K-Ras and c-Myc 3' UTRs. Firefly luciferase (pGL3) was used as a transfection control. Expression was normalized to pGL3 levels and subsequently normalized relative to pRL-CXCR4 expression. Values are mean +/- s.d. (n=6) with propagated error. (C) LKR13 cells were transfected with 2'-O-methyl oligonucleotides complementary to either miR-181a or let-7g and then plated at 5000 cells per well. Cells were counted over time in triplicate. Values are mean +/- s.e.m (n=3).

were both targets of the let-7 family of miRNAs. To evaluate whether elevated K-Ras and c-Myc protein levels were due to let-7 mediated derepression, we selectively overexpressed let-7g and measured the effects on luciferase-based reporter constructs carrying the 3' UTRs of K-Ras and c-Myc (Figure 2.7E). Consistent with previous studies (Johnson et al., 2005), we observed robust repression of the K-Ras 3' UTR with let-7 overexpression (Figure 2.8B). In addition, the c-Myc 3' UTR was also repressed in this setting, suggesting that both oncogenes are let-7 targets. Conversely, 2'-O-methyl oligonucleotides complementary to let-7g caused derepression of the 3' UTRs of K-Ras and c-Myc (Figure 2.7F). Moreover, both oncogenes are direct let-7 targets, as mutation of the let-7 binding sites in the K-Ras and c-Myc 3' UTRs caused derepression comparable to let-7 inhibition (Figure 2.7G). To examine the role of let-7 family members in transformation, LKR13 cells were transfected with 2'-O-methyl oligonucleotides complementary to let-7g. We observed an elevation in growth rate with let-7 inhibition (Figure 2.8C). Moreover, overexpression of let-7g in miR KD LKR13 cells led to significant reduction in low-density colony-forming ability (Figure 2.7H). Finally, in miR KD human cancer cells, we noted an enrichment of let-7 family members among the miRNAs whose repression correlates best with heightened transformation (data not shown). However, it is also possible that other miRNAs whose repression correlates with enhanced transformation might function in a similar way.

To understand the significance of these findings *in vivo*, we assessed the role of impaired miRNA processing in a mouse model of lung tumorigenesis, using the conditional, activatable allele of K-Ras, *LSL-K-Ras*^{G12D} (Tuveson et al., 2004). Intranasal infection of *LSL-K-Ras*^{G12D} mice with adenovirus expressing Cre has been shown to induce non-small cell lung cancer in these mice with complete penetrance and a defined time course (Jackson et al., 2001). By crossing *LSL-K-Ras*^{G12D} mice with mice conditional knock-out allele of *Dicer1* (Harfe et al.,

2005), we generated *LSL-K-Ras*^{G12D} mice that were wild type, heterozygous, or homozygous for the conditional allele for *Dicer1* (hereafter referred to as "KD +/+," "KD flox/+," and "KD flox/flox," respectively).

When this cohort of mice was compared twelve weeks after infection with adenovirus expressing Cre, we observed a significant increase in tumor burden in the KD flox/+ and KD flox/flox mice compared to KD +/+ mice. This tumor burden was reflected in significant increases in tumor/lung area ratio (Figure 2.9A), tumor size (Figure 2.9B), and tumor number (Figure 2.9C). We also performed histological grading analysis of tumors from KD +/+, KD flox/+, and KD flox/flox mice. Overall, we noted a slight shift in grade in the *Dicer1* mutant mice, with the highest-grade lesions only present in the KD flox/+ and KD flox/flox mice (Figure 2.9D). These lesions displayed areas of nuclear pleomorphism, prominent nucleoli, and significant vacuolation (Figure 2.9E). In addition, the KD flox/+ and KD flox/flox mice exhibited more extensive bronchiolar hyperplasia than KD +/+ mice (Figure 2.9F), further demonstrating the enhanced tumor burden in *Dicer1* mutant mice. In total, these results suggest that impaired miRNA processing can enhance tumorigenesis *in vivo* through an enhanced tumor burden.

These studies demonstrate that impaired miRNA biogenesis accelerates oncogenic transformation through the deregulation of target oncogenes. Previous work has shown that both human and murine cancers exhibit a global reduction of mature miRNA levels when compared to normal tissues (Lu et al., 2005). Our work demonstrates that this reduced mature miRNA expression can promote tumorigenesis. Importantly, the loss of miRNA processing, though incomplete, causes a dramatic change in the transformed phenotype of cancer cells. Furthermore, our results are consistent with observations correlating reduced Dicer1 expression in a subset of

Figure 2.9

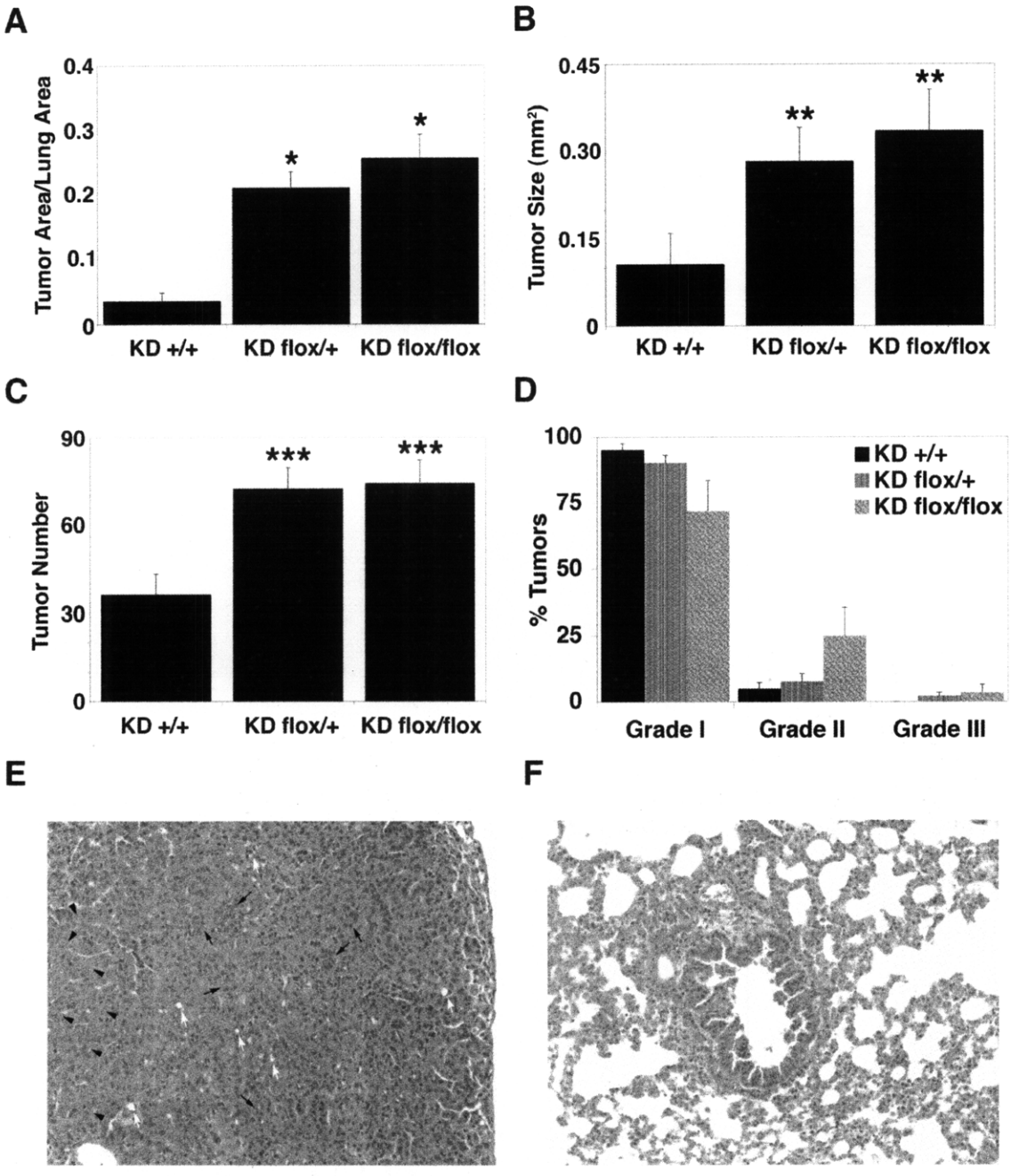

A
Tumor Area/Lung Area
0.4
0.3
0.2
0.1
0
*
*
KD +/+
KD flox/+
KD flox/flox
B
Tumor Size (mm²)
0.45
0.30
0.15
0
**
**
KD +/+
KD flox/+
KD flox/flox
C
Tumor Number
90
60
30
0
***
***
KD +/+
KD flox/+
KD flox/flox
D
% Tumors
100
75
50
25
0
KD +/+
KD flox/+
KD flox/flox
Grade I
Grade II
Grade III
E
F

Figure 2.9 Impaired miRNA processing enhances the *in vivo* tumor burden in a mouse model of lung cancer. (A) *LSL-K-Ras*^{G12D} mice either wild type, heterozygous, or homozygous conditional for *Dicer1* (KD +/+, KD flox/+, and KD flox/flox, respectively) were intranasally infected with adenovirus expressing Cre. Twelve weeks post-infection, animals were sacrificed and tumor and lung areas were quantified with Bioquant software. (B) Tumor sizes were quantified for the animals described above with Bioquant software. (C) Tumor numbers were quantified for the animals described above with Bioquant software. Values are mean +/- s.e.m. (n=11 for KD +/+, n=15 for KD flox/+, and n=10 for KD flox/flox). *: $p<5x10^{-5}$, **: $p<0.05$; ***: $p<0.005$. (D) Tumors from animals of the genotypes described above were scored for histological grade using a grading scheme described previously (see Methods for details). Values are mean +/- s.e.m. (n=5 for each genotype). (E) Hematoxylin/eosin staining of a Grade III tumor from a KD flox/+ animal. Original magnification, 20X. Filled arrows indicate areas of nuclear pleomorphism. Arrowheads indicate areas with prominent nucleoli. Empty arrows indicate areas of vacuolation. (F) Hematoxylin/eosin staining of bronchiolar hyperplasia from a KD flox/flox animal. Original magnification, 20X.

non-small cell lung cancers with poor prognosis (Karube et al., 2005). These findings are also consistent with work showing the let-7 family as a negative regulator of the Ras family of oncogenes (Johnson et al., 2005), while extending its role in oncogenic transformation to c-Myc. Of note, previous studies have implicated specific miRNAs as oncogenes, as their overexpression accelerates tumorigenesis (Hayashita et al., 2005; He et al., 2005; O'Donnell et al., 2005; Ota et al., 2004; Volinia et al., 2006). Our study does not directly contradict these findings, but suggests that overall repression of miRNAs can enhance tumorigenesis. Recent work has also shown that the global repression of miRNAs in human cancers does not coincide with reductions in the primary miRNA transcripts, suggesting that altered regulation of the miRNA processing machinery occurs in human cancers (Thomson et al., 2006). Taken as a whole, these investigations highlight a functional role for miRNA biogenesis in tumorigenesis.

Materials and Methods

Cell culture. Human cancer cell lines (HCA7, MCF7, U2OS) were originally obtained from ATCC. LKR13 cells were generated from a murine lung adenocarcinoma (Johnson et al., 2001; Wislez et al., 2006). Wild type and *LSL-K-Ras*^{G12D} MEFs were generated as described previously (Tuveson et al., 2004). Cells were maintained using standard conditions.

Transformation, immortalization, growth, and tumorigenesis assays. Transformation, immortalization, growth, and tumorigenesis assays were performed essentially as described (Sage et al., 2000).

BrdU and cell cycle profile analysis. Anti-BrdU and 7-AAD staining were analyzed by flow cytometry as per manufacturer's instructions (BD Biosciences).

Invasion assays. Invasion assays were performed using a 24-well collagen-based cell invasion assay as per manufacturer's instructions (Chemicon).

shRNA design and infection. shRNAs were designed, cloned, and infected into cells as described previously (Brummelkamp et al., 2002; Ventura et al., 2004). Subsequent adenovirus infection of shRNA-expressing cells was performed as described (Ventura et al., 2004).

Northern blotting. Small RNA Northern blotting was performed as described (Ventura et al., 2004).

Immunoblotting assays. Immunoblotting was performed as described (Sage et al., 2000).

Quantitative reverse transcription (Q-RT) PCR. Q-RT PCR of murine DGCR8, Drosha, Dicer1, K-Ras, and c-Myc was performed as before (Sweet-Cordero et al., 2005). Q-RT PCR of satellite repeat transcripts was performed as described (Martens et al., 2005).

miRNA overexpression and repression assays. miRNAs let-7g and seed mutant let-7e with approximately 500 basepairs of flanking sequence were cloned into MSCV-neo (Clontech). 2'-O-methyl (2'-O-Me) oligonucleotides complementary to let-7g and miR-181a were synthesized commercially (Integrated DNA Technologies). Overexpression vectors and 2'-O-Me oligonucleotides were transfected into cells using described methods (Hutvagner et al., 2004; Meister et al., 2004).

Luciferase assays. Murine K-Ras (EMBL accession no. BC004642) and c-Myc (EMBL accession no. X01023) 3' UTRs were cloned into *Renilla* luciferase vectors described previously and luciferase assays were performed as before (Doench et al., 2003).

miRNA expression analysis. miRNA expression profiling was performed using a bead-based miRNA detection platform as described with modifications (Lu et al., 2005).

Mice. *LSL-K-Ras^{G12D}* mice (Tuveson et al., 2004) were crossbred to *Dicer1$^{flox/flox}$* mice (Harfe et al., 2005) to generate *LSL-K-Ras^{G12D}*; *Dicer1$^{flox/+}$*mice, which were subsequently interbred to produce the experimental cohort.

Intranasal infection and tumor analysis. Mice were infected intranasally with adenovirus expressing Cre as described (Jackson et al., 2001). Twelve weeks after infection, mice were sacrificed and lungs were collected, fixed in formalin, and stained for histology as described previously (Jackson et al., 2001). Lung and tumor areas were determined using Bioquant Image Analysis software as described previously (Jackson et al., 2005).

Tumor grading analysis. Tumor grading was done without knowledge of genotype as described previously (Jackson et al., 2005). Each tumor was given a score of 1 to 3 based on predetermined criteria.

shRNA design. shRNAs against murine DGCR8, Drosha, and Dicer1 were cloned into pSuper.RETRO.puro (Oligoengine) with the following oligonucleotides:

control shRNA: 5'-CTTACAATCAGACTGGCGA-3'

shDGCR8: 5'-CCAATGATGACCAAGATTA-3'

shDrosha: 5'-CAACAGTCATAGAATATGA-3'

shDicer1: 5'-GCATGGTGGTGTCGATATT-3'

shRNAs against firefly luciferase, murine Dicer1, and human DGCR8, Drosha, and Dicer1 were cloned into pSicoR.pgk.puro with the following oligonucleotides:

shluc: 5'-GAGCTGTTTCTGAGGAGCC-3'

murine shDicer1: 5'-GCATGGTGGTGTCGATATT-3'

human shDGCR8 A: 5'-GAAGCTCATTACTTTATCA-3'

human shDGCR8 B: 5'-GAAAGAGTTTGTTATTAAC-3'

human shDrosha A: 5'-GAAGCTCTTTGGTGAATAA-3'

human shDrosha B: 5'-GAATTCGGCAGCCCAAATA-3'

human shDicer1 A: 5'-GCAGCTCTGGATCATAATA-3'

human shDicer1 B: 5'-GGAAGAGGCTGACTATGAA-3'

BrdU and cell cycle profile analysis. LKR13 cells were grown overnight in media containing reduced serum (1%) and treated with BrdU for 2 hours before preparation for staining and flow cytometry.

Quantitative reverse transcription (Q-RT) PCR. Taqman probes (Applied Biosystems) were as follows: DGCR8 (Mm00473695_s1), Drosha (Mm01310009_m1), Dicer1 (Mm00521734_g1), K-Ras (Mm00517494_m1), c-Myc (Mm00487804_m1), TBP (Mm00446971_m1).

Immunoblotting assays. Primary antibodies used were as follows: K-Ras, N-Ras, H-Ras, E2F-1, c-Myc, β-tubulin (Santa Cruz); phospho- and total ERK1/2, phospho- and total MEK1/2, phospho- and total Akt, phospho- and total eIF2α, phospho- and total ribosomal S6 (Cell Signaling Technology); M2 anti-Flag (Sigma-Aldrich).

cDNA rescue. cDNAs containing Flag-tagged Dicer1, Drosha, and DGCR8 were co-transfected with MSCV-neo into LKR13 cells, selected, and subcloned for uniform cDNA expression. Cells expressing cDNAs were subsequently infected with shRNAs as above.

miRNA overexpression. miRNAs of the following mature sequences, with approximately 500 basepairs of flanking sequence, were cloned into MSCV-neo:

let-7g: 5'-UGAGGUAGUAGUUUGUACAGU-3'

seed mutant let-7e: 5'-UCUGGACGGAGGUUGUAUAGU-3'

miRNA expression analysis. Samples were subjected to total RNA preparation using TRIzol (Invitrogen). Two μg of total RNA from each sample, together with two pre-labeling control

synthetic RNA oligonucleotides (60 fmoles of 5'-pGACCUCCAUGUAAACGUACAA-3' and 10 fmoles of 5'-pUUGCAGAUAACUGGUACAAG-3') were labeled, through adaptor-ligation-mediated amplification. Six mock labeling reactions, in which water was used as the starting material, were also performed for background calculation. Labeled materials were mixed with biotinylated post-labeling control oligonucleotides and were hybridized to miRNA-detection-probes conjugated to colored xMap beads (Luminex Corp.), stained with streptavidin-phycoerythrin, and detected on a Luminex 100S flow cytometer.

Median fluorescence intensity readings from Luminex data collector software were used in subsequent analyses. To eliminate the background signal from each miRNA-detection-probe, average readings from the six mock labeling reactions were subtracted from the sample readings. Data were further normalized following a two -tep procedure, as described. First, data for the same sample were linearly scaled between different bead sets, based on the sum of the two post-labeling control readings. Second, data were further scaled to reflect the amount of miRNA per amount of total RNA. To this end, the sum of both pre-labeling controls from all bead sets for the same sample was used to eliminate weakly labeled outliers (using a cutoff of 3000) and to linearly normalize data (so that the sum becomes 9000 for each sample). Data were then thresholded at 0.1 and control features were removed. To aid further analysis, a new feature (termed TFI) was added to the dataset representing the sum of all miRNA readings from each sample.

For each of the three cell lines (HCA7, MCF7, U2OS), data were thresholded to eliminate features whose maximum expression in all samples of that particular cell line does not exceed 64. The remaining features were used to calculate Pearson correlation between miRNA

expression and soft-agar colony formation. Nominal P-values were calculated after 10000 permutations on the construct labels in the soft-agar colony formation data.

Tumor grading. The criteria for each grade are as follows: Grade 1 tumors have uniform nuclei showing no nuclear atypia. Grade 2 tumors contain cells with uniform but slightly enlarged nuclei that exhibit prominent nucleoli. Grade 3 tumors have cells with enlarged, pleomorphic nuclei showing prominent nucleoli and nuclear molding and/or vacuolation within the tumor.

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CHAPTER 3: *Dicer1* functions as a haploinsufficient tumor suppressor

I contributed to all Figures presented in this chapter. Ryan E. Pester and Cindy Y. Chen assisted in the experiments presented in Figures 3.1, 3.2, 3.3, 3.4, 3.6., 3.7, and 3.8. Christine Chin assisted in the experiments presented in Figures 3.7 and 3.8. Jun Lu performed miRNA profiling on samples. Alison L. Dooley assisted in the intratracheal Lenti-Cre presented in Figure 3.6. David G. Kirsch assisted in the intramuscular Ad-Cre injections presented in Figure 3.2.

Abstract

While the global down-regulation of microRNAs (miRNAs) is a common feature of human tumors, its genetic basis is largely undefined. To address this, we analyzed the consequences of conditional *Dicer1* mutation (*Dicer1* "floxed" or $Dicer1^{f}$) on several mouse models of cancer. Here we show that *Dicer1* functions as a haploinsufficient tumor suppressor. Tumors from $Dicer1^{f/+}$ animals, though exhibiting reduced miRNA processing, maintain the wild type allele, while tumors from $Dicer1^{f/f}$ animals undergo incomplete recombination. When complete loss of *Dicer1* is forced, tumorigenesis is abrogated. In line with these models, genome copy number analysis shows frequent deletion of *DICER1* in human cancer; importantly, *DICER1* never undergoes homozygous deletion, suggesting that *DICER1* is haploinsufficient in human cancer. These findings suggest that haploinsufficient tumor suppressors like *Dicer1*, which are frequently missed by traditional genetic analyses, play an important role in oncogenesis.

Introduction

MicroRNAs (miRNAs) are short, non-coding RNAs that function to suppress post-transcriptionally the expression of target mRNAs, predominately via inhibition of translation. Such translational inhibition relies upon imperfect base pairing between the miRNA and the target transcript, with the interaction at nucleotides 2-8, or the seed region, of the miRNA being required for translational repression. Computational prediction of miRNA targets based upon seed regions and sequence conservation has revealed a widespread potential for miRNA-mediated transcript regulation, with hundreds of putative mRNA targets for an individual miRNA (Bartel, 2004).

In line with this potential, it has been speculated that miRNAs could function as oncogenes or tumor suppressor genes based upon their inhibition of a variety of tumor suppressive and oncogenic mRNAs, respectively (Plasterk, 2006; Ventura and Jacks, 2009). In particular, three distinct mechanisms have been posited. First, oncogenic miRNAs can undergo gain of function in tumors. This has been most clearly demonstrated for the miR-17~92 cluster, whose amplification in B-cell lymphomas promotes their development, potentially through its control of B-cell differentiation (He et al., 2005; Koralov et al., 2008; Ventura et al., 2008). In contrast, tumor suppressive miRNAs can experience loss of function in tumors. This has been shown for the let-7 family, whose expression inhibits lung tumorigenesis by inhibition of oncogenes like the Ras family and HMGA2 (Esquela-Kerscher et al., 2008; Kumar et al., 2008). In particular, let-7 family members are in sites of frequent deletion in human tumors and their processing is inhibited by the oncogenic Lin-28 proteins (Chang et al., 2009; Heo et al., 2008; Newman et al., 2008; Viswanathan et al., 2008). Finally, oncogenes can acquire mutations to

remove miRNA binding sites in tumors. This has been described for HMGA2, whose translocation promotes lipoma development by releasing the transcript from let-7 mediated tumor suppression (Mayr et al., 2007).

Early studies observed a global down-regulation of miRNAs in human cancer (Lu et al., 2005). It was unclear whether this widespread loss of miRNAs was merely a consequence of tumor development or was functionally related to tumorigenesis. We have demonstrated previously that this global loss of miRNAs was functionally relevant to oncogenesis, as impairment of miRNA maturation enhanced transformation both in cancer cells and a K-Ras—driven model of lung cancer (Kumar et al., 2007). While these studies provide a functional basis for inhibition of miRNA biogenesis in cancer, the genetic basis of impaired miRNA processing in human cancer has been largely undefined. For a subset of miRNAs, widespread silencing occurs at the transcriptional level via the c-Myc oncogene (Chang et al., 2008). Yet it has also been shown that such broad reductions in miRNAs can occur post-transcriptionally, since changes in miRNA levels frequently occur without changes in the levels of the primary miRNA transcript (Thomson et al., 2006). Recently, it was shown that mutations in the miRNA processing component *TARBP2* occur frequently in mismatch repair-deficient colon cancer and that these mutations promote tumorigenesis by impaired processing of miRNAs (Melo et al., 2009). However, these limited cases do not resolve the common global reduction of miRNAs in human cancers. Moreover, the precise genetics of such changes in tumors is poorly defined, especially as no components of the miRNA processing pathway have been reported to be completely deleted in human tumors. This is not surprising, since it has been shown that germline deletion of miRNA processing components *Dicer1* and *Dgcr8* in mice fails to produce viable progeny (Bernstein et al., 2003; Wang et al., 2007). Thus, conditional deletion of miRNA

processing components provides a powerful means of examining the role of miRNAs in tumorigenesis.

Results and Discussion

We have previously shown that *Kras*$^{LSL\text{-}G12D}$ mice either heterozygous or homozygous conditional for *Dicer1* (hereafter referred to as KD f/+ and KD f/f), after intranasal infection with adenovirus expressing Cre (Adeno-Cre), experienced a significant increase in lung tumor burden compared to *Kras*$^{LSL\text{-}G12D}$ mice wild type for *Dicer1* (hereafter referred to KD +/+) (Kumar et al., 2007). To extend these observations of *Dicer1*-mediated tumor suppression, we characterized survival of KD +/+, f/+, and f/f animals after intranasal Adeno-Cre infection. Both KD f/+ and f/f animals experienced a significant reduction in survival relative to KD +/+ mice (Figure 3.1). Surprisingly, we found that the KD f/+ cohort had a significant reduction in survival when compared to KD f/f animals. To extend these findings to additional cancer types, we utilized a mouse model of soft-tissue sarcoma generated through intramuscular infection with Adeno-Cre of *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$ mice (Kirsch et al., 2007). When we compared survival of *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$ mice either wild type, heterozygous, or homozygous conditional for *Dicer1* (hereafter referred to as KPD +/+, f/+, and f/f) intramuscularly infected with Adeno-Cre, we observed a reduction in survival only in the KPD f/+ cohort (Figure 3.2). Overall, these results indicate that tumors generated from animals heterozygous conditional for *Dicer1* specifically impact survival.

These findings stand in opposition to traditional tumor suppressors, in which homozygous mutation provides a direct advantage to tumor cells while heterozygous mutation promotes tumorigenesis only after subsequent mutation or inactivation of the remaining wild

Figure 3.1

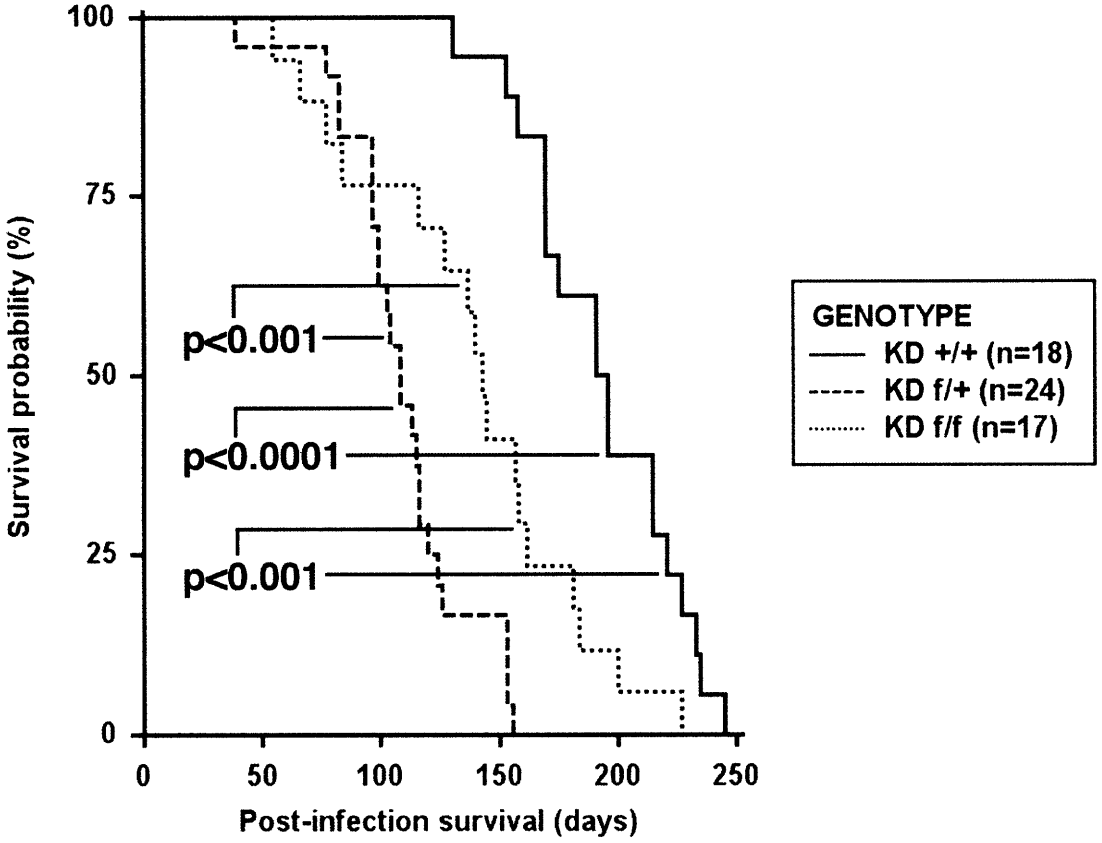
100
75
50
25
0
Survival probability (%)
p<0.001
p<0.0001
p<0.001
0
50
100
150
200
250
Post-infection survival (days)
GENOTYPE
KD +/+ (n=18)
KD f/+ (n=24)
KD f/f (n=17)

Figure 3.1. *Dicer1* mutation reduces post-infection survival in a genetically engineered mouse model of K-Ras driven lung cancer. $Kras^{LSL\text{-}G12D}$ mice either wild type, heterozygous, or homozygous conditional for *Dicer1* (KD +/+, KD f/+, and KD f/f, respectively) were intranasally infected with adenovirus expressing Cre and survival was assessed. Statistical significance was assessed by the log-rank test.

Figure 3.2

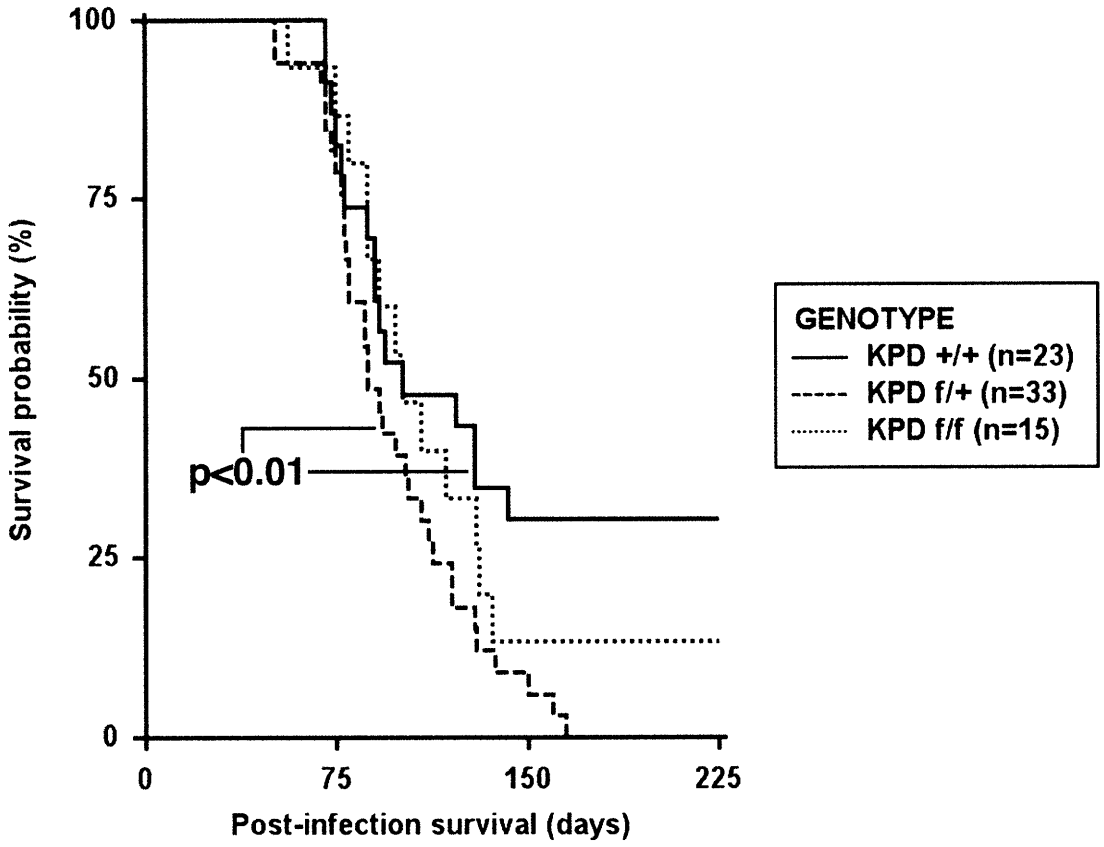
100
75
50
25
0
Survival probability (%)
p<0.01
0
75
150
225
Post-infection survival (days)
GENOTYPE
KPD +/+ (n=23)
KPD f/+ (n=33)
KPD f/f (n=15)

Figure 3.2. *Dicer1* mutation reduces post-infection survival in a genetically engineered mouse model of K-Ras, p53-driven soft-tissue sarcoma. $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ mice either wild type, heterozygous, or homozygous conditional for *Dicer1* (KPD +/+, KPD f/+, and KPD f/f, respectively) were intramuscularly infected with adenovirus expressing Cre and survival was assessed. Statistical significance was assessed by the log-rank test.

type allele. To characterize precisely the mutation status of *Dicer1* conditional tumors, we generated lung cancer cell lines from KPD f/+ animals and examined the *Dicer1* locus for further deletion. We observed recombination of the conditional allele of *Dicer1* and retention of the wild type allele, generating KPD +/- lung cancer cells (Figure 3.3A); similar maintenance of the wild type allele was found in sarcoma cell lines from KPD f/+ mice (Figure 3.4). We further examined *Dicer1* recombination in lung cancer cell lines from KPD f/f animals. In these cells, we again found recombination of the conditional allele. However, recombination was incomplete, generating KPD f/- lung cancer cells (Figure 3.3B); this incomplete loss of *Dicer1* was also observed in sarcoma cell lines from KPD f/f mice (Figure 3.4). In sum, this data indicates that only partial loss of *Dicer1* occurs in our genetically engineered mouse models during tumorigenesis.

Based upon the hemizygous mutation of *Dicer1* in our tumors, we wanted to assess the consequence of partial loss of Dicer1 on the global miRNA profile. When we broadly compared miRNAs in *Dicer1* wild type and heterozygous lung cancer cells, there was a global decrease in steady-state miRNA levels in KPD +/- cells (Figure 3.5A and data not shown). Small RNA Northern analysis verified these reductions in miRNA levels (Figure 3.5B). Taken together, the miRNA profile of *Dicer1* heterozygous lung cancer cells suggests that Dicer1 loss, even when incomplete, functionally affects miRNA processing in tumors.

While the genetic analysis of the *Dicer1* locus in our mouse cancer models suggests that *Dicer1*-mediated tumor suppression is haploinsufficient, it was still formally possible that *Dicer1* mutant tumors eventually undergo complete inactivation of Dicer1 via an alternative mechanism like epigenetic silencing. Thus, we assessed the consequences of complete loss of *Dicer1* in our genetically engineered mouse models. First, to promote complete loss of *Dicer1* in the lung, we

Figure 3.3

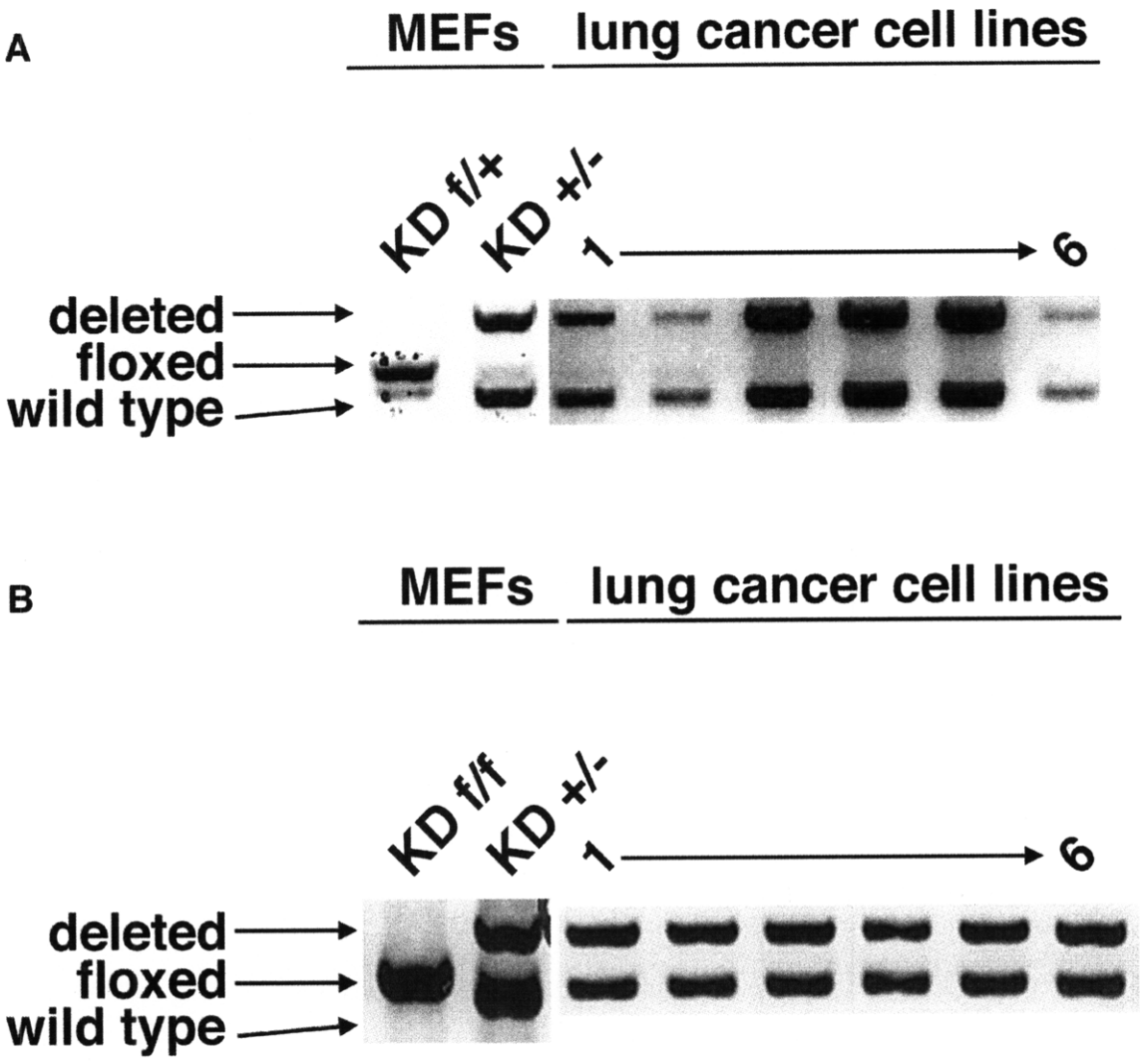
A
MEFs
lung cancer cell lines
KD f/+
KD +/-
1
6
deleted
floxed
wild type
B
MEFs
lung cancer cell lines
KD f/f
KD +/-
1
6
deleted
floxed
wild type

Figure 3.3. *Dicer1* undergoes hemizygous loss in lung tumors. (A) DNA was prepared from $Kras^{LSL\text{-}G12D}$ mouse embryonic fibroblasts (MEFs) either heterozygous conditional or mutant for *Dicer1* (KD f/+ and KD +/-) and lung cancer cell lines from KPD f/+ mice and the *Dicer1* locus was examined by PCR. (B) DNA was prepared from $Kras^{LSL\text{-}G12D}$ MEFs either homozygous conditional or heterozygous mutant for *Dicer1* (KD f/f and KD +/-) and lung cancer cell lines from KPD f/f mice and the *Dicer1* locus was examined by PCR.

Figure 3.4

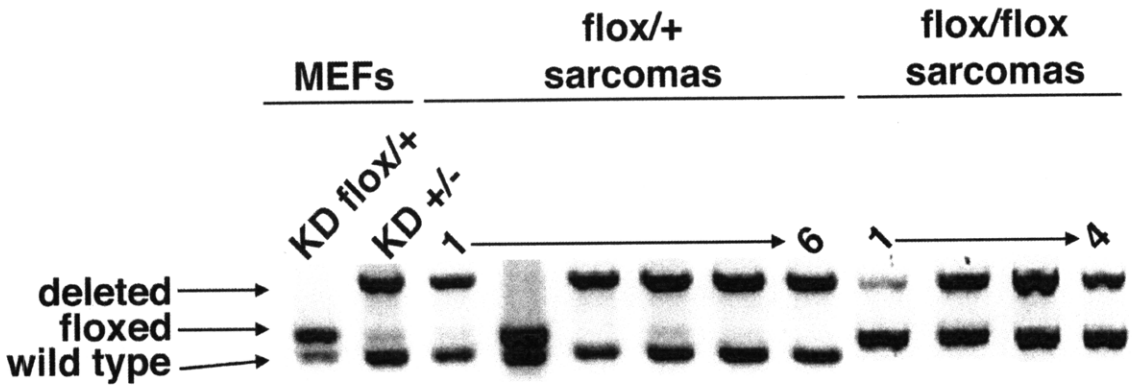
MEFs
flox/+ sarcomas
flox/flox sarcomas
KD flox/+
KD +/-
1
6
1
4
deleted
floxed
wild type

Figure 3.4. *Dicer1* undergoes hemizygous loss in soft-tissue sarcoma. DNA was prepared from $Kras^{LSL\text{-}G12D}$ MEFs either heterozygous conditional or mutant for *Dicer1* (KD f/+ And KD +/-) and sarcoma cell lines from KPD f/+ And f/f mice. The *Dicer1* locus was examined by PCR.

Figure 3.5

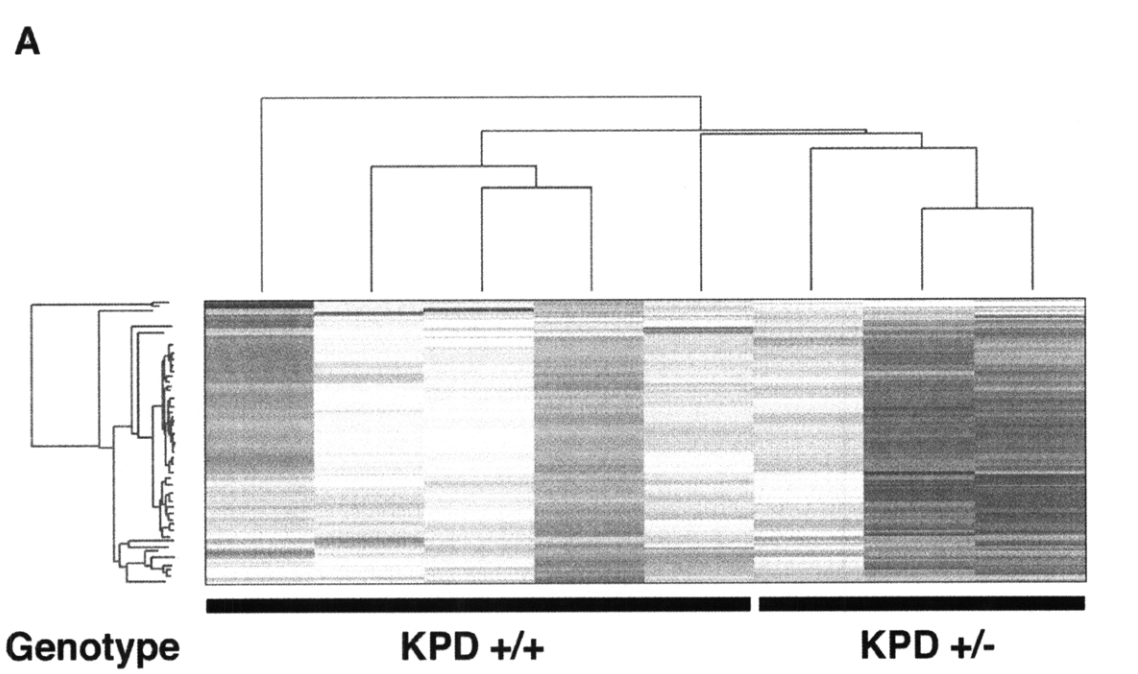
A
Genotype
KPD +/+
KPD +/-
B
miR-29
miR-26
miR-30
let-7
miR-15b
miR-7
Gln tRNA
Genotype
KPD +/+
KPD +/-

Figure 3.5. Hemizygous deletion of *Dicer1* causes a global reduction in steady-state miRNA levels. (A) MiRNA profiling and hierarchical clustering were performed on lung cancer cell lines either wild type or heterozygous for *Dicer1* (KPD +/+ and KPD +/- respectively). (B) Small RNA Northern blotting analysis of miRNAs and Glutamine tRNA was performed on KPD +/+ and KPD +/- lung cancer cell lines.

infected KD f/+ and f/f mice with a lentivirus expressing Cre (Lenti-Cre). In contrast to adenovirus, which transiently expresses Cre in the lung epithelium, lentiviral integration should allow for stable, longer-term expression of Cre, allowing for likelier complete recombination at the *Dicer1* locus. When we compared mice infected with Lenti-Cre, we observed a striking decrease in tumor burden in KD f/f compared to KD f/+ mice (Figure 3.6A-C); this result contrasts sharply with previous studies in which KD f/+ and f/f mice created a similar tumor burden after Adeno-Cre infection. Notably, when we isolated tumors from KD f/f mice and assessed *Dicer1* loss, we found all tumors had incomplete recombination, suggesting that there is active selection against total *Dicer1* loss in lung tumors (Figure 3.6D).

To further examine the effects of complete *Dicer1* loss, we transduced a set of sarcoma cell lines with $CreER^{T2}$ to allow for tamoxifen-dependent Cre activity. Treatment with 4-hydroxytamoxifen (4-OHT) led to efficient deletion of *Dicer1 in vitro* (Figure 3.7A). To assess the effect of this recombination on tumorigenesis, we transplanted untreated KPD f/- sarcoma cells expressing $CreER^{T2}$ into immune competent hosts and recombination was induced by systemic tamoxifen administration. Compared to control treatments, tamoxifen delivery substantially slowed tumor growth in KPD f/- sarcomas (Figure 3.7B). Of note, this system allowed for Cre-mediated deletion of *Dicer1*, as the tumors that developed in tamoxifen-treated animals had extensive *Dicer1* recombination (Figure 3.7C). Importantly, this tumor suppression was not merely an effect of tamoxifen- or Cre-mediated toxicity, as tamoxifen treatment of animals injected with KPD +/+ sarcoma cells expressing $CreER^{T2}$ did not impair tumor growth (Figure 3.8). In total, these studies demonstrate that complete deletion of *Dicer1* is deleterious, strongly suggesting that *Dicer1* is a haploinsufficient tumor suppressor.

Figure 3.6

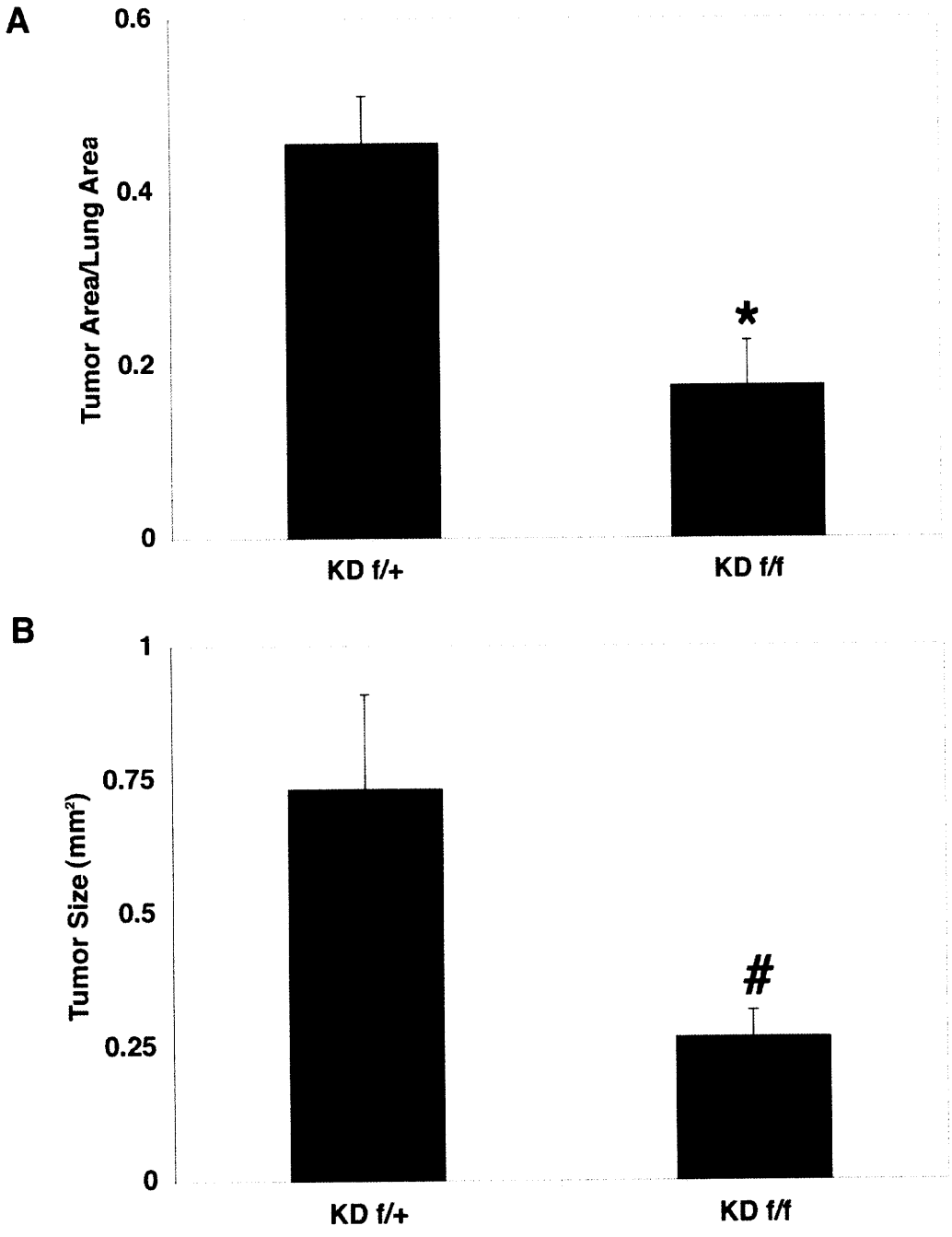
A
0.6
0.4
0.2
0
Tumor Area/Lung Area
*
KD f/+
KD f/f
B
1
0.75
0.5
0.25
0
Tumor Size (mm²)
#
KD f/+
KD f/f

Figure 3.6 continued

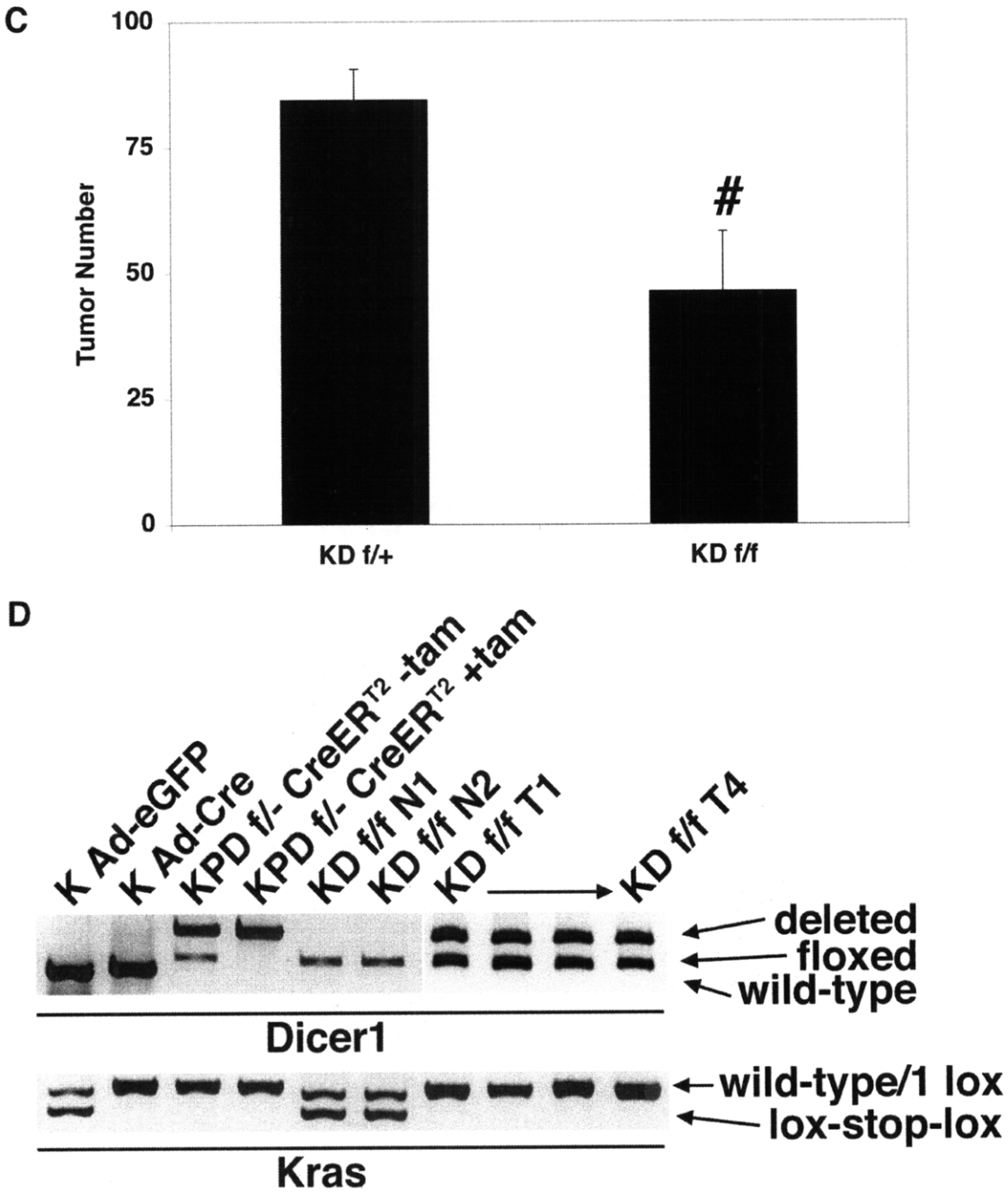

Figure 3.6. Complete deletion of *Dicer1* in murine lung cancers inhibits tumor growth. (A) KD f/+ and f/f mice were intratracheally infected with lentivirus expressing Cre. Twelve weeks post-infection, animals were sacrificed and tumor and lung areas were quantified with Bioquant software. (B) Tumor sizes were quantified for the animals described above. (C) Tumor numbers were quantified for the animals described above. Values are mean +/- s.e.m. (n=7 for KD f/+ and n=8 for KD f/f). *: $p<0.005$, #: $p<0.05$. (D) DNA was prepared from $Kras^{LSL\text{-}G12D}$ MEFs infected with adenovirus expressing eGFP (K Ad-eGFP) or adenovirus expressing Cre (K Ad-Cre); KPD f/- sarcoma cell lines infected with MSCV.CreER^{T2}.puro in the presence or absence of 4-OHT (KPD f/- CreER^{T2} -/+ tam); and thymus and lung tumors from KD f/f mice infected with Lenti-Cre (KD f/f N1-2 and T1-4, respectively). The *Dicer1* locus was examined by PCR, while the *Kras* locus was examined by PCR to assess tumor purity.

Figure 3.7

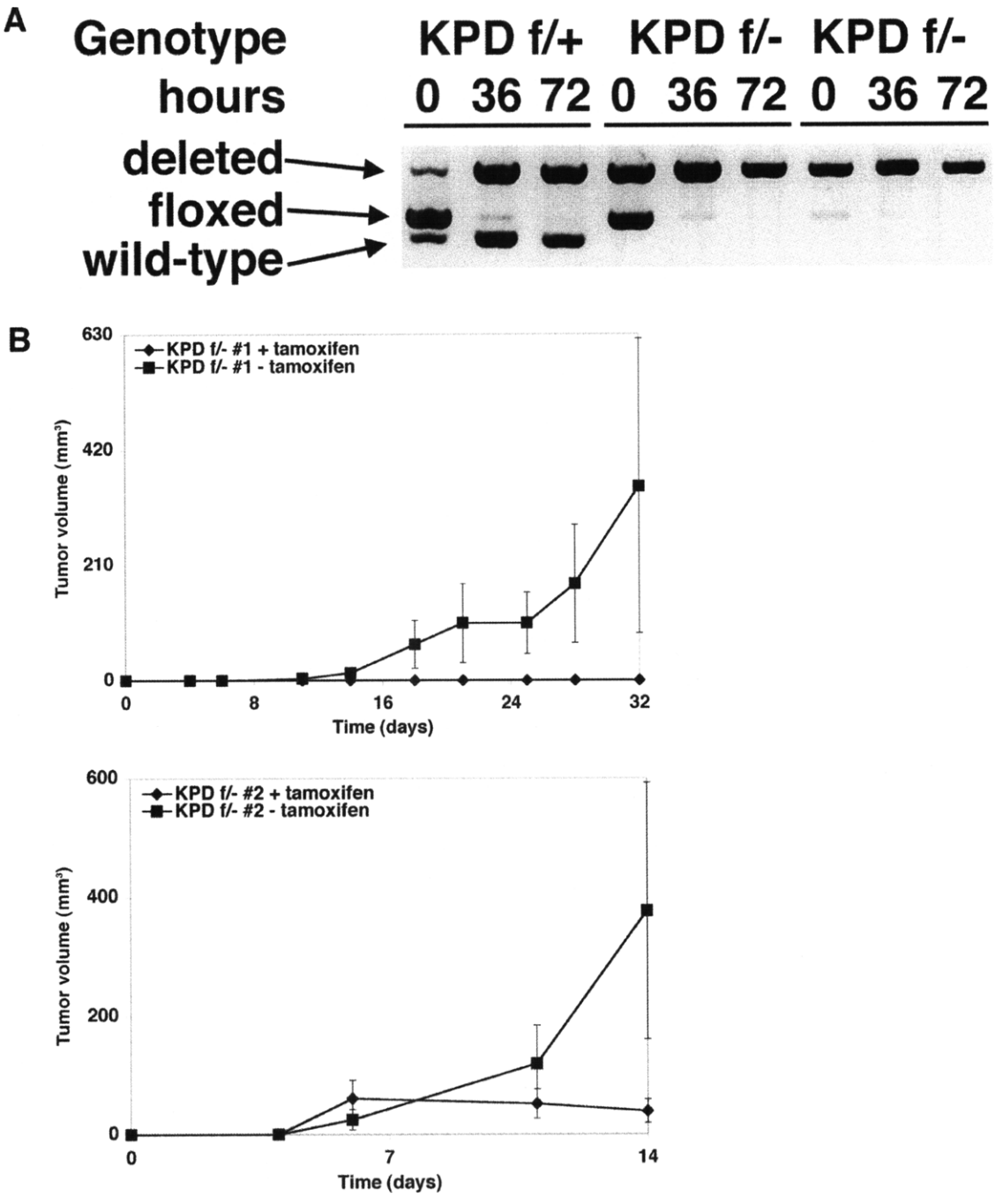
A
Genotype
hours
KPD f/+
KPD f/-
KPD f/-
0 36 72 0 36 72 0 36 72
deleted
floxed
wild-type
B
KPD f/- #1 + tamoxifen
KPD f/- #1 - tamoxifen
Tumor volume (mm³)
630
420
210
0
0
8
16
24
32
Time (days)
KPD f/- #2 + tamoxifen
KPD f/- #2 - tamoxifen
Tumor volume (mm³)
600
400
200
0
0
7
14
Time (days)

Figure 3.7 continued

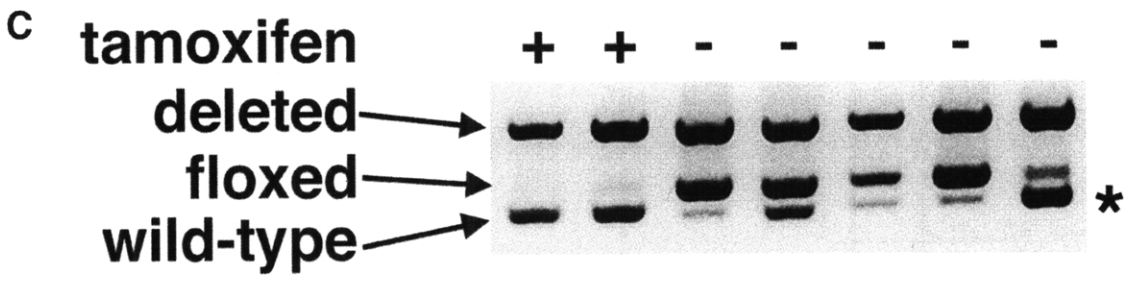

Figure 3.7. Complete deletion of *Dicer1* inhibits sarcoma development. (A) KPD f/+ and f/- sarcoma cell lines were infected with MSCV.CreER^{T2}.puro. Cre-mediated recombination was induced by treatment with 4-OHT for defined time points. DNA was prepared and recombination of the *Dicer1* locus was assessed by PCR. (B) Two independent KPD f/- sarcoma cell lines infected with MSCV.CreER^{T2}.puro were injected subcutaneously into C57Bl6/129SV F1 animals. Animals were treated with or without tamoxifen by intraperitoneal injection and tumor growth was measured over time. Values are mean +/- s.e.m. (n=8 each). (C) Tumors were isolated from KPD f/- sarcoma cell line transplants treated with or without tamoxifen. DNA was prepared and recombination of the *Dicer1* locus was assessed by PCR. Asterisk corresponds to the wild type *Dicer1* locus from the C57Bl6/129SV F1 host animals.

Figure 3.8

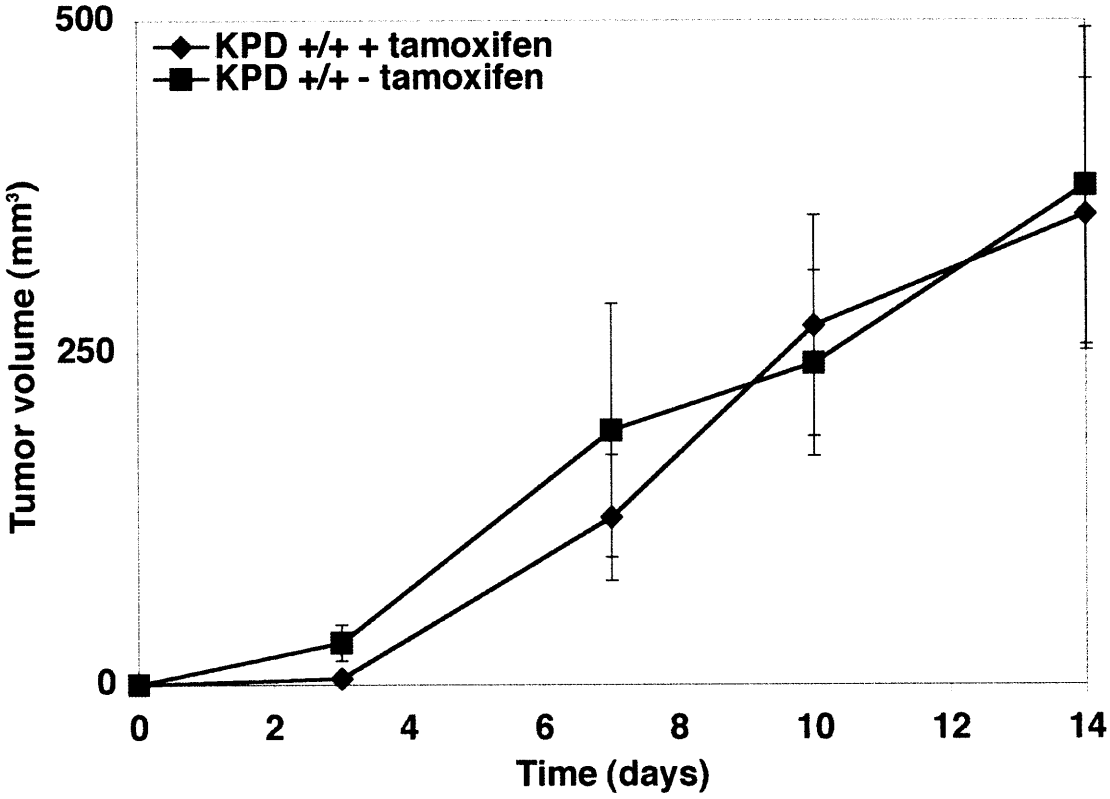
500
250
0
Tumor volume (mm³)
KPD +/+ + tamoxifen
KPD +/+ - tamoxifen
0
2
4
6
8
10
12
14
Time (days)

Figure 3.8. Tamoxifen treatment of KPD +/+ sarcoma cells expressing $CreER^{T2}$ does not impair tumor growth. KPD +/+ sarcoma cell lines infected with MSCV.$CreER^{T2}$.puro were injected subcutaneously into C57Bl6/129SV F1 animals. Animals were treated with or without tamoxifen by intraperitoneal injection and tumor growth was measured over time. Values are mean +/- s.e.m. (n=7 each).

While these findings indicate that partial loss of *Dicer1* promotes tumor development, it was not clear whether comparable deletions are seen in human cancer. To explore this, we assessed *DICER1* copy number data from the Cancer Genome Project at the Sanger Institute (Forbes et al., 2008). In their data sets, there was frequent loss of one allele of *DICER1* in several different tumor types (Table 3.1). Of note, there were never high-level amplifications of the *DICER1* locus in these tumor sets, suggesting these losses were not due to random genome instability. More importantly, homozygous deletion of *DICER1* was never observed, in line with our findings of *Dicer1* functioning as a haploinsufficient tumor suppressor. When combined with the promotion of tumorigenesis by frequent heterozygous point mutation of *TARBP2* (which promotes miRNA processing by stabilizing Dicer1) in mismatch-repair deficient colon cancer, the *DICER1* hemizygous loss observed here provides one reasonable mechanism by which miRNA processing becomes impaired in human cancer (Melo et al., 2009).

The findings presented here provide substantial evidence for *Dicer1* as a haploinsufficient tumor suppressor. These findings are in line with earlier observations of global down-regulation of miRNAs in human cancer and the functional relevance of this down-regulation in tumorigenesis (Kumar et al., 2007; Lu et al., 2005). These results further clarify the genetic basis for this global loss of miRNAs, via partial loss of function of the miRNA processing machinery in human tumors. Although the frequent *DICER1* single-copy deletion noted in human cancers provides a relevant mechanism of impairing miRNA biogenesis, additional mechanisms may occur. In particular, in light of the frequent mutation of *TARBP2* in mismatch-repair deficient colon cancer, it is possible that point mutation of other components of the miRNA processing machinery can occur, as has been described for *DICER1* and *DROSHA* in a small number of ovarian cancer cell lines (Merritt et al., 2008).

Table 3.1. *DICER1* is frequently deleted in various human cancers.

Tumor Type	Fraction of Tumors with DICER1 hemizygous loss
Breast	17/45
Kidney	13/21
Large Intestine	14/39
Liver	3/9
Lung	37/149
Ovary	6/22
Pancreas	6/16
Stomach	9/21

Beyond such mutational analyses, these results represent an expansion of the list of haploinsufficient tumor suppressors to include components of the miRNA processing machinery. The breadth of tumor suppressor genes that function via haploinsufficiency is only beginning to be appreciated (Santarosa and Ashworth, 2004). In fact, the traditional reliance on complete loss of tumor suppressor genes by heterozygous mutation and subsequent LOH is likely to miss factors whose effects do not require complete loss like $p27^{Kip1}$ and Dmp1. Moreover, such analyses are certain to ignore genes whose partial loss is tumor suppressive while their complete loss is deleterious to tumor growth, such as *Dicer1*. As genome-wide studies begin to explore the functional role of chromosomal deletions in human cancer, it will be important to consider haploinsufficiency of deleted genes in such contexts.

Materials and Methods

Mice. $Kras^{LSL\text{-}G12D}$; $Dicer1^{+/+,f/+,f/f}$ animals were generated as described previously (Kumar et al., 2007). $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ animals were bred with $Kras^{LSL\text{-}G12D}$; $Dicer1^{f/f}$ animals to produce $Kras^{LSL\text{-}G12D}$; $Trp53^{f/+}$; $Dicer1^{f/+}$ animals. These animals were backcrossed to $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ animals to produce $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$; $Dicer1^{f/+}$ animals, which were interbred to produce the experimental cohort.

Intranasal, intratracheal, and intramuscular infection. Mice were infected intranasally, intratracheally, and intramuscularly with adenovirus and lentivirus expressing Cre as described (Jackson et al., 2001; Kirsch et al., 2007; Kumar et al., 2008). Lung and tumor areas were determined using Bioquant Image Analysis software as described previously (Kumar et al., 2007).

Cell line injection and tamoxifen treatment. C57Bl6/129SV F1 animals were subcutaneously injected with $2.5*10^4$ sarcoma cells. Mice were subsequently treated with corn oil with and without tamoxifen as described previously (Ventura et al., 2007). Tumors were measured as described (Kumar et al., 2007).

Cell culture. Primary sarcoma and lung cancer cell lines were generated by dissection of tumors from mice infected with adenovirus expressing Cre, digestion with trypsin and plating and propagation in serum-containing media. *Kras*$^{LSL\text{-}G12D}$; *Dicer1*$^{f/+,f/f}$ mouse embryonic fibroblasts were generated as described (Tuveson et al., 2004). Sarcoma cell lines were infected with MSCV.CreER^{T2}.puro and treated with 4-OHT for defined time points as described (Ventura et al., 2007). Cells were maintained using standard conditions.

Recombination analysis. DNA was prepared from MEFs, tumors, and cancer cell lines. *Dicer1* recombination was assessed by PCR using previously described primers (Harfe et al., 2005). *Kras* recombination primer information is provided below.

Northern blotting. Small RNA Northern blotting was performed as described (Ventura et al., 2004). Probe information is provided in Supplemental Materials and Methods.

miRNA expression analysis. miRNA expression profiling was performed using a bead-based miRNA detection platform as described with modifications (Lu et al., 2005).

PCR genotyping. PCR genotyping of *Dicer1* was performed as described previously (Harfe et al., 2005). PCR genotyping of *Kras* was performed using standard conditions with the following primers:

Forward #1: 5'-GTC TTT CCC CAG CAC AGT GC-3'

Reverse #1: 5'-CTC TTG CCT ACG CCA CCA GCT C-3'

Reverse #2: 5'-AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A-3'

Northern blotting. Small RNA Northern blotting was performed using the following probes (all 5' to 3'):

miR-29: AYCGATTTCARATGGTGCTA

miR-26: CCTATCCTGRATTACTTGAA

miR-30: AGTSDRGGATGTTTACA

let-7 (combined at an even stoichiometric ratio): AACTATACAACCTACTACCTCA, AACTATACAATCTACTACCTCA, AACTATGCAACCTACTACCTCT, AACCATACAACCTACTACCTCA, ACTGTACAAACTACTACCTCA

miR-15b: TGTAAACCATGATGTGCTGCTA

miR-7: ACAACAAAATCACWAGTCTTCCA

Glutamine tRNA (Gln tRNA): TGGAGGTTCCACCGAGAT

MSCV.CreER^{T2}.puro. CreER^{T2} was generated from the *Rosa26-CreER^{T2}* targeting vector described previously (Ventura et al., 2007). CreER^{T2} was amplified with the primers 5'-GCA GTC GAC ACC ATG TCC AAT TTA CTG ACC-3' And 5'-GAA GAA TTC TCA AGC TGT GGC AGG GAA ACC-3'. The PCR product was digested with SalI and EcoRI and ligated to XhoI/EcoRI-cut MSCV.puro (from Clontech).

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CHAPTER 4: Suppression of non-small cell lung tumor development by the *let-7* microRNA family

The work presented in this chapter is largely taken from a paper published in *The Proceedings of the National Academy of Sciences* (Kumar et al., 2008). I contributed to Figures 4.3, 4.4, 4.7, 4.8A, 4.9-4.13. Stefan J. Erkeland contributed Figures 4.1A and B, 4.2, 4.5, 4.6, and 4.8B. Ryan E. Pester and Cindy Y. Chen assisted in the experiments presented in 4.3, 4.4, 4.7, 4.8A, 4.9-4.13. Margaret S. Ebert contributed Figure 4.1C.

Abstract

Many microRNAs (miRNAs) target mRNAs involved in processes aberrant in tumorigenesis, such as proliferation, survival, and differentiation. In particular, the *let-7* miRNA family has been proposed to function in tumor suppression, as reduced expression of let-7 family members is common in non-small cell lung cancer (NSCLC). Here we show that let-7 functionally inhibits non-small cell tumor development. Ectopic expression of let-7g in K-Ras^{G12D}-expressing murine lung cancer cells induced both cell-cycle arrest and cell death. In tumor xenografts, we observed significant growth reduction of both murine and human non-small cell lung tumors when overexpression of let-7g was induced from lentiviral vectors. In let-7g expressing tumors, reductions in Ras family and HMGA2 protein levels were detected. Importantly, let-7g—mediated tumor suppression was more potent in lung cancer cell lines harboring oncogenic K-Ras mutations than in lines with other mutations. Ectopic expression of K-Ras^{G12D} largely rescued let-7g mediated tumor suppression, while ectopic expression of HMGA2 was less effective. Finally, in an autochthonous model of NSCLC in the mouse, let-7g expression substantially reduced lung tumor burden.

Introduction

MicroRNAs (miRNAs) are a class of short, highly conserved, noncoding RNAs known to play important roles in numerous developmental processes. MiRNAs are initially transcribed as longer primary transcripts that undergo sequential processing by the RNase III-like enzymes Drosha and Dicer (Bartel, 2004). After maturation, miRNAs regulate gene expression through incomplete basepairing to a complementary sequence in the 3' untranslated region (UTR) of a target mRNA. The miRNA-mRNA interaction results in translational repression and accelerated turnover of the target transcript (Valencia-Sanchez et al., 2006). Computational analyses predict that mammalian miRNAs regulate approximately 30% of all protein-coding genes, as an individual miRNA can target many different mRNAs and an individual mRNA can be regulated by several different miRNAs (Lewis et al., 2005; Lewis et al., 2003).

Numerous findings suggest that miRNAs undergo aberrant regulation during tumorigenesis. MicroRNA genes are frequently located in genomic regions gained and lost in mammalian cancers (Calin et al., 2004; Sevignani et al., 2007). Functionally, several miRNAs have been described as oncogenes. For example, the miRNA cluster miR-17~92 is amplified in human B-cell lymphomas and was found to cooperate with c-Myc to accelerate lymphomagenesis in the mouse (He et al., 2005). The *BIC* transcript, which was isolated from a common retroviral insertion site that cooperates with c-Myc in lymphomagenesis and is highly upregulated in Burkitt's lymphoma, encodes a primary miRNA transcript for miR-155 (Lagos-Quintana et al., 2002; Metzler et al., 2004). Moreover, miR-372 and miR-373 were shown to be oncogenic in an expression screen and were implicated in testicular cancer through inactivation of the p53 pathway (Voorhoeve et al., 2006). Other miRNAs have been described as tumor

suppressors. Intriguingly, miRNA expression profiling has shown that miRNAs are globally downregulated in tumors relative to normal tissue (Lu et al., 2005). Recent work from our group demonstrated that global downregulation can promote tumorigenesis (Kumar et al., 2007). MiR-15 and miR-16 are located in chromosome 13q14, a region frequently deleted in chronic lymphocytic leukemia (CLL) (Cimmino et al., 2005). Furthermore, recent studies have shown that p53 transcriptional activation targets the miR-34 family in a step important for cell cycle control (Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007). In total, these findings provide several lines of evidence for the importance of miRNA in tumorigenesis.

Let-7 was originally identified in *C.elegans* as a regulator of developmental timing and cellular proliferation (Reinhart et al., 2000). The discovery of mammalian let-7 family members prompted speculation that these miRNAs might be tumor suppressors (Pasquinelli et al., 2000). There are at least nine individual members of the let-7 family in mammals, and several let-7 genes are located in regions frequently deleted in human cancer (Calin et al., 2004). Moreover, let-7 expression is reduced in a subset of non-small cell lung cancer (NSCLC) patients, and this reduction is correlated with poor prognosis (Takamizawa et al., 2004; Yanaihara et al., 2006). When ectopically expressed in cancer cell lines, let-7 miRNA can repress cellular proliferation (Johnson et al., 2007; Lee and Dutta, 2007). Finally, let-7 family members functionally inhibit the mRNAs of well characterized oncogenes, such as the Ras family (Johnson et al., 2005), HMGA2 (Hebert et al., 2007; Lee and Dutta, 2007; Mayr et al., 2007; Wang et al., 2007), c-Myc (Koscianska et al., 2007; Kumar et al., 2007), and cell-cycle regulators like CDC25A, CDK6, and Cyclin D2 (Johnson et al., 2007).

The two best-characterized let-7 targets are the Ras family and HMGA2. Activating mutations in Ras family members (H-ras, K-ras and N-ras) are found in many human tumors

including ~30% of NSCLCs (Bos, 1989). The let-7 family has been shown to regulate both N-Ras and K-Ras mRNAs via their 3' UTRs (Johnson et al., 2005). Notably, all previous studies reporting let-7—mediated repression of proliferation have been performed in cells expressing mutant forms of N- and K-Ras (Johnson et al., 2007; Lee and Dutta, 2007). The high mobility group A (HMGA) proteins are major nonhistone chromosomal proteins involved in transcriptional regulation controlling proliferation and differentiation. HMGA2 is implicated in tumorigenesis via chromosomal translocations and transcriptional upregulation in several tumor types, although the function of this upregulation in tumorigenesis is unclear (Meyer et al., 2007; Sarhadi et al., 2006; Young and Narita, 2007). The HMGA2 3' UTR contains seven let-7 target sites and disruption of these sites enhances oncogenic transformation (Mayr et al., 2007). Finally, let-7 expression is inversely correlated with expression of HMGA2 in NSCLC and ectopic overexpression of HMGA2 promotes cellular proliferation in the presence of let-7 (Lee and Dutta, 2007).

The above findings suggest that the let-7 miRNA family functions in tumor suppression. However, studies to date have not demonstrated that let-7 miRNA can suppress tumorigenesis *in vivo*. Moreover, there is a lack of functional data related to the regulation of individual let-7 targets on tumorigenesis *in vivo*. Finally, no studies to date have examined let-7 function in autochthonous tumors, which allows the evaluation of the roles of let-7 in tumor initiation and progression. Here we have used constitutive and inducible expression of let-7 to examine its effect on tumor development.

Results

Let-7g Impairs Tumor Cell Proliferation and Promotes Tumor Cell Death *in vitro*. To assess the roles of the let-7 miRNA on cell cycle control and cell death, we transfected a let-7g miRNA duplex into murine K-Ras^{G12D}-expressing lung adenocarcinoma cells (LKR13). Consistent with previous studies (Johnson et al., 2007; Lee and Dutta, 2007), transfected let-7g triggered a significant shift in the cell-cycle distribution, with an accumulation of G_0/G_1- and G_2/M-phase cells and a corresponding reduction of S-phase cells (Figure 4.1A). In addition, transfection of let-7g caused significant cell death in LKR13 cells (Figure 4.1B). To extend these findings, we developed a doxycycline (dox)-regulated expression system to induce miRNA expression in cell lines. Using the inducible vector system, we observed a substantial (approximately five-fold) induction of let-7g in the presence of dox (Figure 4.2B). Furthermore, induction of let-7g in LKR13 cells caused a robust decrease in cell density (Figure 4.2A). Overall, these results indicate that let-7g can restrict cellular proliferation and induce cell death.

Let-7g Suppresses Tumorigenesis *in vivo*. The LKR13 cells with inducible let-7g were transplanted into immune-compromised mice to which dox was administered in the drinking water. Using this system, we observed a substantial reduction in tumor growth in mice following induction of let-7g compared to controls; induction of miR-15b, though putatively described as a tumor suppressor in CLL (Cimmino et al., 2005), did not alter tumor growth in this system (Figure 4.3A). Importantly, this reduction in tumor growth was dependent upon induction of let-7g, as transplantation of the same cells into animals without dox treatment led to rapid tumor development (Figure 4.3B). Interestingly, tumors with ectopic let-7g expression, although

Figure 4.1

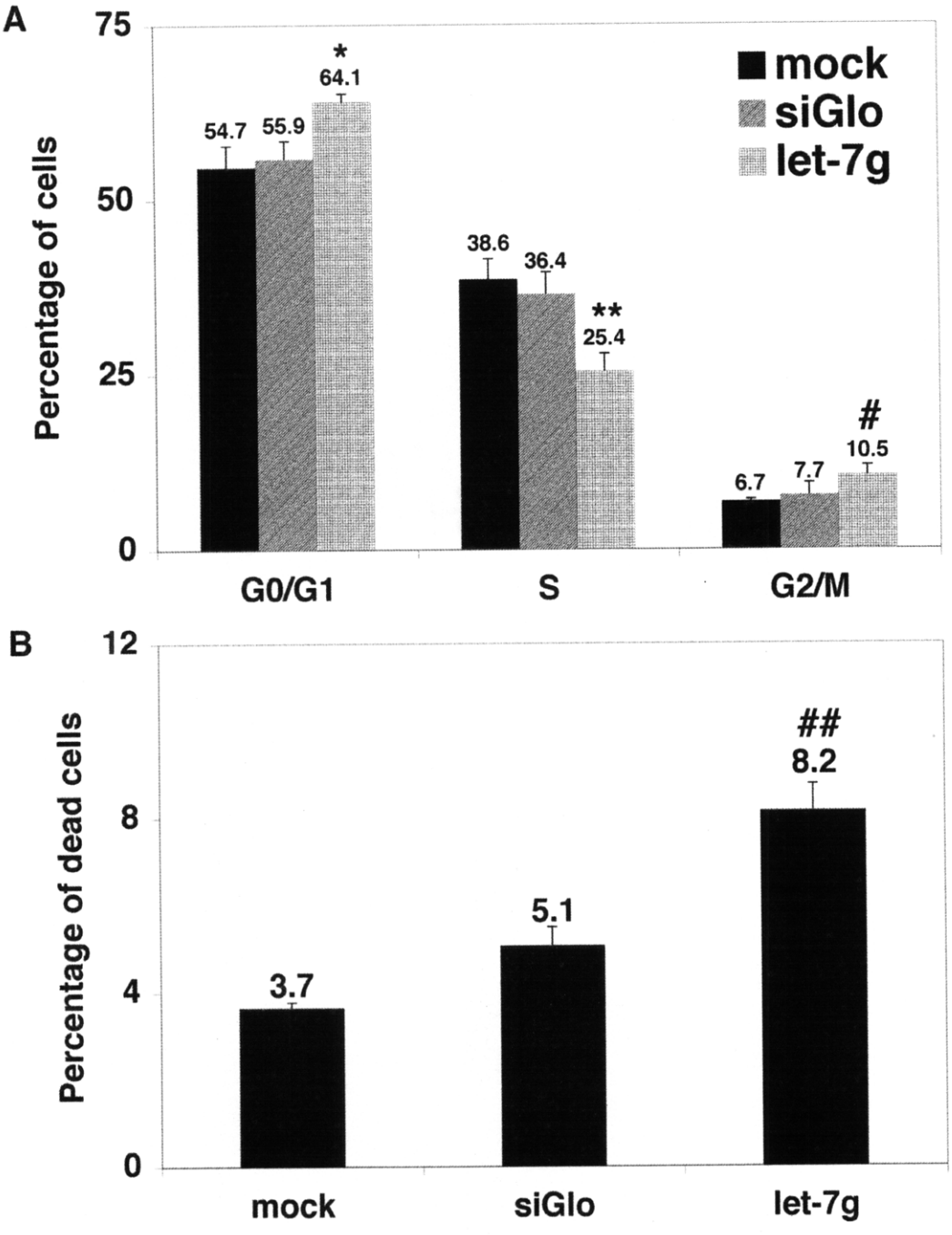
A
75
50
25
0
Percentage of cells
mock
siGlo
let-7g
54.7
55.9
*
64.1
38.6
36.4
**
25.4
6.7
7.7
#
10.5
G0/G1
S
G2/M
B
12
8
4
0
Percentage of dead cells
3.7
5.1
##
8.2
mock
siGlo
let-7g

Figure 4.1 continued

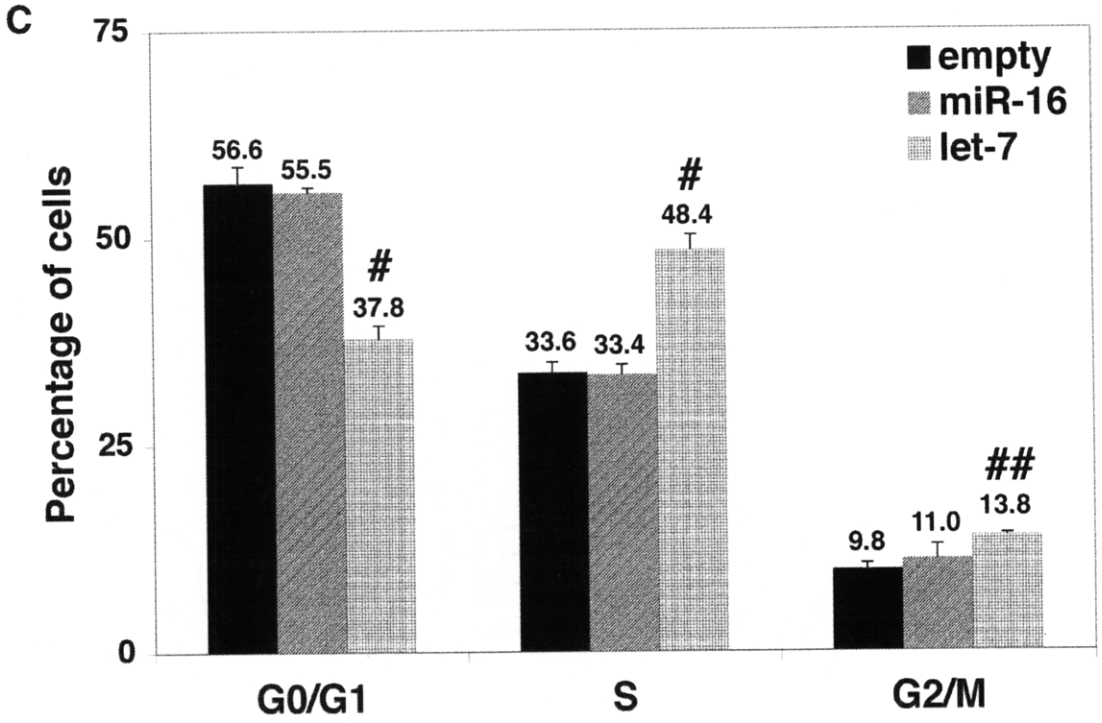

Figure 4.1 Let-7 activity impairs proliferation and enhances cell death *in vitro*. (A-B) LKR13 cells were transfected with duplex small RNA corresponding to controls or mmu-let-7g (see Supporting Information for details), fixed and stained with 7-AAD (A) or the Apoptest^{TM}-FITC kit (B), and analyzed by flow cytometry. Values are mean +/- s.d. *: $p<0.0005$, **: $p<5x10^{-5}$; #: $p<0.01$; ##: $p<0.05$. (C) LKR13 cells were transfected with miRNA sponges corresponding to no sites (empty), miR-16, or the let-7 family. Cells were then fixed and stained with propidium iodide and analyzed by flow cytometry. Values are mean +/- s.d.

Figure 4.2

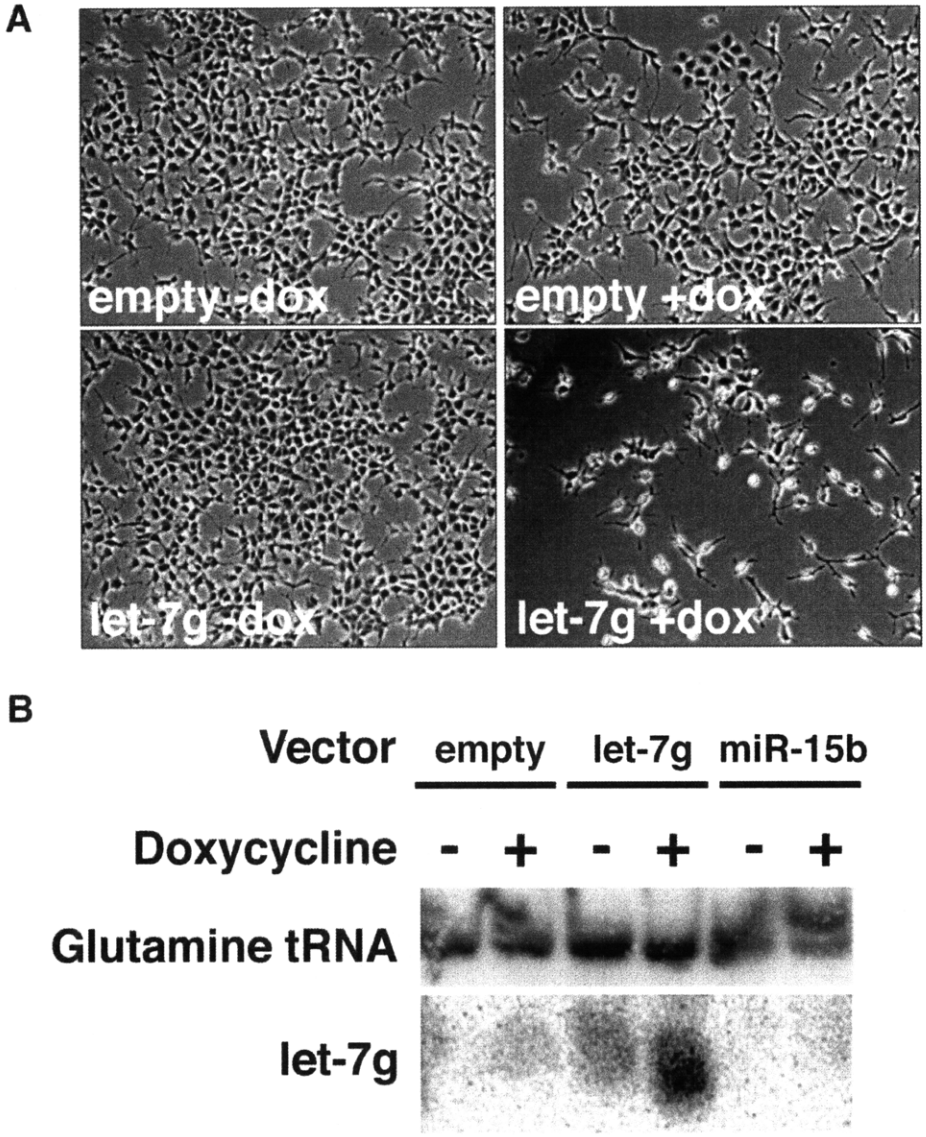
A
empty -dox
empty +dox
let-7g -dox
let-7g +dox
B
Vector
empty
let-7g
miR-15b
Doxycycline
- + - + - +
Glutamine tRNA
let-7g

Figure 4.2 Let-7g impairs proliferation and enhances cell death. (A) LKR13-Tet-On-KRAB-TE-empty and -let-7g cells were plated ($5x10^5$ cells/plate). Twelve hours later, cells were placed in the presence/absence of 5 μg/ml doxycycline. Forty-eight hours later, images were taken by phase contrast microscopy. (B) Small RNA Northern blotting was performed against let-7g and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g (see Supporting Information for details) cells in the presence/absence of 5 μg/mL doxycycline.

Figure 4.3

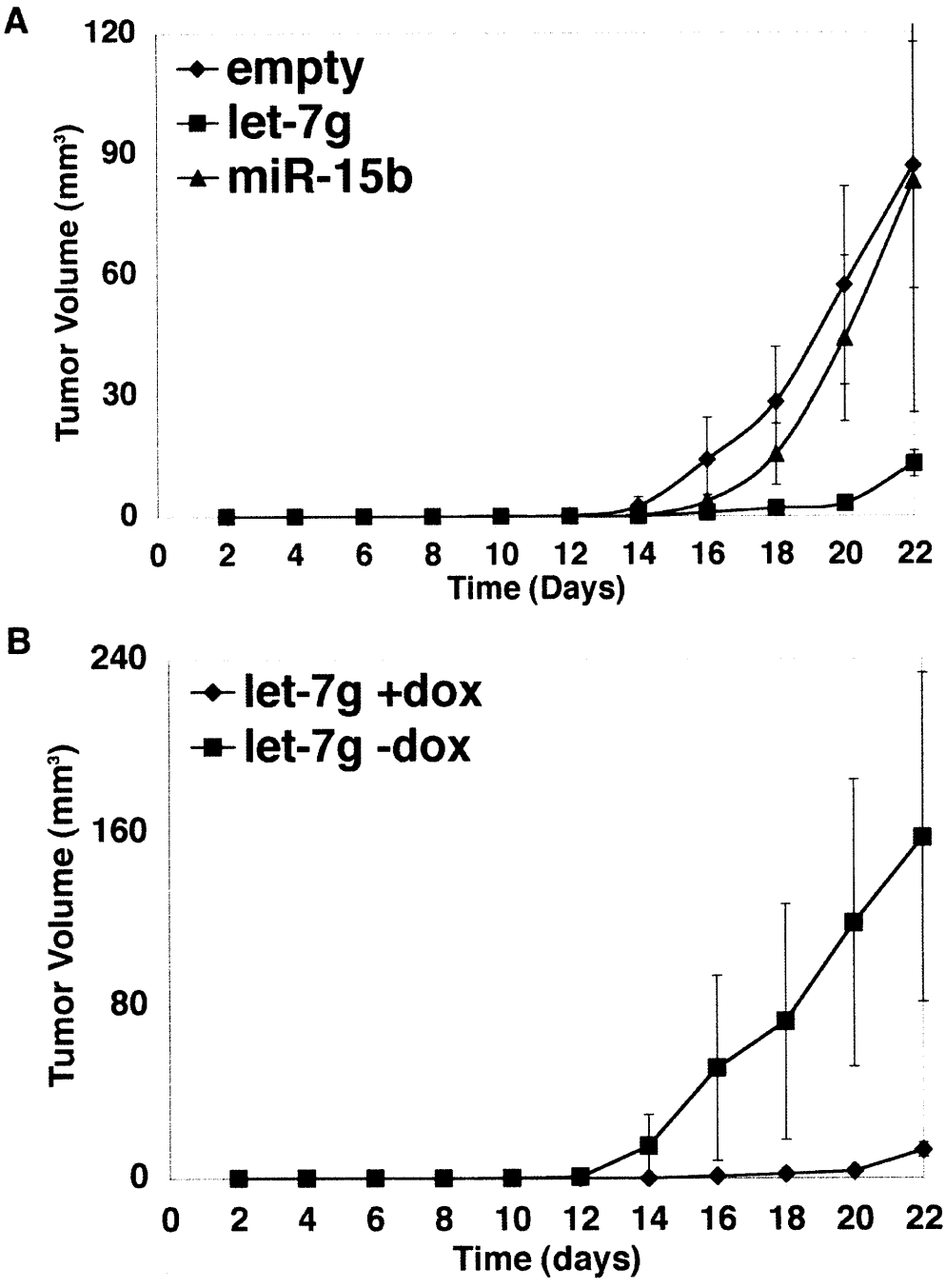
A
120
90
60
30
0
Tumor Volume (mm³)
empty
let-7g
miR-15b
0 2 4 6 8 10 12 14 16 18 20 22
Time (Days)
B
240
160
80
0
Tumor Volume (mm³)
let-7g +dox
let-7g -dox
0 2 4 6 8 10 12 14 16 18 20 22
Time (days)

Figure 4.3 continued

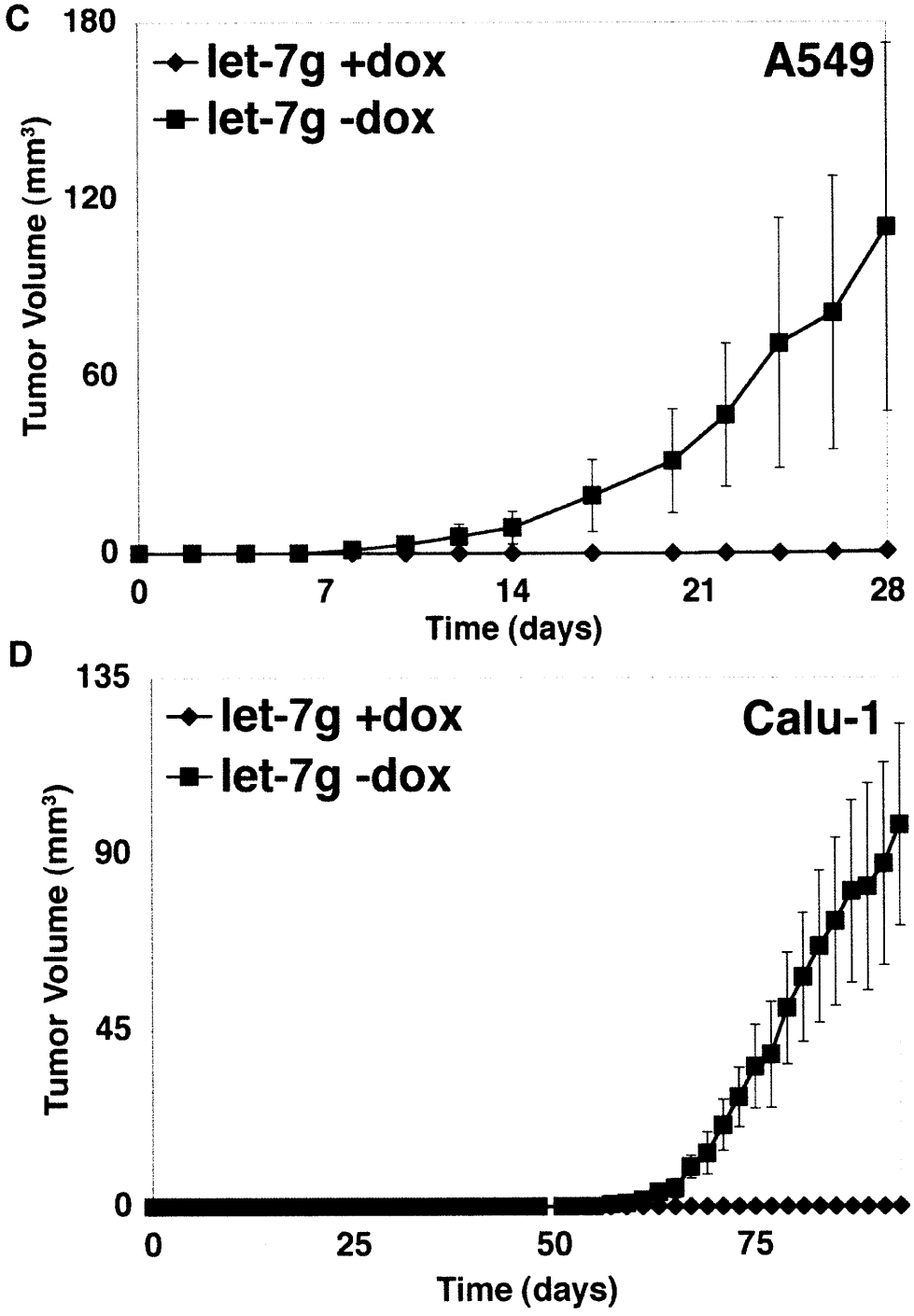

Figure 4.3 continued

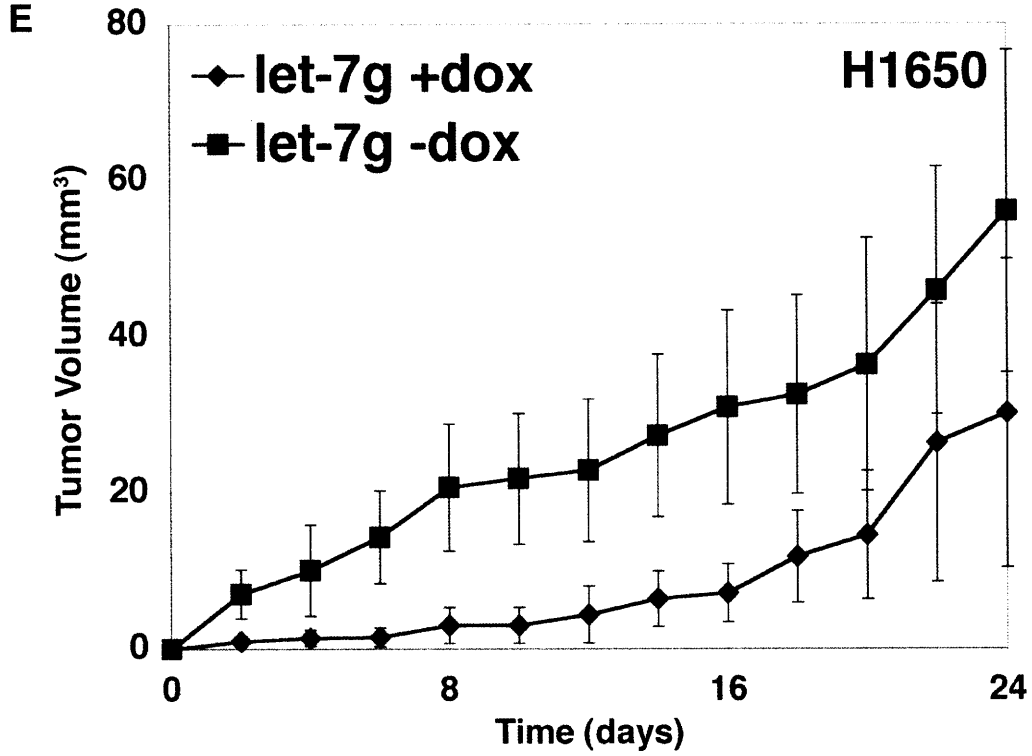

Figure 4.3 Let-7g suppresses tumorigenesis *in vivo*. (A) LKR13 cells with Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g cells were plated in the presence of 5 μg/ml doxycycline. Twenty-four hours later, cells were sorted and injected subcutaneously into immune-compromised mice ($2.5x10^4$ cells/injection). Two days later, mice were treated with drinking water containing doxycycline (2 mg/mL) and sucrose (4% w/v) and tumor values were measured over time. Values are mean +/- s.e.m. (n=6). (B) LKR13-Tet-On-KRAB-TE-let-7g cells were treated with doxycycline, sorted, and injected as described above. Two days later, mice were treated with either drinking water containing doxycycline (2 mg/mL) and sucrose (4% w/v) or drinking water containing sucrose alone. Tumor values were measured over time. Values are mean +/- s.e.m. (n=6). (C-E*)* Tet-On-KRAB-TE-let-7g cells were generated in A549 (C), Calu-1 (D), and H1650 (E) cells. Cells were prepared and injected (10^6 cells/injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean +/- s.e.m. (n=6).

smaller in size, were more advanced than controls, demonstrating widespread invasion into surrounding muscle (Figure 4.4). The steady-state levels of let-7g and miR-15b miRNAs were substantially induced in tumors grown in the presence of dox (Figure 4.5A and Figure 4.6). This induction was correlated with a decrease in Ras family members and HMGA2, previously characterized targets of the let-7 family (Figure 4.5B) (Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007).

While let-7g induction suppressed tumor growth, tumors did eventually form in the presence of dox. In order to assess the growth of let-7g—expressing tumors, we explanted tumors and tested them in secondary transplants. Secondary transplants of let-7g tumors that developed in the presence of dox grew at a similar rate as controls (Figure 4.7A). These tumors generally had significant levels of let-7g, though the degree of induction varied (Figure 4.7B). These results demonstrate that while let-7g expression suppresses tumor growth, tumors can form in the presence of high levels of let-7g. Furthermore, the tumors eventually propagate similarly to controls, suggesting that cancer cells may become resistant to the tumor suppressive functions of let-7g.

We then examined the effect of let-7g induction on established tumors. Tumors were allowed to grow to 30 mm^3 prior to addition of dox. This treatment led to a slight reduction in tumor growth rate but did not cause tumor regression (Figure 4.8A). As expected, there was a substantial induction of the corresponding miRNAs in these tumors, suggesting that the absence of tumor regression was not due to the failure to induce let-7g (Figure 4.8B).

Let-7g Potently Suppresses Mutant K-Ras—Driven Non-Small Cell Lung Tumorigenesis *in vivo*. To extend let-7g—mediated tumor suppression to human cells, we introduced the dox-

Figure 4.4

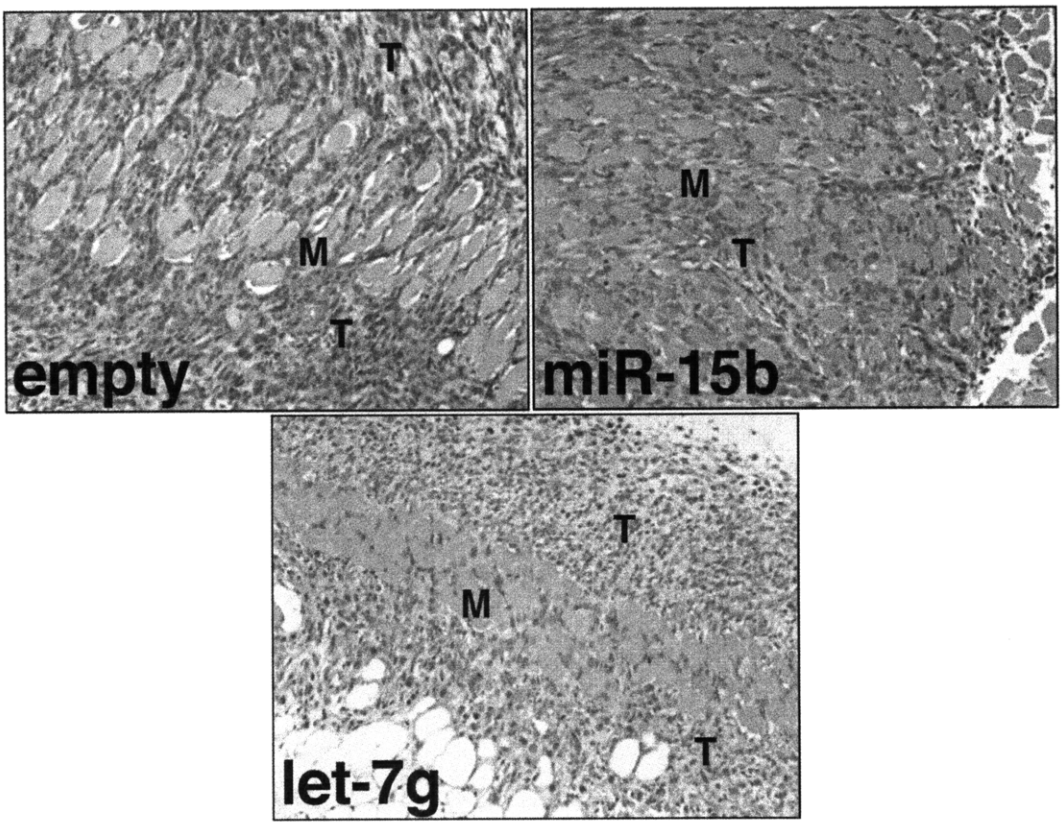

Figure 4.4 Let-7g does not alter tumor invasion. Hematoxylin/eosin staining of LKR13-Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g tumors treated with doxycycline in the drinking water as described in Figure 4.3A. Original magnification, 20X. M, skeletal muscle. T, tumor cells.

Figure 4.5

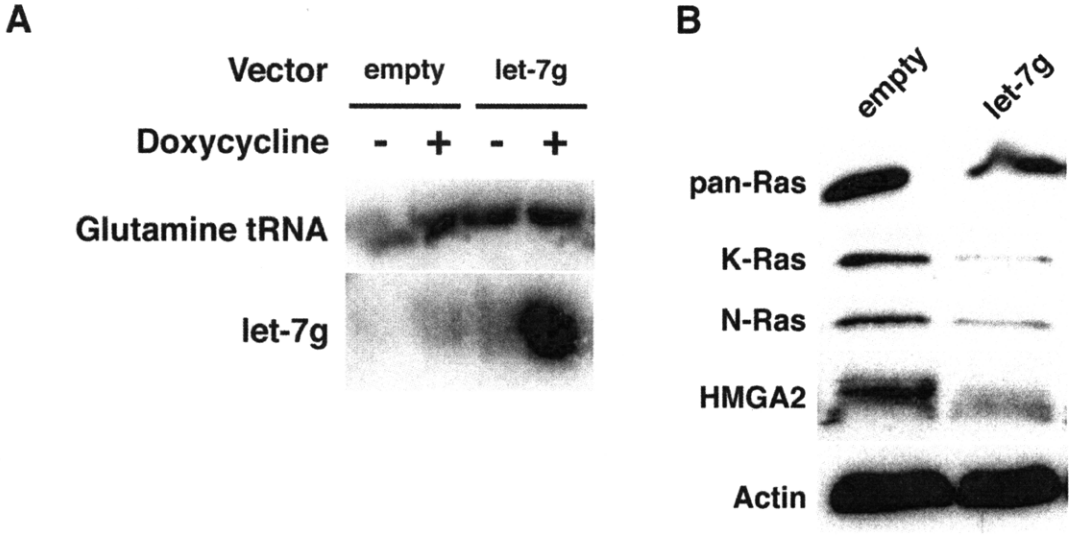
A
Vector
empty
let-7g
Doxycycline
- + - +
Glutamine tRNA
let-7g
B
empty
let-7g
pan-Ras
K-Ras
N-Ras
HMGA2
Actin

Figure 4.5 Let-7g induced tumors maintain overexpression and target suppression. (A) Small RNA Northern blotting was performed against let-7g and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-empty and -let-7g tumors generated from mice treated with or without doxycycline in the drinking water as described above. (B) Western blotting was performed in LKR13-Tet-On-KRAB-TE-empty and -let-7g tumors generated from mice treated with doxycycline in the drinking water as described above.

Figure 4.6

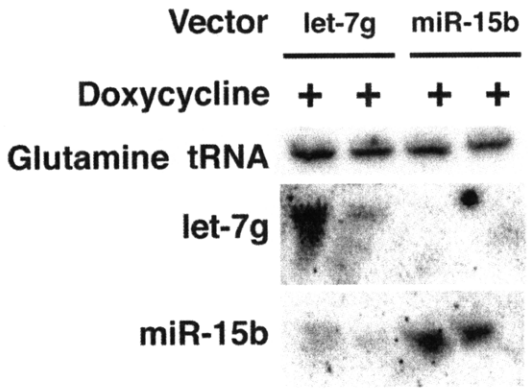
Vector
let-7g
miR-15b
Doxycycline + + + +
Glutamine tRNA
let-7g
miR-15b

Figure 4.6 Induced tumors maintain miRNA overexpression. Small RNA Northern blotting was performed against let-7g, miR-15b, and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-miR-15b and -let-7g tumors generated from mice treated with doxycycline in the drinking water as described in Figure 4.3A.

Figure 4.7

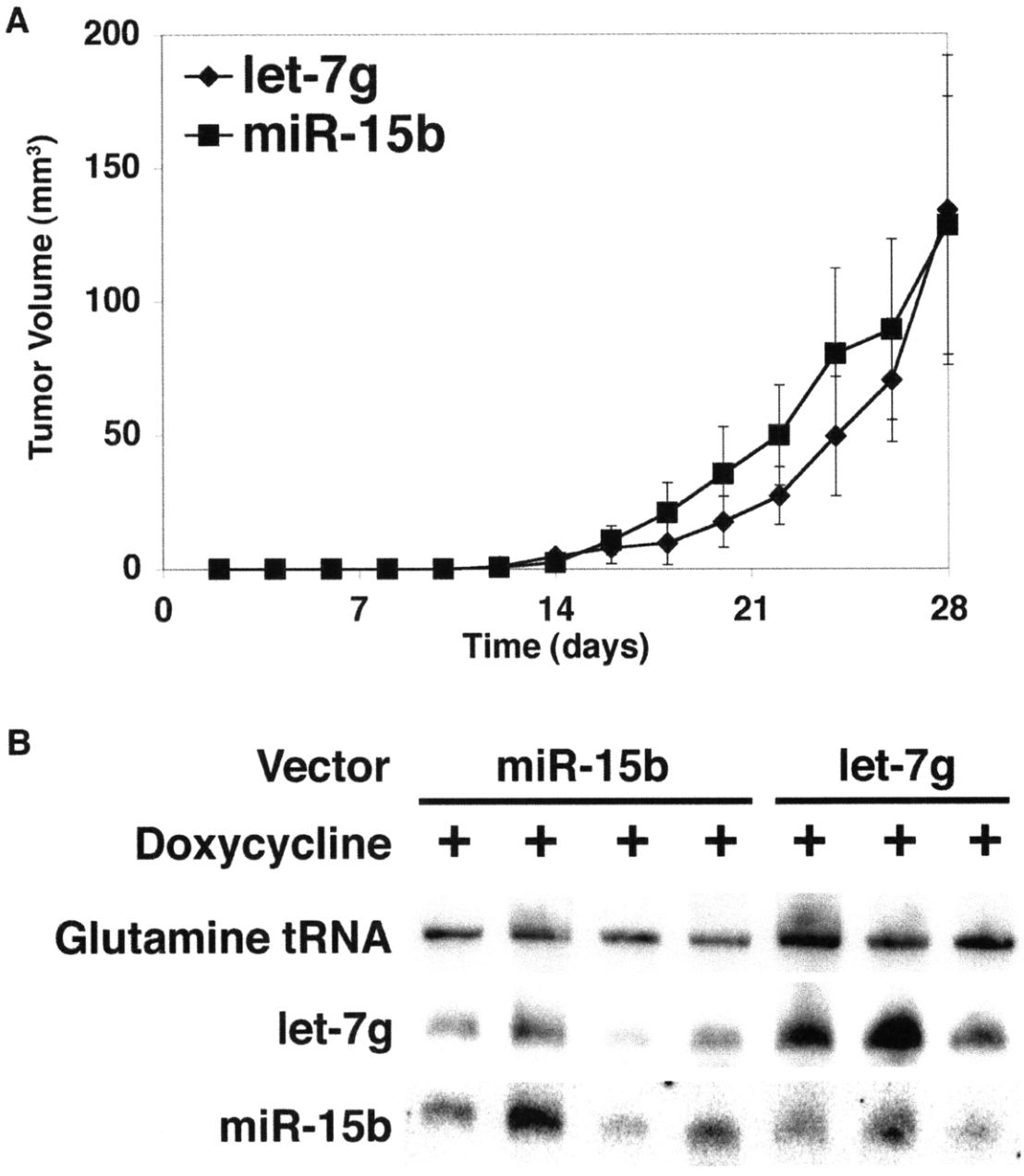
A
200
150
100
50
0
let-7g
miR-15b
Tumor Volume (mm3)
0
7
14
21
28
Time (days)
B
Vector
miR-15b
let-7g
Doxycycline
+ + + + + + +
Glutamine tRNA
let-7g
miR-15b

Figure 4.7 Let-7g does not suppress growth of induced tumors in secondary transplants.

(A) Mice were injected ($2.5x10^4$ cells/injection) with LKR13 cells with Tet-On-KRAB-TE-miR-15b and -let-7g cells, and treated with doxycycline in the drinking water as previously described. Tumors were then explanted, re-treated for 24 hours with doxycycline (5 μg/mL), sorted, and injected ($2.5x10^4$ cells/injection) and mice were treated with doxycycline in the drinking water as previously described. Tumors were then measured over time. Values are mean +/- s.e.m. (n=6). (B) Small RNA Northern blotting was performed against let-7g, miR-15b and Glutamine tRNA in LKR13-Tet-On-KRAB-TE-miR-15b and -let-7g tumors generated as described above.

Figure 4.8

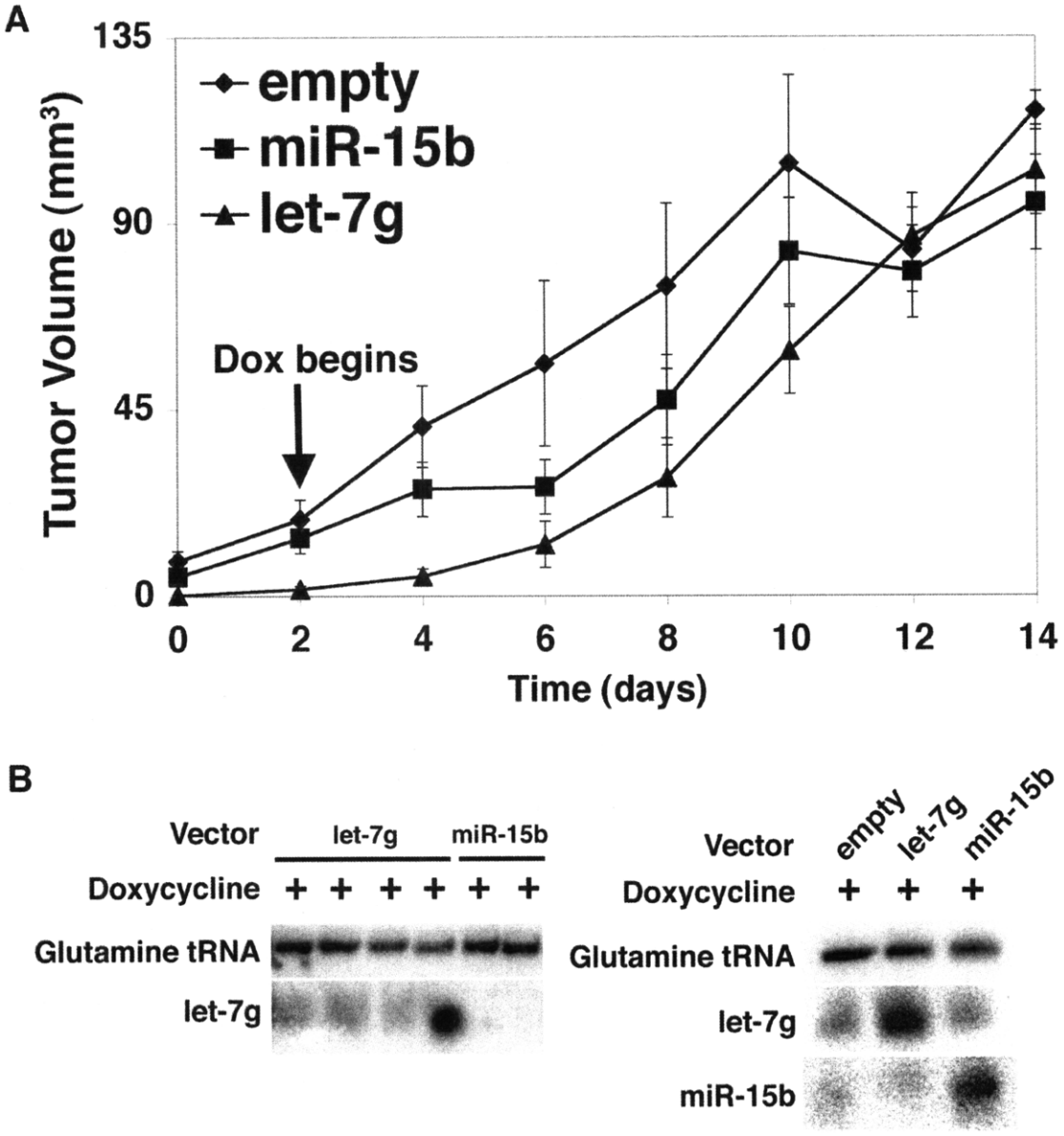
A
135
90
45
0
Tumor Volume (mm³)
empty
miR-15b
let-7g
Dox begins
0
2
4
6
8
10
12
14
Time (days)
B
Vector
let-7g
miR-15b
Doxycycline + + + + + +
Glutamine tRNA
let-7g
Vector
empty
let-7g
miR-15b
Doxycycline + + +
Glutamine tRNA
let-7g
miR-15b

Figure 4.8 Let-7g expression in established tumors slows tumor growth without regression. (A) Mice were injected with LKR13-Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g cells ($2.5x10^4$ cells/injection) and monitored for tumors. Once tumors were greater that 2 mm in diameter, mice were intraperitoneally injected with doxycycline (40 mg/kg) and tumors were measured over time. Values are mean +/- s.e.m. (n=6). (B) Small RNA Northern blotting was performed against let-7g, miR-15b and Glutamine tRNA in LKR13 cells with Tet-On-KRAB-TE-empty, -miR-15b, and -let-7g tumors generated from mice treated with doxycycline in the drinking water as described above.

inducible miRNA system in a series of human NSCLC lines. Two of the NSCLC lines contain activating mutations in K-Ras (A549 and Calu-1), while one does not (H1650). In all three NSCLC lines, control miRNA induction had no effect on tumor growth (Figure 4.9). In the K-Ras mutant NSCLC lines, let-7g induction triggered near-complete suppression of tumor formation (Figure 4.3C and D). In contrast, let-7g induction caused only a partial reduction in the rate of tumor growth in the non K-Ras mutant NSCLC line (Figure 4.3E). This indicates that tumor suppression is somewhat mutation-specific, as the let-7g—mediated reduction in tumor growth is stronger in tumors driven by mutant K-Ras than those containing wild-type K-Ras.

Let-7g-Mediated Tumor Suppression is Rescued Substantially by Mutant K-Ras. In order to determine which mRNA targets of let-7 are germane to its ability to suppress non-small cell lung tumorigenesis, we ectopically expressed mutant K-Ras (K-Ras^{G12D}) and HMGA2 in LKR13 cells containing the dox-inducible miRNA system (Figure 4.10A). Of note, each protein was upregulated modestly in the presence of enhanced expression of the other protein, suggesting mutual activation of these let-7 targets. Importantly, the expression vectors for K-Ras^{G12D} and HMGA2 lack their corresponding 3' UTRs, uncoupling the mRNA targets from direct let-7—mediated repression.

When these cells were transplanted into immune compromised mice, we observed potent let-7g-mediated tumor suppression in controls (Figure 4.11A). As shown in Figure 4.10B, ectopic K-Ras^{G12D} led to substantial, though not complete, rescue of tumor growth in the face of increased let-7g levels. In cells expressing ectopic HMGA2, there was a less robust rescue of tumor growth (Figure 4.10C). There were also no changes in tumor growth rate in cells over-expressing miR-15b upon ectopic expression of activated K-Ras or HMGA2 (Figures 4.11B and

Figure 4.9

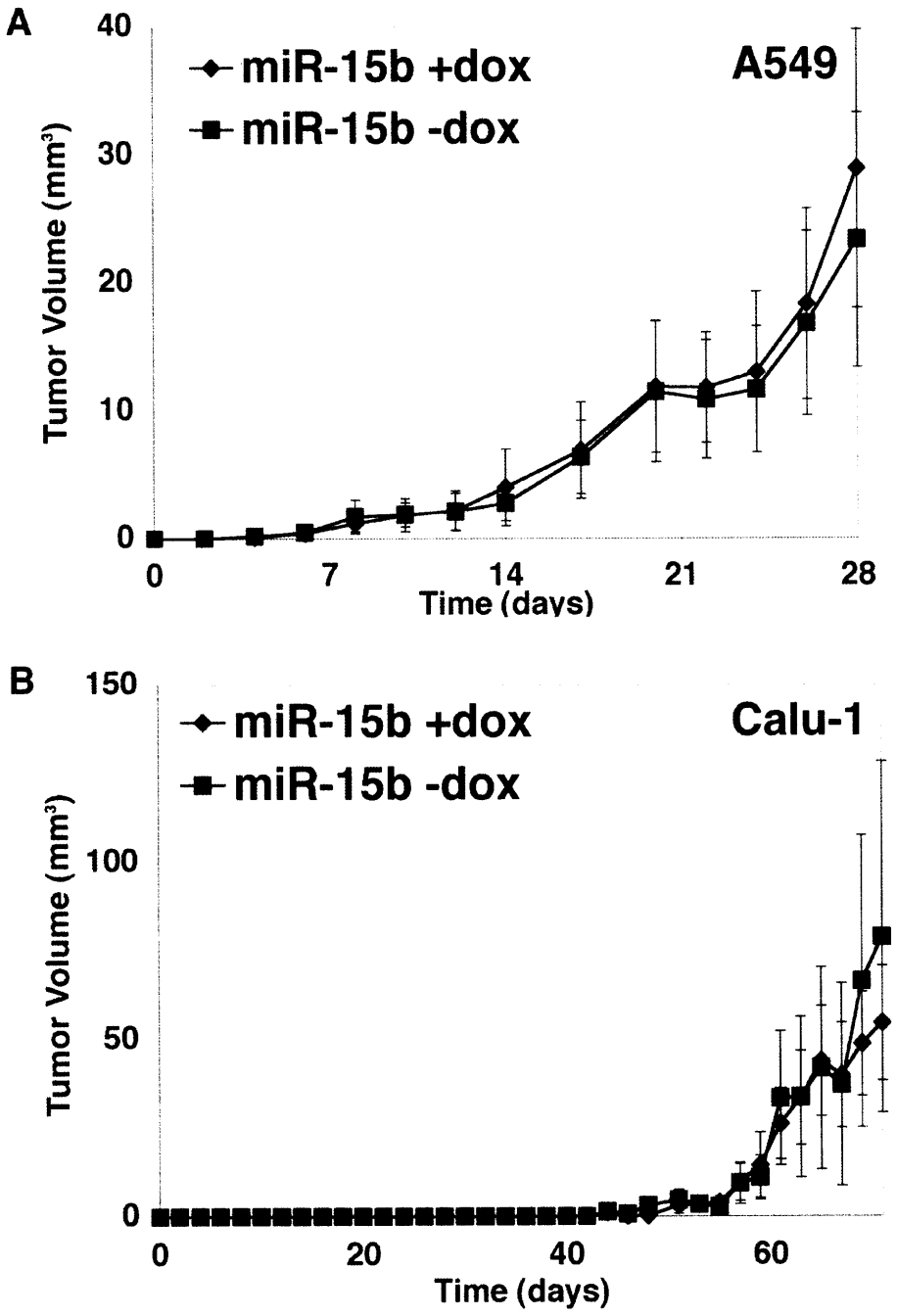
A
40
30
20
10
0
Tumor Volume (mm³)
miR-15b +dox
miR-15b -dox
A549
0
7
14
21
28
Time (days)
B
150
100
50
0
Tumor Volume (mm³)
miR-15b +dox
miR-15b -dox
Calu-1
0
20
40
60
Time (days)

Figure 4.9 continued

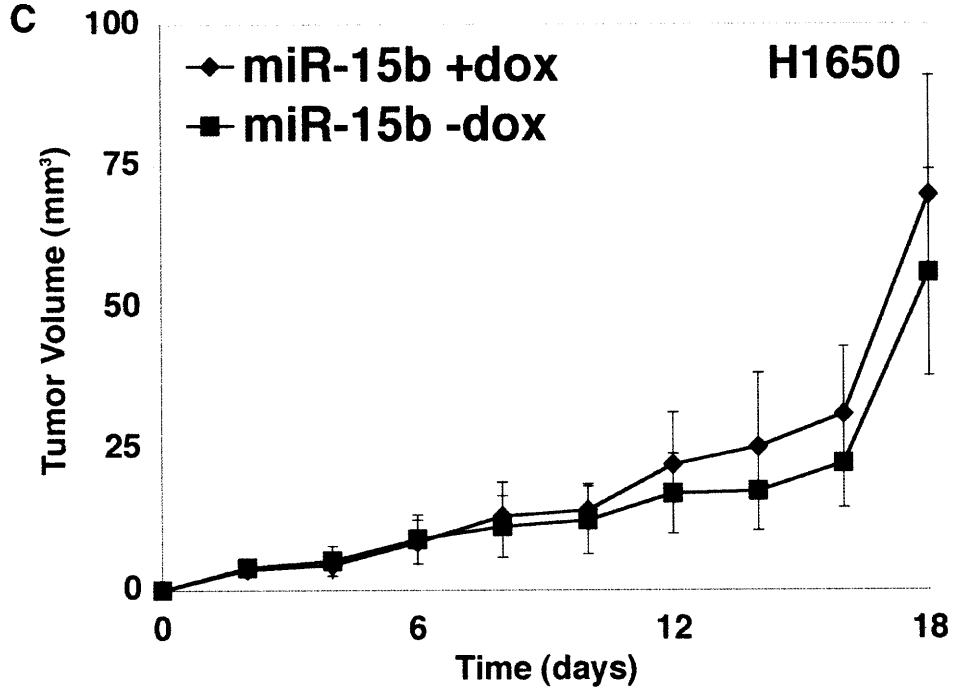

Figure 4.9 MiR-15b does not suppress non-small cell lung tumorigenesis. (A-C) Tet-On-KRAB-TE-miR-15b cells were generated in A549 (A), Calu-1 (B), and H1650 (C) cells. Cells were prepared and injected (10^6 cells/injection) into immune compromised mice. Mice were treated and tumors were measured as above. Values are mean +/- s.e.m. (n=6).

Figure 4.10

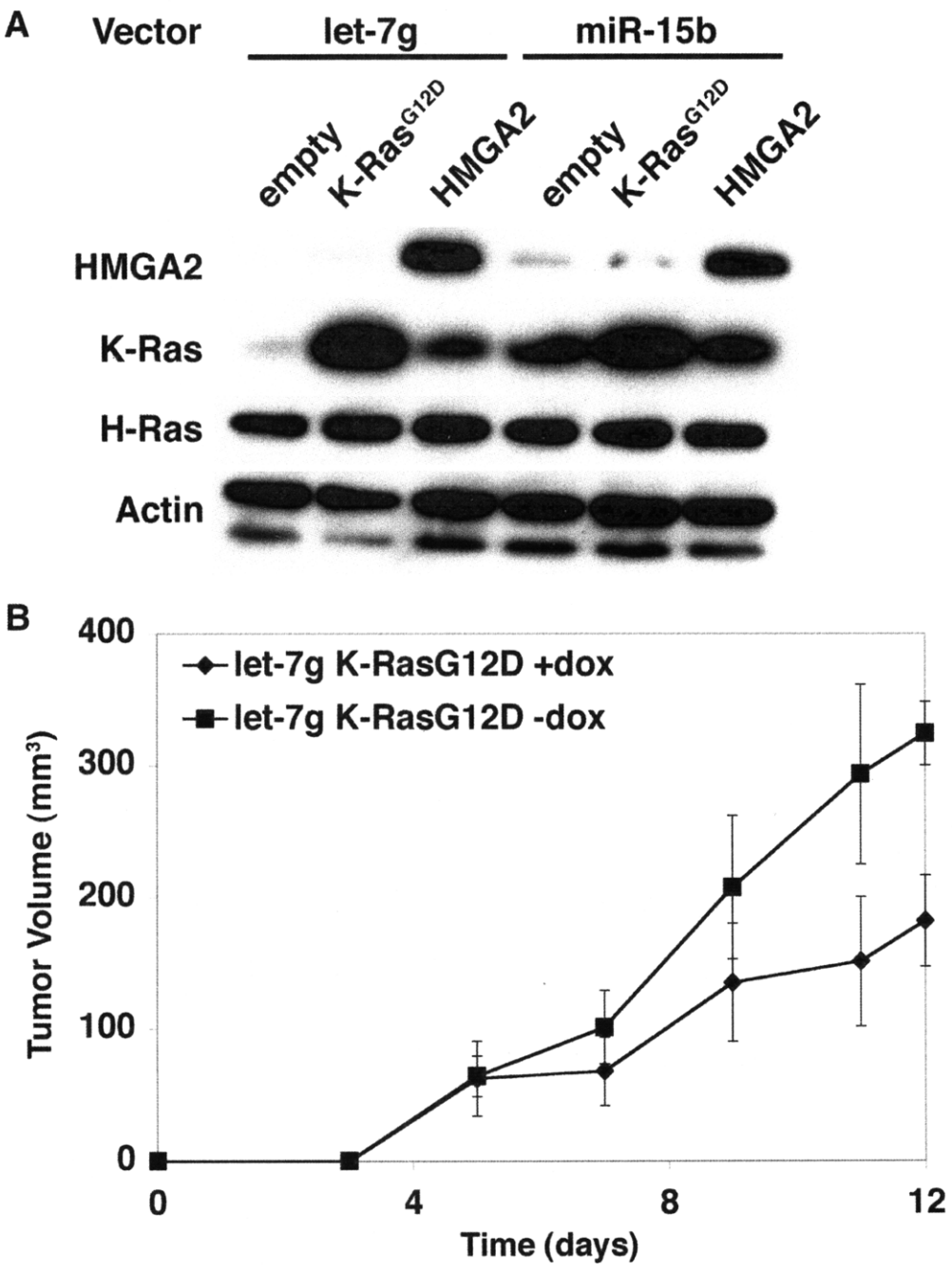
A
Vector
let-7g
miR-15b
empty
K-RasG12D
HMGA2
empty
K-RasG12D
HMGA2
HMGA2
K-Ras
H-Ras
Actin
B
let-7g K-RasG12D +dox
let-7g K-RasG12D -dox
Tumor Volume (mm3)
400
300
200
100
0
0
4
8
12
Time (days)

Figure 4.10 continued

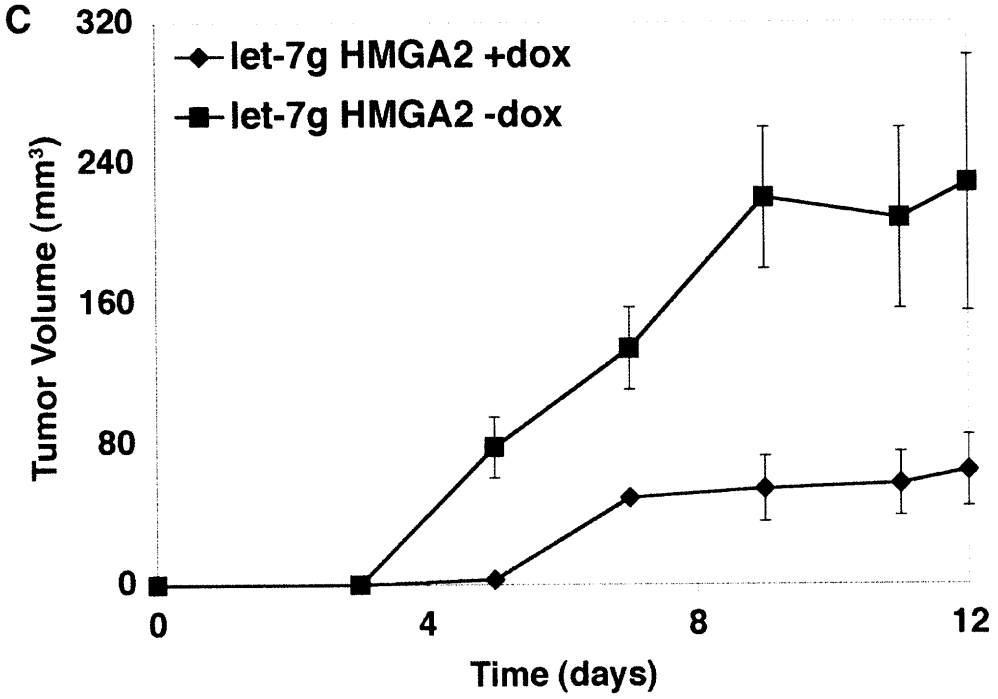

Figure 4.10 K-Ras^{G12D} substantially rescues let-7g mediated tumor suppression. (A) LKR13-Tet-On-KRAB-TE-miR-15b and -let-7g cells were infected with pBabe.Zeo.empty, K-Ras^{G12D}, and HMGA2 and Western blotting was performed. (B-C) LKR13-Tet-On-KRAB-TE-let-7g cells infected with pBabe.Zeo.K-Ras^{G12D} (B) or HMGA2 (C) were prepared and injected (10^6 cells/injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean +/- s.e.m. (n=6).

Figure 4.11

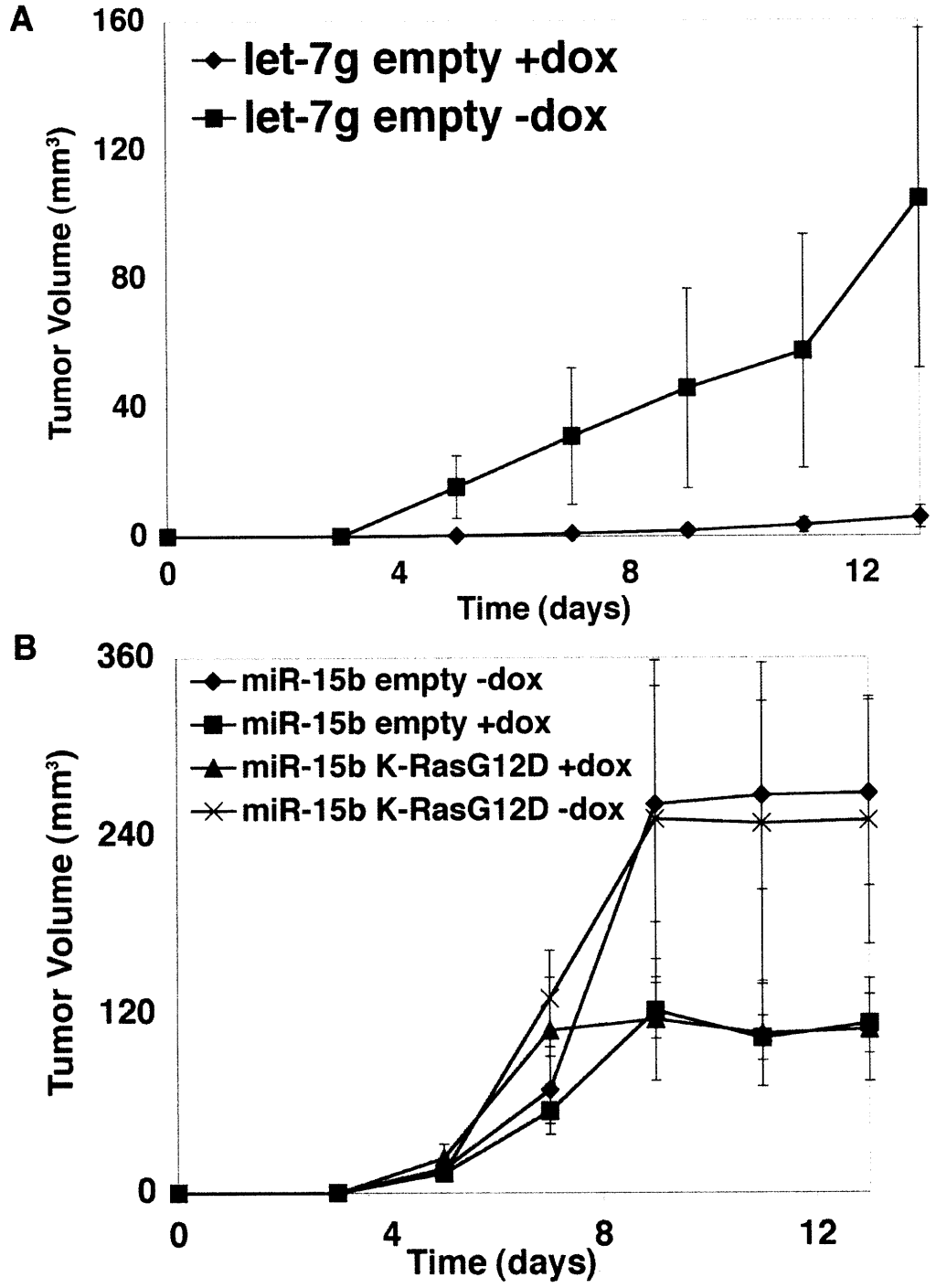
A
160
120
80
40
0
Tumor Volume (mm³)
let-7g empty +dox
let-7g empty -dox
0
4
8
12
Time (days)
B
360
240
120
0
Tumor Volume (mm³)
miR-15b empty -dox
miR-15b empty +dox
miR-15b K-RasG12D +dox
miR-15b K-RasG12D -dox
0
4
8
12
Time (days)

Figure 4.11 continued

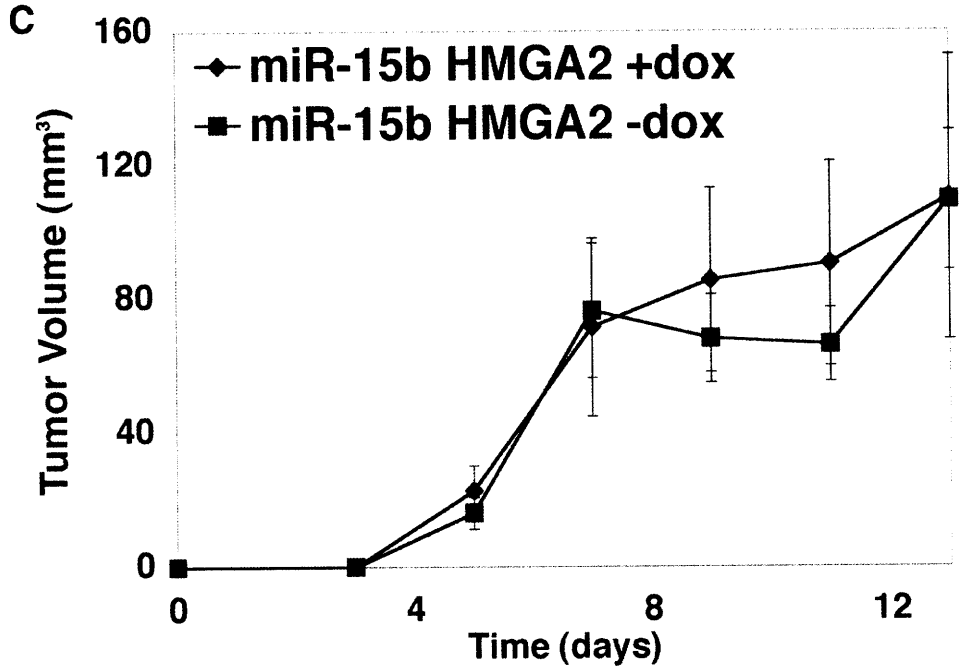

Figure 4.11 K-Ras^{G12D} does not alter miR-15b mediated tumor growth. (A) LKR13-Tet-On-KRAB-TE-let-7g cells infected with pBabe.Zeo.empty were prepared and injected (10^6 cells/injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean +/- s.e.m. (n=6). (B-C) LKR13-Tet-On-KRAB-TE-miR-15b cells were infected with pBabe.Zeo.empty (B), K-Ras^{G12D} (B), or HMGA2 (C). Cells were prepared and injected (10^6 cells/injection) into immune-compromised mice. Mice were treated and tumors were measured as above. Values are mean +/- s.e.m. (n=6).

C). Overall, these results suggest that mutant K-Ras plays a key role in let-7g—mediated tumor suppression. However, the rescue of tumor growth was incomplete, suggesting that other let-7 targets, likely including HMGA2, are also relevant for let-7g—mediated tumor suppression.

Let-7g Suppresses Tumor Initiation in an Autochthonous NSCLC Model. We next examined the tumor suppressive effect of let-7g in naturally arising lung tumors in the mouse. When infected with Cre-expressing virus in the lung epithelium, $Kras^{LSL\text{-}G12D}$; $Trp53^{flox/flox}$ mice develop highly aggressive NSCLC following a well-defined time course (Jackson et al., 2005). These lesions recapitulate advanced human lung adenocarcinoma in many ways, including stromal desmoplasia. To deliver let-7g to the lung epithelium, we generated a lentiviral vector co-expressing let-7g or a seed-mutant version of let-7g (let-7g sm) with Cre using a dual-expression system (Figure 4.12A). This vector generated substantial expression of let-7g and let-7g sm relative to controls in cultured cells (Figure 4.12B). In addition, let-7g expressed from this vector caused robust repression of a reporter through the K-Ras 3' UTR, which was not observed with the seed mutant of let-7g (Figure 4.13).

$Kras^{LSL\text{-}G12D}$; $Trp53^{flox/flox}$ mice were infected with let-7g- and let-7g sm-containing lentiviruses, and effects on tumor development were determined by visual inspection and histological analysis. We observed a significant reduction in both lung tumor number and tumor area following infection with the vector expressing let-7g versus controls (Figure 4.12C and D). In addition, there was a slight decrease in average tumor size in let-7g expressing mice (Figure 4.12E). The levels of let-7g and let-7g sm expression in these tumors were determined by Northern blotting. Consistent with findings in xenograft tumors, there was sustained

Figure 4.12

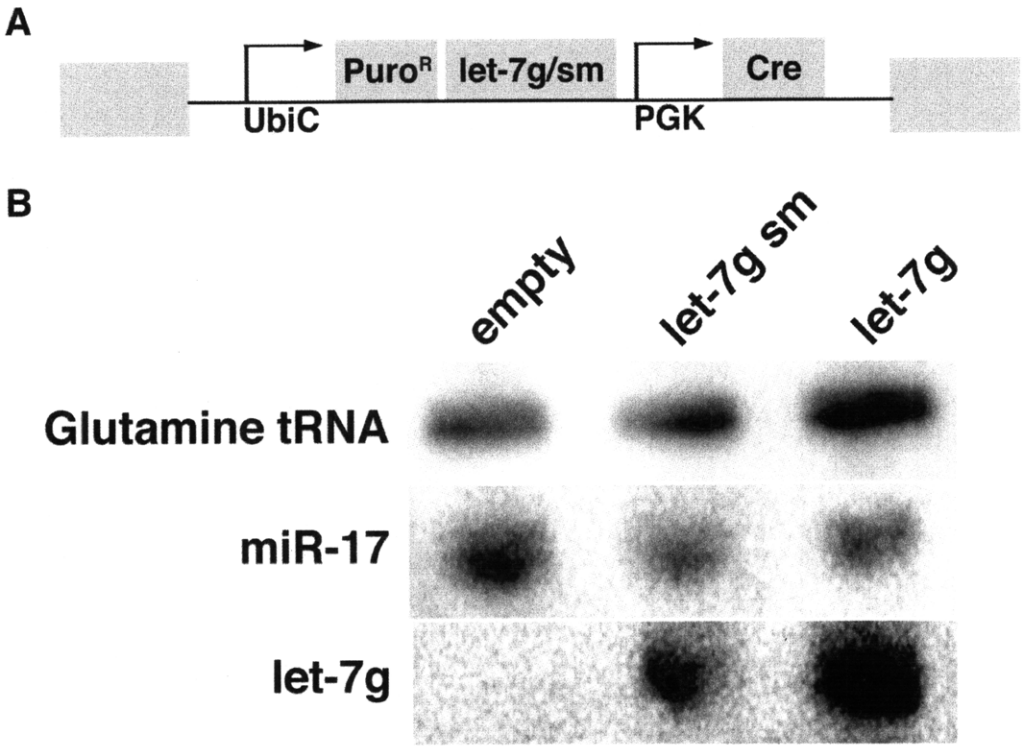
A
Puro^R
let-7g/sm
Cre
UbiC
PGK
B
empty
let-7g sm
let-7g
Glutamine tRNA
miR-17
let-7g
C
60
40
20
0
Tumor Number
*
empty
let-7g
let-7g sm

Figure 4.12 continued

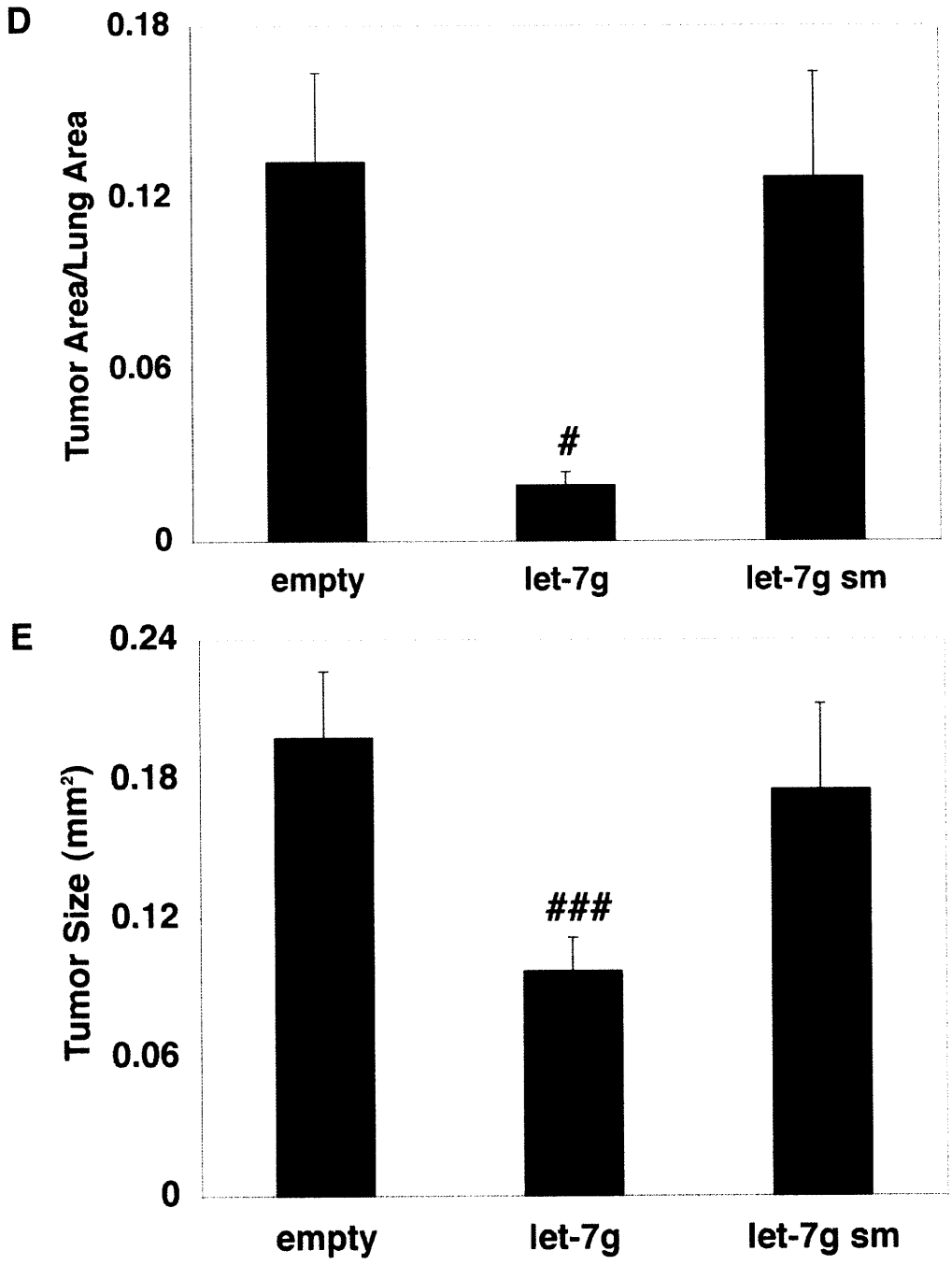

Figure 4.12 continued

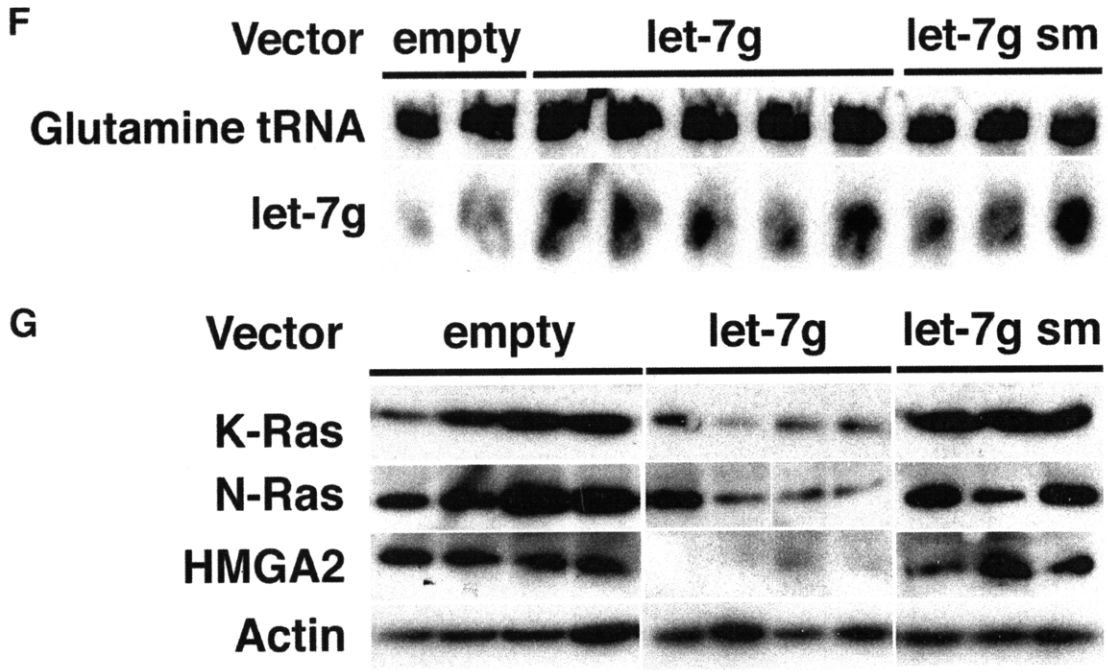

Figure 4.12 Let-7g suppresses tumor initiation in an autochthonous NSCLC model. (A) Diagram of the Puro.Cre lentiviral vector for co-expression of let-7g/let-7g sm with Cre recombinase. (B) Small RNA Northern blotting was performed against let-7g (both wild type and seed mutant), miR-17 and Glutamine tRNA in HEK293 cells infected with Puro.Cre (empty), Puro.let7gsm.Cre (let-7g sm), and Puro.let7g.Cre (let-7g). (C-E) *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice were intratracheally infected with the Puro.Cre lentiviral vectors described above. Twelve weeks post-infection, animals were sacrificed and tumor number (C), tumor and lung area (D), and tumor size (E) were quantified with Bioquant software. Values are mean +/- s.e.m. (n=9 for empty, n=11 for let-7g, and n=11 for let-7g sm). *: $p<0.0005$, #: $p<0.01$; ###: $p<0.1$. (F) Small RNA Northern blotting was performed against let-7g (both wild type and seed mutant) and Glutamine tRNA on lung tumors generated from *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice infected with Puro.Cre (empty), Puro.let7g.Cre (let-7g), and Puro.let7gsm.Cre (let-7g sm). (G) Western blotting was performed on lung tumors generated from *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice infected with Puro.Cre (empty), Puro.let7g.Cre (let-7g), and Puro.let7gsm.Cre (let-7g sm).

Figure 4.13

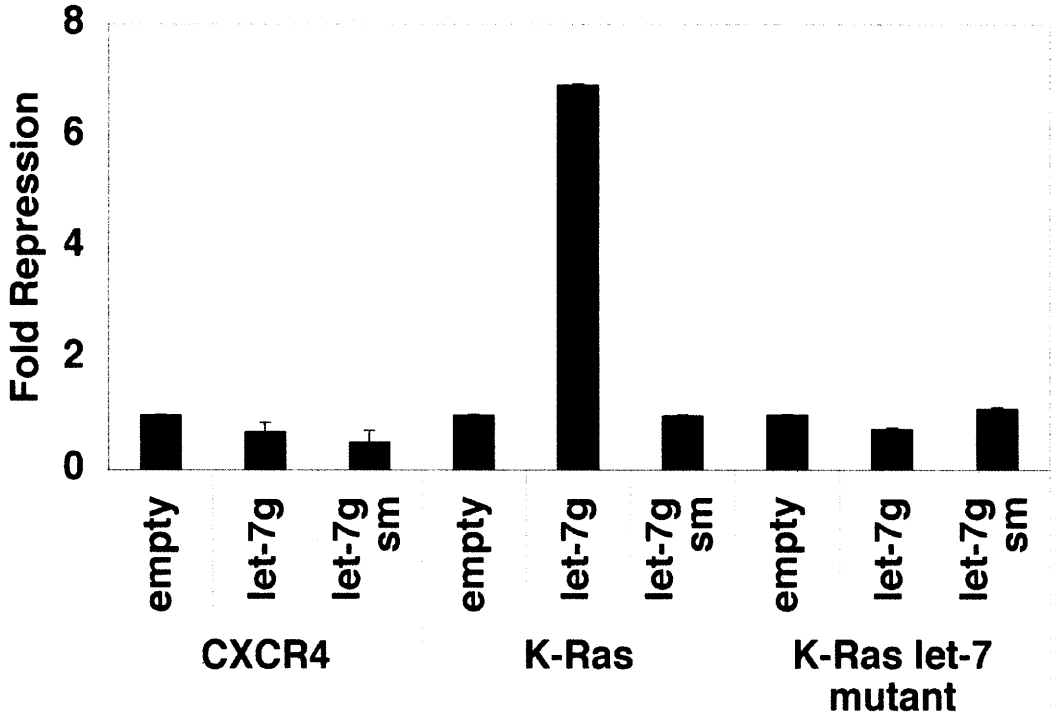
Fold Repression
8
6
4
2
0
empty
let-7g
let-7g sm
empty
let-7g
let-7g sm
empty
let-7g
let-7g sm
CXCR4
K-Ras
K-Ras let-7 mutant

Figure 4.13 Let-7g-expressing lentiviral vectors functionally repress the K-Ras 3' UTR. Luciferase assays were performed in HEK293 cells transfected with Puro.Cre (empty), Puro.let7g.Cre (let-7g), and Puro.let7gsm.Cre (let-7g sm) and pRL-TK containing bulged siCXCR4 binding sites (CXCR4), a wild-type murine K-Ras 3' UTR (K-Ras), or a murine K-Ras 3' UTR with mutations in the let-7 binding site (K-Ras let-7 mutant) (Kumar et al., 2007). Firefly luciferase (pGL3) was used as a transfection control. Expression was normalized to pGL3 levels and subsequently normalized relative to CXCR4 and empty expression. Values are mean +/- s.d. (n=6) with propagated error.

overexpression of let-7g and let-7g sm in tumors from mice infected with the corresponding lentiviruses (Figure 4.12F). Moreover, these let-7g overexpressing tumors have reduced protein levels of N-Ras, K-Ras, and HMGA2, indicating functional repression of known let-7 targets (Figure 4.12G). In total, these findings suggest that let-7g effectively suppresses tumor initiation in an autochthonous mouse model of mutant K-Ras—driven NSCLC. However, the tumors that do form continue to express let-7g and suppress known targets, supporting the conclusion that naturally arising tumors may gain resistance to let-7g.

Discussion

In this study we investigated the functional consequence of let-7g expression on non-small cell lung tumorigenesis. Utilizing both inducible and constitutive expression systems, we observed substantial tumor suppression by let-7g both in xenografts and in a mouse lung tumor model. Two lines of evidence suggest that let-7g—mediated tumor suppression is representative of the let-7 family. First, transfection of other let-7 family members caused comparable induction of cell death to let-7g (data not shown). Second, transfection of a miRNA sponge (Ebert et al., 2007) targeting the entire let-7 family shifted the cell cycle distribution opposite to let-7g overexpression, with a significant reduction of G_0/G_1-phase cells and a corresponding increase in S- and G_2/M-phase cells (Figure 4.1C).

Previous studies of the effect of let-7 on cellular proliferation have used cells containing activating mutations in K-Ras (A549) and N-Ras (H1299 and HepG2) (Johnson et al., 2007; Lee and Dutta, 2007). As both K-Ras and N-Ras are previously characterized targets of the let-7 family, it was possible that the effects of let-7 on proliferation in these cell lines were due largely

to downregulation of the Ras family. In the present study, we determined that K-Ras^{G12D}-mediated rescue of tumor growth was not complete, suggesting that other let-7 targets are also relevant to tumor suppression. In sum, these findings suggest that let-7-mediated tumor suppression occurs largely, though not completely, through regulation of the Ras family.

While let-7g expression potently suppressed non-small cell lung tumorigenesis, tumors inevitably formed in the presence of sustained let-7g induction. Notably, these tumors continued to express let-7g and actively repressed let-7 targets, suggesting the tumors that form do not propagate because of silencing of the let-7g vector. Moreover, this continued let-7g expression occurred both in xenograft models and autochthonous lung tumors expressing let-7g. This apparent resistance to let-7 was not observed in previous studies (Johnson et al., 2007; Lee and Dutta, 2007), as they relied upon transient delivery of let-7 family members. This distinction highlights the importance of stable induction of let-7 when analyzing its role in tumorigenesis, since transient expression of let-7 does not recapitulate the long-term effects of let-7 on tumorigenesis *in vivo*.

While we cannot exclude the possibility that the let-7g target repression observed here was insufficient to suppress tumorigenesis, the apparent resistance to let-7g expression has significant implications for the use of let-7 miRNAs as a therapeutic agent. Our data indicate that sustained let-7 delivery might lead to initial suppression of tumor growth but that let-7—resistant tumors might eventually emerge. Using the systems described here, one could probe downstream pathways from let-7 targets, including Ras, HMGA2, and others, to assess their roles in let-7—resistant tumors. Additionally, expression analysis of sensitive and resistant tumors might reveal novel pathways functionally related to let-7 resistance.

Here we describe one of the first cases of a miRNA family functioning as a tumor suppressor *in vivo*. Our findings make clinically relevant predictions related to the use of let-7—based therapeutic agents in NSCLC. The systems outlined in this study provide insight into let-7—mediated tumor suppression and also establish unique tools for understanding the basis for resistance of cancer cells to miRNA-mediated control of tumorigenesis. Moreover, the doxycycline-based and lentiviral systems described could be applied to examine other small RNAs suggested to function in tumorigenesis.

Materials and Methods

Cell Culture. Human cell lines (HEK293, A549, Calu-1, and H1650) were originally obtained from ATCC. 3TZ cells were described previously (Psarras et al., 2004). LKR13 cells have been described previously (Wislez et al., 2006). Cells were grown under standard conditions. After introduction of pTE vectors, cells were maintained under standard conditions in Tet-Free Serum per manufacturer's instructions (Clontech).

Cell Cycle and Cell Death Analysis. MicroRNA duplex sequences were transfected in triplicate into LKR13 cells with DharmaFECT-1 (Dharmacon) according to manufacturer's protocol. Cells were harvested after 48 and 72 hours and either fixed in methanol and stained with 7-AAD (Stem-Kit reagent, Beckman Coulter, Miami, FL) or stained using the Apoptest^{TM}-FITC kit (Nexins Research, Kattendijke, Netherlands) and analyzed by flow cytometry.

Lentivirus Production and Infection. Lentivirus production was performed as described (Rubinson et al., 2003).

Allograft/Xenograft Studies. Tet-On-KRAB-TE cells were treated for 24 hours with doxycycline (5 μg/mL) and sorted by flow cytometry. BalbC/Nu males (Taconic) were injected with cells as described (Sage et al., 2000). Two days after injection, mice were treated with drinking water containing doxycycline (2 mg/mL) and sucrose (4% w/v). Tumor sizes were measured every two days. After indicated days, mice were sacrificed and tumors were isolated for histology, Western and Northern blot analysis.

Intervention Studies. Mice were injected with LKR13-Tet-On-KRAB-TE cells and monitored for tumors. Once tumors were greater that 2 mm in diameter, mice were intraperitoneally injected with doxycycline (40 mg/kg) and tumors were measured as above.

Secondary Transplant Studies. Mice were injected with LKR13-Tet-On-KRAB-TE cells and treated with doxycycline in the drinking water as above. Tumors were then explanted, re-treated for 24 hours with doxycycline (5 μg/mL), and sorted by flow cytometry. Cells were then injected and mice were treated with doxycycline in the drinking water as above. Tumor sizes were measured every two days. After indicated days, mice were sacrificed and tumors were isolated for Northern blot analysis.

Genetically Engineered Mice. *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice were generated as described previously (Jackson et al., 2005).

Intratracheal Infection and Tumor Analysis. *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{flox/flox}$ mice were infected intratracheally with Puro.Cre lentivirus essentially as described (Murphy et al., 2006). Tumor analysis was performed as described (Kumar et al., 2007).

Cell-Cycle and Cell Death Analysis. MicroRNA duplex sequences were designed for mmu-let-7g (miRBase); the siGLO Cyclophilin B siRNA; and the miRIDIAN mimic control microRNA #1 (Dharmacon, Perbio Science, Erembodegem, Belgium).

Sponge treatment and Cell-Cycle Analysis. One day after plating in 24-well dishes, LKR-13 cells were transfected in triplicate with MSCV-puro empty vector, miR-16 sponge, or let-7 sponge using Lipofectamine2000. The next day the samples were enriched for transfected cells by treatment with 5 μg/ml puromycin for 32 hours. The samples were placed in DMEM without puromycin for 16 hours and harvested for fixation with Triton X-100 and staining with propidium iodide at 72 hours post-transfection. Stained samples were analysed by flow cytometry. Cell cycle profiles were analysed with ModFit software. The experiment was performed three independent times.

Western Blotting. Tumor cell extracts were fractionated on 4%–20% SDS-polyacrylamide gradient gels (Biorad) and transferred to Hybond-C membranes (Amersham). Membranes were blocked with 5% milk in PBS and then incubated with the indicated antibody. Antibodies K-Ras (sc30), N-Ras (sc31) from Santa Cruz Biotechnology, Pan-Ras (Upstate), HMGA2 (kind gift of Dr. M. Narita), and β-Actin (A5441) from Sigma were detected using HRP-conjugated antisera (Amersham) and chemiluminescence.

Northern Blotting. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Samples containing 20 μg total RNA and diluted in Gel loading buffer II (Ambion, Applied Biosystems, Austin, TX) were loaded on a 15% denaturing polyacrylamide gel (National Diagnostics) and separated at 35 Watt for 2 h. RNA was semi-dry blotted using Brightstar Plus positively charged nylon membranes (Ambion, Applied Biosystems, Austin, TX) at 350 mA for 2 hours. The blots were UV cross-linked and hybridized with 5'-end γ-radiolabeled antisense probes to let-7g (5'-ACTGTACAAACTACTACCTCA-3'), let-7g sm (5'-ACTGTACAAACTACTAGGTCA-3'), miR-15b (5'-TGTAAACCATGA-TGTGCTGCTA-3'), miR-17 (5'-CTACCTGCACTGTAAGCACTTTG-3'), and Glutamine

tRNA (5'-TGGAGGTTCCACCGAGAT-3') at 37°C in ULTRAhyb®-Oligohybridization buffer (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer's protocol. All Northern blots involving let-7g with Puro.Cre lentiviruses used let-7g sm probe mixed equally with let-7g probe. Signals were visualized on a Phospho-Imager.

Lentivirus Production and Infection. Lentiviral pTE-empty, pTE-miR-15b, pTE-let-7g, Puro.Cre, Puro.let-7g.Cre, and Puro.let-7gsm.Cre vectors were co-transfected with packaging vectors into HEK293 cells with Fugene-6 transfection reagent (Roche Applied Sciences, Indianapolis, IN). Virus was concentrated as described (Kissler et al., 2006). For pTE vectors, virus was diluted in media containing polybrene (10 μg/mL) and added to Tet-On-KRAB cells. Tet-On-KRAB cells were selected on puromycin to generate Tet-On-KRAB-TE-empty, Tet-On-KRAB-TE-miR-15b, and Tet-On-KRAB-TE-let-7g cells. For Puro.Cre vectors, virus was resuspended in HBSS and titrated by infection onto 3TZ cells and subsequent LacZ staining as described previously (Psarras et al., 2004). Puro.Cre viral titers were normalized before intratracheal delivery.

Luciferase Assays. Luciferase assays were performed as described previously (Doench et al., 2003).

Tet-On-KRAB Cells. Retrovirus was produced as previously described (Cherry et al., 2000). LKR13, A549, Calu-1, and H1650 cells were infected with pRevTet-On (BD Biosciences Clontech, Carlsbad, CA) virus and selected on 500 μg/mL G418 to generate Tet-On cells. Tet-On cells were infected with pBABE-CMVtetRKRAB virus and selected on 100μg/mL hygromycin to generate Tet-On-KRAB cells.

Tet-On-KRAB-TE-Zeo Cells. pBabe.Zeo.empty, K-Ras^{G12D}, and HMGA2 retroviruses were produced and infected into LKR13-Tet-On-KRAB-TE-miR-15b and -let-7g cells and selected on 500μg/mL zeocin to generate Tet-On-KRAB-TE-Zeo cells.

Construct Design

Sponge vectors. Sponge MicroRNA binding sites for miR-16 or let-7 were cloned into MSCV-puro with XhoI and HpaI as described previously (Ebert et al., 2007). The miR-16 sponge contains nine bulged sites of sequence CGCCAAUAUUCUAUGCUGCUA; the let-7 family sponge contains six bulged sites with alternating sequences AACUAUACAAGGACUACCUCA and AACUAUACAAUGACUACCUCA.

pBabe-CMVtetRKRAB. The CMVtetRKRAB vector (Deuschle et al., 1995) was cut with EcoRI and HpaI and the insert fragment was blunted by Klenow and cloned in the SnabI-site of pBabe-Hygro generating pBabe-CMVtetRKRAB.

pTE-miR. PGK-PURO was amplified from pSM2C (Paddison et al., 2004) with pgkpuro-f (5'-AGCGGCCGCAATTCTACCGGGTAGGGGA-3') and pgkpuro-r (5'-ACAATTGTCAGGCACCGGGCTTGC-3'), cut with NotI and MfeI and cloned in the NotI-MfeI fragment of the PUV1 lentiviral vector (courtesy of Dr. P. Stern). EGFP was amplified with EGFPSalIkozak: 5'-AGTCGACGCCACCATGGTGAGCAAGGGCGAGGA-3' and EGFPBXM-linker: 5'-TTCAATTGTGTGCTCGAGTGTGGGATCCTTACTTGTACAGCTCG-TCCATGCCG-3' and cloned in pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). PCR2.1-TOPO-EGFP was cut with EcoRV and SpeI, and the EGFP-fragment was cloned in SmaI-NheI sites of pTRE-tight (BD Biosciences Clontech, Carlsbad, CA). pTRE-tight-EGFP was cut with SSP1, blunted by Klenow and digested with NotI. This fragment was cloned in the blunted BamHI-site and NotI sites of PUV1-Puro resulting in pTE-EGFP. MicroRNA sequences were

amplified with Let-7gF (5'-CACACTCGAGCAATTCTCCAAATATGGTAAAGATGAGGC-3'), Let-7gR (5'-CACAGAATTCAAACGGTTTATCTGAACAACTCCAAGC-3') and miR-15bF (5'-CACACTCGAGGGAGATGATTACGAAGTCCTTCCTAACA-3'), miR-15bR (5'-CACAGA-ATTCCCCTGTCACACTAAAGCAGCACAAT-3'), cut with EcoRI and XhoI and cloned in equivalent sites of pTE-EGFP.

pBabe.Zeo.K-Ras^{G12D}/HMGA2. The ORF of murine K-Ras4B^{G12D} was amplified from a luciferase fusion expression vector (courtesy of P. Sandy) using K-RasF (5'-GACGGATCCATGACTGAGTATAAACTTGTG-3') and K-RasR (5'-GAAGAATTCTCACATAACTGTACACCTTGTCC-3'). The ORF of human HMGA2 was amplified from a pcDNA expression vector provided by C. Mayr (Mayr et al., 2007) using HMGA2F (5'-TAAGGATCCATGAGCGCACGCGGTGAG-3') and HMGA2R (5'-GCGGAATTCCTAATCCTCCTCTGCGGACTCTTG-3'). Both products were cut with BamHI and EcoRI and cloned into pBabe.Zeo (Addgene).

Puro.Cre. Puro.Cre was initially generated by amplification of Puro^{R} from MSCV.Puro (Clontech) with PuroF (5'-CGCTGCGCCGAATTCATGACCGAGTACAAGCCCACG-3') and PuroR (5'-TAAGCGGCCGCTCGAGTCAGGCACCGGGCTTGCGGGTC-3'), digestion with EcoRI and NotI and cloning into UbC.Luciferase.PGK.Cre (courtesy of M. Dupage) to generate Puro.Cre. Let-7g was amplified from murine genomic DNA with purolet7gF (5'-GTGGCGCTCGAGAATATATATAAATGACTGGTGTATTTC-3') and purolet7gR (5'-TAGCGGCCGCGAACCCTTCTATATATTCTG-3'), digested with XhoI and NotI and cloned into Puro.Cre to generate Puro.let7g.Cre. Puro.let7gsm.Cre was generated by two rounds of site-directed mutagenesis of Puro.let7g.Cre with mut1F (5'-GATTCCAGGCTGACCTAGTAGTTTGTACAGTTTGAGGGTCTATGATAC-3'), mut1R (5'-

GTATCATAGACCCTCAAACTGTACAAACTACTAGGTCAGCCTGGAATC-3'), mut2F (5'-CAGGAGATAACTGTACAGGCCACTGGGTTGCCAGGAAC-3'), and mut2R (5'-GTTCCTGGCAACCCAGTGGCCTGTACAGTTATCTCCTG-3').

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CHAPTER 5: Discussion

Overview

Here I have described a series of observations on the widespread and specific role of microRNAs (miRNAs) in cancer. First, we found that the global loss of miRNAs in mammalian cancers is not merely a consequence of altered differentiation, but that this global loss of miRNAs functionally enhances cellular transformation and tumorigenesis. Second, one putative mechanism by which this loss of miRNAs occurs is incomplete loss of *Dicer1*, as it functions as a haploinsufficient tumor suppressor in mammalian cancers. Finally, a specific miRNA family called let-7 plays an important role in the development of non-small cell lung cancer by suppressing a wide collection of mRNA targets, including K-Ras and HMGA2. Overall, these data suggest that miRNAs have the potent capacity to inhibit tumorigenesis, and that individual miRNA families can act specific tumor suppressors via suppression of discrete sets of oncogenes. Moreover, they suggest that enhancing either the miRNA processing pathway or ectopic expression of known tumor suppressive miRNAs might provide a novel therapeutic avenue.

Global loss of miRNAs in cancer

Using a bead-based miRNA profiling strategy, it was shown that, when compared to normal tissues, the steady-state levels of many miRNAs were reduced in human cancers (Lu et al., 2005). Moreover, this global down-regulation of miRNAs in tumors also occurred in a mouse model of mutant K-Ras—driven lung cancer, suggesting that this effect was generally relevant to mammalian cancer. Initially, this widespread loss of miRNAs was believed to be a mere

consequence of altered differentiation in cancer cells. This possibility was based upon changes in miRNA abundance being reversible in response to differentiation *in vitro*. Notably, this reversibility suggests that the loss of miRNAs could not be due to a complete loss of miRNA biogenesis in tumors, as this miRNA production could be induced. In total, these findings led to the idea that the loss of miRNAs in cancer was only an effect of transformation and not functionally important for the disease.

In Chapter 2, I have shown that the global loss of miRNAs does play a functionally relevant role in oncogenesis, as impaired processing of miRNAs enhanced cellular transformation and tumorigenesis (Kumar et al., 2007). This was a general phenomenon of mammalian cancers, as it occurred in both mouse and human cancer cells exhibiting a variety of genetic changes. Moreover, it appeared to be an effect of the miRNA processing pathway and not simply individual genes, as depletion of three distinct components of the pathway (Dicer1, Drosha, and DGCR8) produced comparable effects on transformation. Notably, impaired processing of miRNAs only cooperated with cells that were fully transformed; partially transformed cells received no benefit from impaired miRNA processing. In fact, untransformed cells actually exhibited proliferative defects in response to inhibited miRNA maturation. This likely explains why germline heterozygous mutations of miRNA processing components are neither tumor prone in animal models nor observed in human cancer predisposition syndromes, since mutation of these components as an initiating event would likely be deleterious to tumors.

The enhanced transformation seen with impaired miRNA maturation led to overexpression of several oncogenes, including K-Ras and c-Myc, known to be regulated by miRNAs like let-7. While several miRNAs were shown to be specifically down-regulated in cells depleted for processing, it was unclear which miRNAs contribute to the tumor suppressive effect

observed. Most likely, the combined loss of multiple miRNA families plays a role in the enhanced tumorigenesis observed, complicating traditional loss of function genetic analyses.

Beyond this, it was found that inhibiting miRNA biogenesis caused an increased tumor burden in an autochthonous model of K-Ras driven lung cancer, as $Kras^{LSL\text{-}G12D}$ mice either heterozygous or homozygous conditional for *Dicer1* had an elevated tumor area as a fraction of total lung compared to $Kras^{LSL\text{-}G12D}$ mice wild type for *Dicer1*. This increased tumor burden was largely due to increases in tumor number in *Dicer1* mutant animals, suggesting that *Dicer1* mutation, when combined with known oncogenes, promotes tumor initiation. Notably, while *Dicer1* mutant animals had a modest increase in average tumor grade, this was likely due to the increased number of lung tumors. Overall, these data provide important support for the role of miRNA processing in suppressing tumor development in naturally arising tumors.

One concern about these findings is the series of observed miRNAs which appear to function as oncogenes (He et al., 2005; Ma et al., 2007; Tam et al., 2002; Voorhoeve et al., 2006). The results presented do not conflict with these reports, as not all miRNAs exhibit comparable reductions with impaired processing. Moreover, none of the miRNAs described as oncogenes were significantly repressed with inhibition of miRNA processing. It is intriguing to speculate that oncogenic miRNAs might actually be up-regulated in tumors with impaired miRNA processing, as reduced miRNA biogenesis might lead to more efficient processing of oncogenic pre-miRNAs. However, the data presented in Chapter 3 suggests that there is not such up-regulation of these oncogenic miRNAs with impaired processing. Overall, these results indicate that, while some miRNAs likely function as oncogenes, the widespread loss of miRNAs in cancer is tumor suppressive.

Dicer1 haploinsufficiency

As described above, it was previously shown that the global loss of miRNAs in human cancer is not merely a consequence of tumorigenesis, but in fact functionally promotes oncogenic transformation. However, in Chapter 3, I examined survival in K-Ras—driven lung tumor-bearing mice either wild type, heterozygous, or homozygous conditional for *Dicer1* and made a surprising observation. While both heterozygous and homozygous *Dicer1* conditional mice exhibited reduced survival compared to wild type mice, animals heterozygous for conditional *Dicer1* had a further reduction in survival compared to homozygous conditional animals. This was notable, as traditional tumor suppressor genes work via inactivation of both gene copies, leading to complete loss of function. In this situation, it appeared that such complete loss of function was actually being selected against, since animals that should produce tumors with partial loss of *Dicer1* fared worse than those animals that should produce tumors with complete loss of *Dicer1*. In total, these results suggested that *Dicer1*, unlike traditional tumor suppressor genes, inhibited tumorigenesis via haploinsufficiency.

This haploinsufficiency was verified by genetic analysis of both lung tumor and sarcoma cell lines. In these cells, tumors from *Dicer1* conditional heterozygous animals failed to lose the wild type allele and tumors from *Dicer1* conditional homozygous animals underwent only partial deletion of *Dicer1*. Moreover, we found that heterozygosity was necessary for *Dicer1* to function as a tumor suppressor, since forced homozygous deletion of *Dicer1* was deleterious to tumor growth. Finally, the haploinsufficiency of *Dicer1* found in genetically engineered mouse models of cancer was in line with human cancer genome analyses, in which *DICER1* undergoes frequent

hemizygous deletion in a variety of tumor types but never undergoes homozygous loss. Thus, *DICER1* functions as a haploinsufficient tumor suppressor gene.

While hemizygous deletion of *DICER1* provides one mechanism by which miRNA processing is impaired during tumorigenesis, several other possible mechanisms exist. First, there might be comparable hemizygous deletions of additional members of the miRNA processing pathway. Unfortunately, the deletions seen for *DICER1* are usually extremely large (>1 megabase) and include several genes, making it difficult to ascribe functional importance to loss of an individual gene's deletion. One way to show the specific contribution of a gene to tumor suppression is evidence for specific mutations within the gene in cancers. This has been shown for both *DICER1* and *RNASEN* (the gene encoding Drosha) in a limited number of human ovarian cancers (Merritt et al., 2008). A more interesting example was seen in mismatch-repair deficient colon cancers, in which *TARBP2* (the gene encoding TRBP) frequently contains heterozygous frameshift mutations (Melo et al., 2009). These mutations create premature stop codons and cause a loss of TRBP function. Importantly, this loss of TRBP specifically promotes tumorigenesis via loss of miRNA processing caused by destabilization of the Dicer1 protein.

Beyond mutations, these components could undergo epigenetic changes within human tumors, such as silencing via DNA methylation. Furthermore, inhibition of many miRNAs can occur at the level of transcription. For example, the oncogenic transcription factor c-Myc was shown to inhibit a variety of miRNAs by silencing of the promoter (Chang et al., 2008). However, it must be noted that many miRNAs down-regulated in cancer do so without changes to the levels of their primary miRNA transcripts, indicating changes in miRNA processing or stability in human cancers (Thomson et al., 2006). Moreover, the miRNA processing pathway could be inhibited post-transcriptionally, such as the mutual regulation of Drosha and DGCR8

gene expression and the inhibition of Argonaute by prolyl hydroxylation (Han et al., 2009; Qi et al., 2008). In the future, robust resequencing and copy-number analysis of miRNA maturation pathway components will be necessary to establish firmly the mechanisms of global miRNA loss in human cancers; however, studies should also be done to look at alternative means of inhibiting miRNA processing in cancer as well.

In addition to understanding the mechanism of miRNA loss in human tumors, these studies suggest a novel therapeutic target in human cancer. Specifically, activators of miRNA processing have the potential to act as robust anti-cancer agents. In particular, since such compounds would only target cells with inhibited miRNA processing, which has been shown to occur in cancer cells relative to normal tissue, they would likely be well-tolerated and of limited toxicity. One limitation to such small molecule screens is that many compounds able to activate miRNA processing in a given cell type will do so only indirectly, via induction of differentiation; thus, screening in a variety of cell types will be necessary. With that noted, a recent study has discovered a small molecule that is able to activate the processing of small RNAs (Shan et al., 2008). In this study, they found that the compound enoxacin promotes both siRNA-mediated degradation of target mRNAs and endogenous miRNA maturation. Importantly, the compound works via interaction with TRBP, demonstrating activation of the miRNA processing pathway, and not simply cell type-specific differentiation. Overall, this compound indicates that it is possible to find chemical enhancers of miRNA processing. Future studies should both perform additional screening for small molecule miRNA processing activators and test these molecules in cancer cells for the ability to inhibit transformation.

Let-7 and non-small cell lung cancer

As one of the initially discovered miRNAs, *let-7* was shown to regulate proliferation of seam cells in the developing *C. elegans* embryo (Pasquinelli et al., 2000; Reinhart et al., 2000). When orthologs of *let-7* were discovered in mammals, it was posited that they could play an important role in cancer development. In support of this, several let-7 family members were shown to be down-regulated in a subset of non-small lung cancers, and it was shown that this reduced let-7 expression correlated with a worse prognosis (Takamizawa et al., 2004; Yanaihara et al., 2006). This notion was strengthened by results showing that let-7 regulates a variety of oncogenes, including the Ras family, HMGA2, c-Myc, and numerous cell-cycle regulators (Johnson et al., 2007; Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Sampson et al., 2007). However, there was a dearth of functional data showing that the let-7 family suppressed tumorigenesis. This is largely due to the nature of the *let-7* gene family in mammals, of which there are twelve different let-7 family members at eight different locations in the genome; this makes loss of function genetics practically impossible.

In Chapter 4 (with additional data in Appendix 1), I demonstrated that induced overexpression of one of the let-7 family members, let-7g, potently suppressed tumor growth in xenografts generated from several non-small cell lung cancer cell lines (Kumar et al., 2008). Similar results were obtained by others using siRNA mediated delivery of let-7 to non-small cell lung cancer cells (Esquela-Kerscher et al., 2008). In both studies, let-7 overexpression was additionally able to suppress tumorigenesis in an autochthonous mouse model of K-Ras driven lung cancer.

Notably, the findings in Chapter 4 indicate that let-7 mediated tumor suppression is significantly more robust in non-small lung cancer cells driven by oncogenic K-Ras, suggesting

the potential for genotype specificity. This idea is in line with the notion of oncogene addiction, in which tumors require the function of a driving oncogenic lesion not only for tumor development but for sustained tumor maintenance (Sharma and Settleman, 2007). In contrast, while expression of an oncogenic K-Ras unable to be suppressed by let-7 partially rescued tumor growth in response to let-7, it was not complete, and additional targets, like HMGA2, also provide an incomplete rescue. Overall, this indicates that while mutant K-Ras is necessary for potent let-7—mediated tumor suppression, it is not sufficient to bypass said tumor suppression.

Though the above studies indicate that let-7 overexpression can suppress tumorigenesis in xenografts and tumor initiation in a genetically engineered mouse model of lung cancer, it was not clear whether let-7 induction in established, naturally arising lung tumors would be able to suppress tumor growth. To address this, I used a lentiviral vector to induce let-7 with doxycycline in the above autochthonous lung cancer model (see Appendix 1). Using this system, I observed potent tumor suppression at all time points after tumor initiation. Surprisingly, there was a comparable degree of tumor suppression at all time points, but the form of tumor suppression depended on the time of induction. Overall, these studies indicate that let-7 can inhibit the growth of established lung cancers *in vivo*.

These findings provide significant evidence for let-7 functioning as a tumor suppressor in non-small cell lung cancer. However, several important questions remain. First, the precise mechanism by which let-7 becomes inhibited in cancer has not been determined. It has been shown that let-7 family members are frequently in sites of deletion in human cancer, suggesting the mechanism of DNA loss in tumorigenesis (Calin et al., 2004). In addition, c-Myc has been shown down-regulate several let-7 family members (Chang et al., 2008). Interestingly, this Myc-mediated repression of let-7 sets up a positive feedback loop, as let-7 has been shown to inhibit

c-Myc. Finally, a series of reports has shown that the let-7 family is regulated at the level of pre-miRNA processing by the Lin-28 family of oncogenic proteins (Heo et al., 2008; Newman et al., 2008; Viswanathan et al., 2008). In fact, recent data suggests that Myc-mediated repression of let-7 is in fact due to Myc-mediate up-regulation of Lin-28, which subsequently blocks let-7 processing (Chang et al., 2009). Taken together, these findings provide several ways by which let-7 can be inhibited during tumorigenesis.

Beyond the mechanism of let-7 loss, it will be important to examine the loss of function of let-7 in cancer models. As mentioned above, there are twelve let-7 family members within eight gene loci in mammals. Thus, traditional gene targeting approaches appear impractical towards the assessment of loss of function. An alternative approach to examining let-7 loss of function is that of miRNA sponges, in which a synthetic miRNA binding site is overexpressed in cells (Ebert et al., 2007). By binding endogenous miRNAs to the synthetic target, endogenous mRNAs are released from miRNA-mediated repression, recapitulating loss of miRNA function. Future studies should work towards development of let-7 family consensus sponges in order to examine loss of let-7 function in development and tumorigenesis.

While such studies will provide insights into the role of let-7 in tumor development, the described results make clear the potential for let-7 to be used as a therapeutic, as let-7 delivery to established tumors is clearly able to cause suppression of tumorigenesis. In this therapeutic context, miRNAs like the let-7 family have several distinct advantages. First, their synthesis is relatively standardized and is comparable for any potential miRNA. Second, unlike many small molecules, miRNAs have a clearly demonstrated mechanism of action. Third, unlike many small molecules, a given miRNA has a computationally predictable set of predicted targets, even if the

relevant targets may not be obvious. Finally, the capacity of a miRNA to suppress multiple targets makes resistance at the level of individual targets significantly less likely.

MiRNA therapeutics in general, and let-7 in particular, also suffer a set of disadvantages. First, there is the fundamental challenge of delivering small RNAs to certain tissues and tumors, which has not yet been resolved. Second, if targeted delivery of a miRNA to a tumor is impossible, it is not clear what the systemic consequence of miRNA overexpression will be in normal tissues. Finally, in Chapter 4 and Appendix 1, I have shown that while let-7 suppresses tumor growth, tumors do eventually form and that these tumors exhibit resistance to further let-7—mediated target suppression. Thus, there are both advantages and disadvantages to the development of miRNA therapeutics.

Future studies can address many of these disadvantages. First, technological and chemical development should aid our ability to deliver small RNAs, including miRNAs, to defined tissues and tumors. Second, the development of transgenic mice (ideally in a doxycycline-regulatable system) will allow one to examine the consequences of systemic induction of let-7 in tumor-bearing animals. Third, the use of existing and transgenic let-7 induction systems should ideally allow for a closer examination of the resistance to let-7 induction and the mechanisms which underlie it. Overall, such studies provide for the exciting possibility of translating our knowledge of miRNAs and cancer into novel therapeutic agents.

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APPENDIX 1: Induction of let-7g in established non-small cell lung cancer suppresses tumorigenesis

I contributed all figures presented here. Cindy Y. Chen assisted in the experiments presented in A1.1, A1.2B, and A1.3. Christine Chin assisted in the experiments presented in A1.2C.

Introduction

As a microRNA (miRNA) shown to regulate proliferation in *C. elegans*, orthologs of the let-7 miRNA family in mammals have been shown to be differentially expressed in lung cancers. In particular, several let-7 members have been shown to be down-regulated in a subset of non-small cell lung cancers (NSCLCs) and this down-regulation correlates with a poorer prognosis (Takamizawa et al., 2004; Yanaihara et al., 2006). Of note, let-7 has been shown to suppress a variety of oncogenes, including the Ras family, HMGA2, c-Myc, and numerous cell-cycle regulators (Johnson et al., 2007; Johnson et al., 2005; Lee and Dutta, 2007; Mayr et al., 2007; Sampson et al., 2007).

There was, however, a dearth of functional data demonstrating that the let-7 family functionally altered tumorigenesis. In two recent reports (one of which is described in detail in Chapter 4), it was shown that let-7, when overexpressed in lung cancer cell lines, potently suppressed tumor development (Esquela-Kerscher et al., 2008; Kumar et al., 2008). Furthermore, this suppression was more robust in lung cancer cells driven by oncogenic mutations in K-Ras, suggesting some degree of genotype specificity. However, oncogenic K-Ras expression was not sufficient to rescue cancer cells overexpressing let-7, indicating the role of multiple let-7 targets in tumor suppression. Finally, in two related genetically engineered mouse models of oncogenic K-Ras driven NSCLC, expression of let-7 family members in the lung potently inhibited tumor initiation. Taken together, these studies provided definitive evidence that the let-7 miRNA family could functionally suppress NSCLC development.

While these reports supported a role for let-7 in lung tumor development, the contribution of let-7 to inhibition of tumor initiation versus tumor maintenance was unclear. In particular, if

let-7 family members were to be developed as therapeutic agents, it would be necessary to establish a role for let-7 in suppressing the growth of established tumors. Moreover, genetically engineered mouse models of NSCLC provide a powerful tool for examining the effect of let-7 induction in established cancers.

Therefore, to induce let-7 specifically within established lung tumors, it would be necessary to generate a system for expression of a miRNA in a regulatable manner, such that let-7 can be induced at a defined time after tumors have initiated. Here we describe such a system and show that expression of let-7g inhibits tumor growth in both human NSCLC xenografts and in an autochthonous mouse model of NSCLC. Surprisingly, induction of let-7g at different times after tumor initiation caused a comparable reduction in tumor burden; this reduced tumor burden, however, is caused by distinct effects of let-7g on tumors at different time points. Overall, these results indicate that let-7 delivery to established lung tumors could serve as an effective therapeutic approach.

Results and Discussion

To examine the role of let-7 in tumor maintenance, we first applied the dox-regulatable miRNA system used previously (see Chapter 4 for details) within a human NSCLC cell line (Kumar et al., 2008). When these cells are both transplanted and induced to express either let-7g or miR-15b in tumor xenografts, we observed robust suppression of tumor growth. To measure the effect of these miRNAs in established tumors, we first transplanted the dox-regulatable cells and only induced the miRNA after tumors had formed. Upon let-7g expression, we observed a strong slowing of tumor growth relative to induction of miR-15b (Figure A1.1). However, while

Figure A1.1

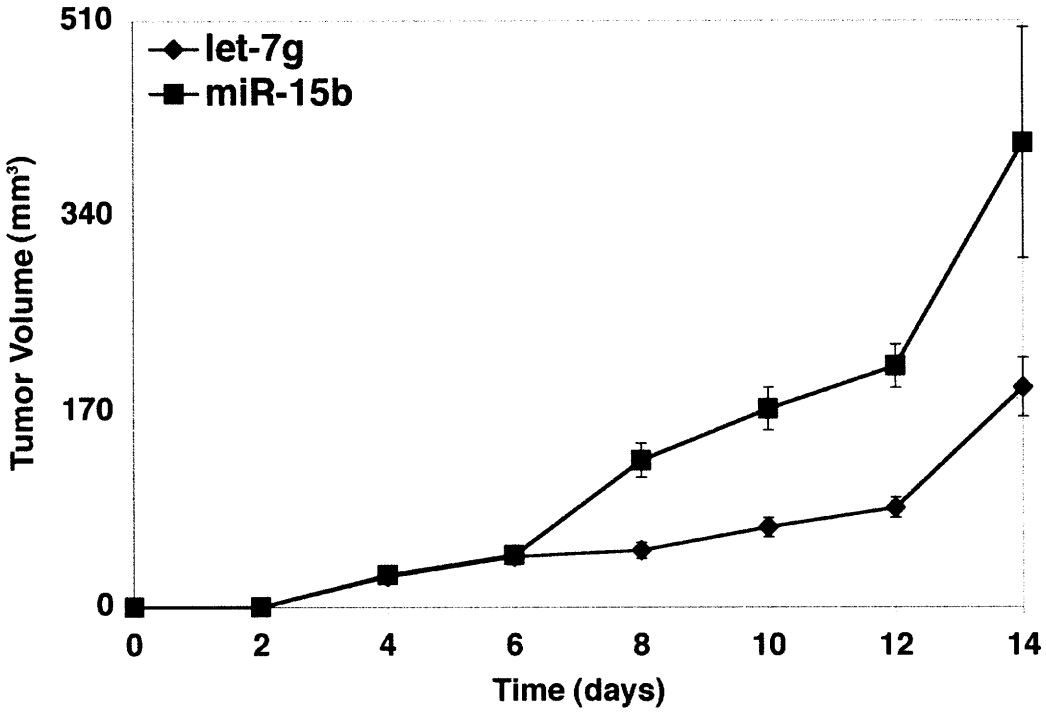
510
340
170
0
let-7g
miR-15b
Tumor Volume (mm3)
0
2
4
6
8
10
12
14
Time (days)

Figure A1.1. Let-7g expression in established tumors slows tumor growth without regression. Mice were injected with either A549-Tet-On-KRAB-TE-miR-15b or A549-Tet-On-KRAB-TE-let-7g cells (10^6 cells/injection) and monitored for tumors. Once tumors were greater that 2 mm in diameter, mice were intraperitoneally injected with PBS with and without doxycycline (40 mg/kg) and tumors were measured over time. Values are mean +/- s.e.m. (n=6).

tumor growth did slow with let-7g expression, these tumors did not exhibit regression. In total, these results suggest that let-7g expression can alter the growth of established lung tumors.

To examine further the role of let-7 in established lung tumors, we developed a bifunctional lentiviral vector (Figure A1.2A). In this system, Cre recombinase, which is essential for tumor development in *Kras*$^{LSL\text{-}G12D}$-driven cancers, is expressed from a constitutive promoter (Jackson et al., 2001). To express let-7 in an inducible manner, we placed both mCherry, a RFP analog, and let-7g behind a doxycycline-regulatable promoter similar to the one used in lung cancer cell lines (Kumar et al., 2008). To induce let-7g in the genetically engineered mouse model, we combined the *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{f/f}$ NSCLC model used previously with the *CCSP-rtTa* transgenic mouse, which can induce gene expression in response to doxycycline throughout the lung epithelium (Perl et al., 2002). These compound mutant animals will hereafter be referred to as "KPR" mice. Using this system, we were able to achieve potent and specific induction of let-7g in established lung tumors from animals treated with doxycycline (Figure A1.2B). Moreover, in lung tumors induced to express let-7g, there was substantial suppression of known let-7 targets like K-Ras, HMGA2, and c-Myc (Figure A1.2C). Thus, this system provides a useful means of inducing let-7g in lung tumors initiated by Cre activity.

Using this system, we devised an experimental scheme for induction of let-7g at different time points after tumor initiation. In this approach, we either never induced let-7g or induced let-7g four, eight, or twelve weeks after lentiviral infection of KPR mice. When we examined tumor burden in these same groups, we observed a robust decrease in tumor area as a fraction of lung area in all sets of induced animals (Figure A1.3A). Surprisingly, this decrease in total tumor area was similar in all sets of induced animals, suggesting that let-7—mediated tumor suppression was equally efficacious over all durations of induction. As we further examined tumor burden in

Figure A1.2

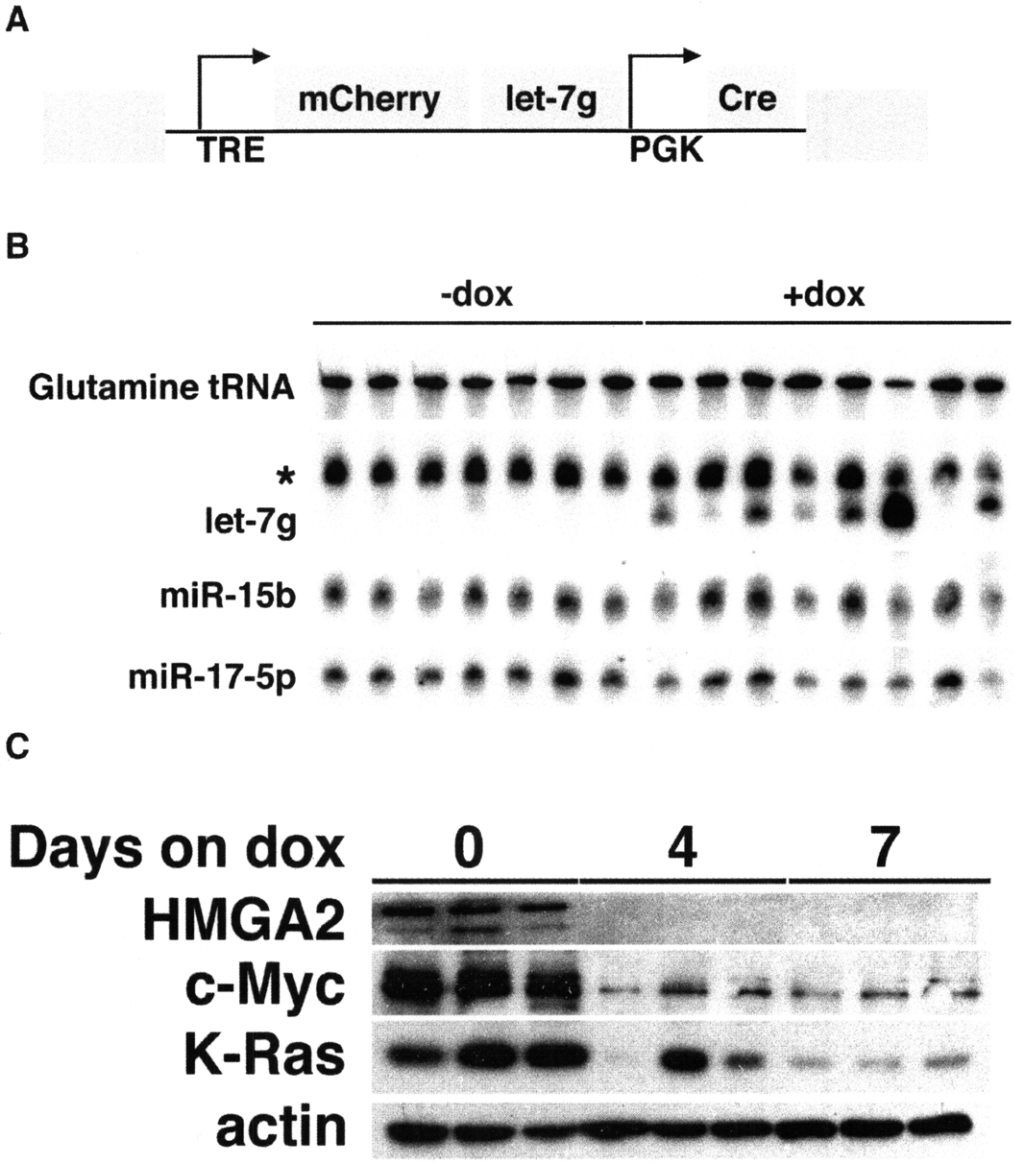
A
mCherry
let-7g
Cre
TRE
PGK
B
-dox
+dox
Glutamine tRNA
*
let-7g
miR-15b
miR-17-5p
C
Days on dox
0
4
7
HMGA2
c-Myc
K-Ras
actin

Figure A1.2. Generation of a bifunctional, doxycycline-inducible let-7g lentiviral vector. (A) Diagram of the lentiviral vector for doxycycline-regulatable expression of let-7g with constitutive expression of Cre recombinase. (B) KPR mice were infected with TRE.mCherry.let-7g.PGK.Cre. Twelve weeks post-infection, animals were given feed containing or lacking doxycycline for three days. Animals were sacrificed, tumors were collected and small RNA Northern blotting was performed against let-7g, miR-15b, miR-17-5p, and Glutamine tRNA. (C) KPR mice were infected with TRE.mCherry.let-7g.PGK.Cre. Twelve weeks post-infection, mice were given feed containing doxycycline for either zero, four, or seven days. Animals were sacrificed, tumors were collected, and Western blotting was performed against K-Ras, HMGA2, c-Myc, and actin.

Figure A1.3

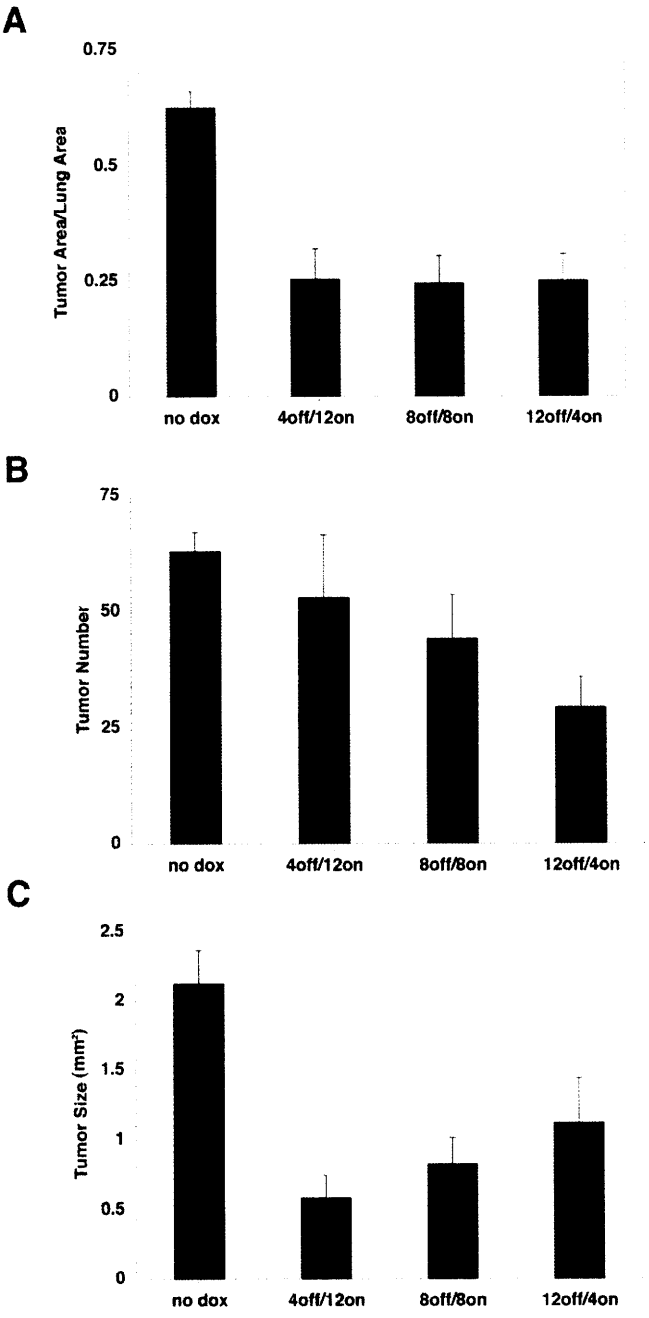
A
0.75
0.5
0.25
0
Tumor Area/Lung Area
no dox
4off/12on
8off/8on
12off/4on
B
75
50
25
0
Tumor Number
no dox
4off/12on
8off/8on
12off/4on
C
2.5
2
1.5
1
0.5
0
Tumor Size (mm²)
no dox
4off/12on
8off/8on
12off/4on

Figure A1.3. Let-7g induction suppresses tumorigenesis in an autochthonous NSCLC model. KPR mice were intratracheally infected with TRE.mCherry.let-7g.PGK.Cre. Animals were either given feed lacking doxycycline or were switched to feed containing doxycycline four, eight, or twelve weeks post-infection. Sixteen weeks post-infection, animals were sacrificed and lungs were examined by histology. (A-C) Tumor burden, measured by tumor area as a fraction of total lung area (A), tumor number (B), and average tumor size (C) was quantified by Bioquant software. Values are mean +/- s.e.m. (n=10 for each group).

these animals, distinctions between these groups emerge. First, induction of let-7g at twelve weeks post-infection caused a more substantial decrease in tumor number than induction at eight or four weeks (Figure A1.3B). In contrast, induction of let-7g at four weeks post-infection led to a greater reduction in average tumor size than induction at eight or twelve weeks (Figure A1.3C). Taken together, these results suggest that delivery of let-7g in established tumors can suppress tumor growth. Furthermore, the effects of suppression depend upon the time of delivery relative to tumor initiation.

Overall, these findings provide important support for the development of let-7 family members as therapeutic agents. In both human NSCLC xenografts and in an autochthonous mouse model of NSCLC, let-7g induction in fully formed tumors was able to suppress tumorigenesis. However, while induction of let-7g in the xenograft model merely slowed the growth of tumors, let-7g overexpression in naturally arising tumors actually triggered regression of tumors, as evinced by the substantial reduction in tumor number in animals induced at later time points. In addition, an intriguing finding from these studies was the distinctive effect of let-7g induction at different times after tumor initiation. While let-7g induction caused greater decreases in average tumor size when induced early after tumor initiation, it caused a more potent decline in tumor number when induced late. It is unclear whether these effects are caused by differences in the duration of let-7g induction or in the stages of tumor development at these time points. Moreover, this potent reduction in tumor number at late time points allows for the characterization of let-7 mediated tumor clearance. A more detailed time course of let-7g induction at later time points should allow for some insight into this process. Additionally, examination of survival in tumor-bearing animals induced at different time points will allow for better understanding of let-7g delivery and its potential in cancer therapy.

Materials and Methods

Cell culture. A549-Tet-On-KRAB-TE-let-7g and A549-Tet-On-KRAB-TE-miR-15b cells were described previously (Kumar et al., 2008). Cells were grown under standard conditions in Tet-Free Serum as per manufacturer's instructions (Clontech).

Tumor xenografts. Tumor xenografts of A549 cells were performed as described (Kumar et al., 2008). In short, mice were injected with A549-Tet-On-KRAB-TE-let-7g and A549-Tet-On-KRAB-TE-miR-15b cells and monitored for tumors. Once tumors were greater that 2 mm in diameter, mice were intraperitoneally injected with PBS with and without doxycycline (40 mg/kg), and tumors were measured.

Mice. *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$ animals were bred with *CCSP-rtTA* animals to produce *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/+}$*; CCSP-rtTA* animals. These animals were backcrossed to *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$ animals to produce *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$*; CCSP-rtTA* animals, which were interbred to produce the experimental cohort of *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$*; CCSP-rtTA* animals.

Construction of TRE.mCherry.let-7g.PGK.Cre. TRE.Puro.let-7g.PGK.Cre was initially generated by amplification of TRE from pTRE-Tight (Clontech) with 5'-GACTCTAGACACGAGGCCCTTTCGTCTTCACACGAG-3' and 5'-GAAGCTAGCTTCTCCAGGCGATCTGACGGTTCACTAAAC-3', digestion with XbaI and NheI and cloning into UbC.Puro.let-7g.PGK.Cre to generate TRE.Puro.let-7g.PGK.Cre. TRE.mCherry.let-7g.PGK.Cre was initially generated by amplification of mCherry from UbC.mCherry.PGK.Cre (a kind gift of M. Winslow) with 5'-CAAGAATTCACCATGGTGAGCAAGGGCGAG-3' and 5'-

GGACTCGAGTTACTTGTACAGCTCGTCCATGC-3', digestion with EcoRI and XhoI and cloning into TRE.Puro.let-7g.PGK.Cre to generate TRE.mCherry.let-7g.PGK.Cre.

Lentivirus Production and Infection. Lentivirus production was performed as described (Rubinson et al., 2003).

Intratracheal Infection and Tumor Analysis. *Kras*$^{LSL\text{-}G12D}$*; Trp53*$^{f/f}$*; CCSP-rtTA* mice were infected intratracheally with TRE.mCherry.let-7g.PGK.Cre lentivirus essentially as described (Murphy et al., 2006). Doxycycline treatment was performed via doxycycline-impregnated food pellets as described previously (Politi et al., 2006). Tumor analysis was performed as described (Kumar et al., 2007).

Western Blotting. Western blotting was performed as described previously (Kumar et al., 2008; Kumar et al., 2007).

Small RNA Northern Blotting. Small RNA Northern blotting was performed as described previously (Kumar et al., 2008).

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APPENDIX 2: Coordinate loss of miR-145 and RPS14 contribute to the pathogenesis of 5q- syndrome

I contributed figures A2.1, A2.3, and A2.4. Anupama Narla contributed figures A2.2, A2.5, and A2.6.

Introduction

Changes in copy number for whole chromosomes or chromosomal regions can cause substantial changes in gene expression that contribute to the pathogenesis of many diseases, including cancer. An interesting example is 5q- syndrome, a clinical subtype of myelodysplastic syndrome (MDS) typified by a combination of impaired erythroid production and frequent elevation of platelet counts, suggesting an imbalance in the erythroid-megakaryocytic lineage pathway (Giagounidis et al., 2006; Heaney and Golde, 1999; Van den Berghe et al., 1974). We have recently described an RNA interference screen in which we knocked down all protein-coding genes within the common deletion region of 5q- syndrome (Boultwood et al., 2002). In this screen, we found that partial knockdown of the ribosomal protein S14 (RPS14) strongly recapitulated the defects in erythroid differentiation seen in 5q- syndrome (Ebert et al., 2008). While hemizygous loss of RPS14 clearly plays an important role in the erythroid differentiation problems exhibited in 5q- syndrome, other features of the disease, such as increased platelet production, are not affected by RPS14 loss.

MicroRNAs (miRNAs) are a novel class of small, non-coding RNAs that regulate target mRNAs via imperfect base pairing and consequent inhibition of translation (Bartel, 2004). Computational prediction of miRNA targets has predicted that the majority of protein-coding genes might be regulated by miRNAs (Bartel, 2009). Moreover, a series of genetic analyses has revealed that specific miRNAs play an important role in hematopoeitic development (Johnnidis et al., 2008; Koralov et al., 2008; Rodriguez et al., 2007; Thai et al., 2007; Ventura et al., 2008; Vigorito et al., 2007; Xiao et al., 2007). In particular, certain miRNAs shown to be regulated during myeloid development play an important role in the normal erythroid-megakaryocyte

lineage decision (Lu et al., 2008). Taken together, these findings indicate that miRNAs have the potential to regulate target mRNAs important for hematopoeitic lineage choice.

A recent study examining miRNAs differentially expressed in response to specific oncogenes revealed potent silencing of two adjacent miRNAs, miR-143 and miR-145, in cells expressing the E1A oncoprotein (He et al., 2007). Moreover, these miRNAs are both down-regulated in a variety of tumor types (Akao et al., 2007; Michael et al., 2003; Wang et al., 2008). Furthermore, both miR-143 and miR-145 are overexpressed in polycythemia vera, a hematologic disorder characterized by over-production of erythroid cells (Bruchova et al., 2008). Of note, both miR-143 and miR-145 are within the common deletion region for 5q- syndrome. Thus, we were interested in the possibility that loss of these miRNAs might, in combination with partial loss of RPS14, contribute to the pathogenesis of 5q- syndrome.

Results and Discussion

To initially examine the role of miR-143 and miR-145 in the development of 5q-syndrome, we generated lentiviral vectors for their overexpression (Figure A2.1). When introduced into human CD34+ hematopoeitic stem cells (HSCs) and triggered to undergo differentiation, overexpression of miR-145, but not miR-143, led to a significant decrease in the ratio of megakaryocyte to erythroid cells (Figure A2.2A). Since 5q- syndrome manifests in an increase in the ratio of megakaryocyte to erythroid cells, one would expect factors that suppress 5q- pathogenesis to act in the manner of miR-145 overexpression. Thus, we focused our efforts on miR-145.

Figure A2.1

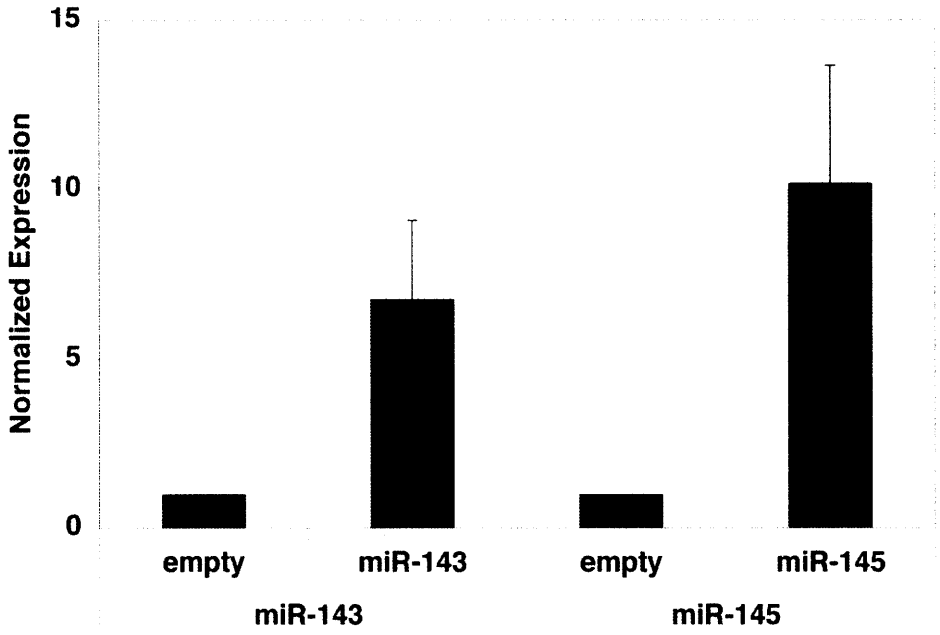

15
10
5
0
Normalized Expression
empty
miR-143
empty
miR-145
miR-143
miR-145

Figure A2.1. Overexpression of miR-143 and miR-145. HEL cells were infected with control lentiviruses or lentiviruses overexpressing miR-143 or miR-145. Quantitative real-time polymerase chain reaction (Q-RT PCR) was performed with probes for miR-143 and miR-145. Expression levels were initially normalized to snoRNA-142 and subsequently normalized relative to the HEL cells infected with the control lentivirus. Values are mean +/- s.d. (n=4) with propagated error.

Figure A2.2

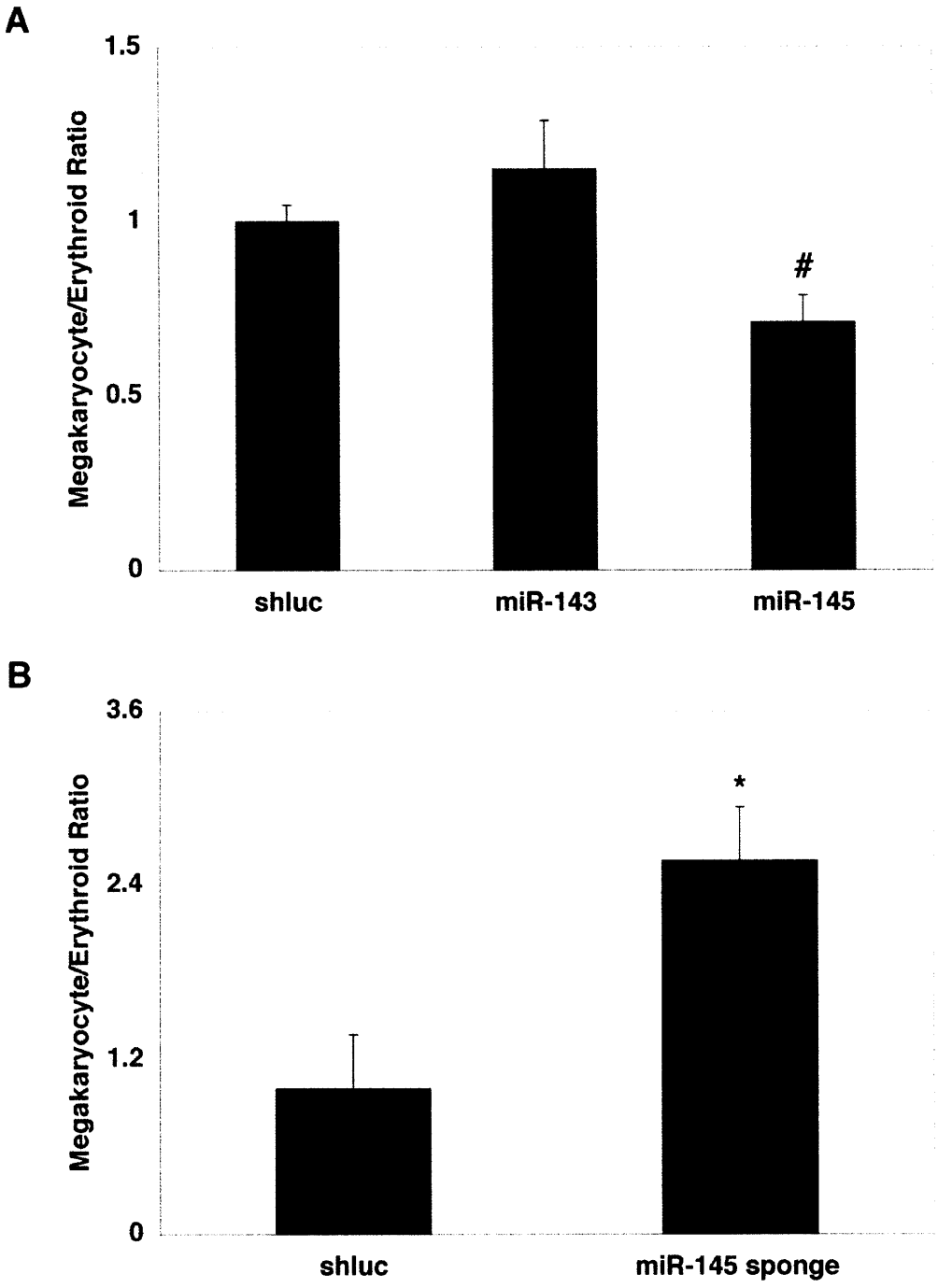

A
1.5
1
0.5
0
Megakaryocyte/Erythroid Ratio
#
shluc
miR-143
miR-145
B
3.6
2.4
1.2
0
Megakaryocyte/Erythroid Ratio
*
shluc
miR-145 sponge

Figure A2.2. MiR-145 regulates the megakaryocyte-erythroid lineage decision. (A) Relative to an shRNA targeting *Renilla* luciferase (shluc), overexpression of miR-145, but not overexpression of miR-143, promotes erythroid relative to megakaryocytic differentiation in CD34+ HSCs. The ratios of cells from the megakaryocytic and erythroid lineages, indicated on the y-axis, were assessed by flow cytometry with antibodies against CD41 and GlyA, respectively. Values are mean +/- s.e.m. with propagated error. #: $p<0.01$. (B) Relative to an shRNA targeting *Renilla* luciferase (shluc), expression of a miRNA sponge targeting miR-145, promotes megakaryocytic relative to erythroid differentiation in CD34+ HSCs as assessed above. Values are mean +/- s.e.m. with propagated error. *: $p<0.05$.

In order to stably inhibit miR-145 in CD34+ HSCs, we generated a lentiviral vector encoding a miRNA sponge to miR-145 (Ebert et al., 2007). When transduced into CD34+ HSCs, inhibition of miR-145 by its sponge caused a significant increase in the ratio of megakaryocyte to erythroid cells, as expected for a factor whose loss contributes to 5q- syndrome (Figure A2.2B). Moreover, when compared to MDS patient samples wild type for 5q (5q+ MDS), samples from 5q- syndrome patients (5q- MDS) have reduced levels of miR-145 (Figure A2.3A). Taken together, these results suggest that miR-145 regulates the megakaryocyte-erythroid lineage decision in a manner consistent with its role in 5q- syndrome.

We then examined which predicted targets of miR-145 could contribute to its effects on differentiation using the miRNA target prediction program TargetScan (Friedman et al., 2009). One of the highest scoring predictions was Fli-1, as it had multiple binding sites highly conserved across mammalian species. Fli-1, an ETS-family transcription factor originally found as an insertion site of the Friend leukemia virus, has been previously shown to play a role in megakaryocytic and erythroid differentiation. First, overexpression of Fli-1 in leukemia cells induces megakaryocyte differentiation while suppressing erythroid differentiation (Athanasiou et al., 1996; Athanasiou et al., 2000). Moreover, in *Fli1*$^{-/-}$ embryos, there is a severe defect in megakaryocyte differentiation, resulting in thrombocytopenia (Hart et al., 2000).

We thus wanted to explore the relationship of miR-145 and Fli-1. When we overexpressed miR-145 in human erythroleukemia (HEL) cells, we observed a substantial decrease in Fli-1 protein expression; conversely, inhibition of miR-145 by its sponge caused a substantial increase in Fli-1 protein levels (Figure A2.4A). To determine whether miR-145 regulated Fli-1 via its 3' UTR, we generated a reporter in which the Fli-1 3' UTR is placed behind *Renilla* luciferase. We found that overexpression of miR-145 caused a significant

Figure A2.3

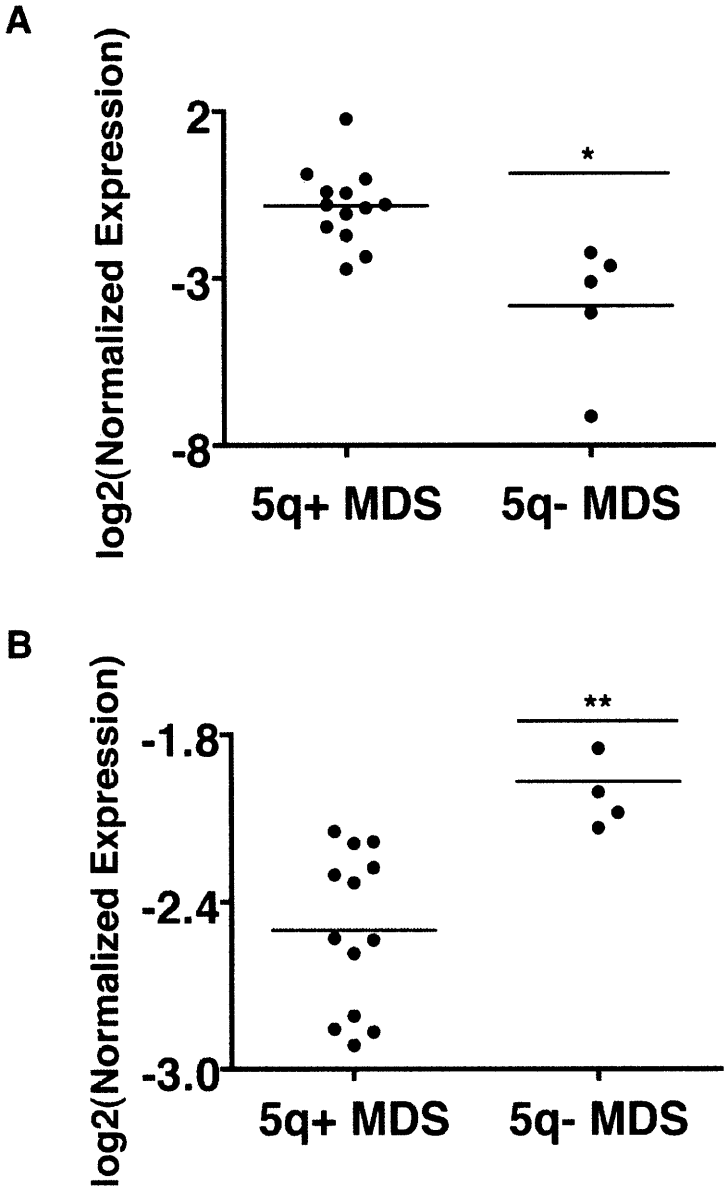
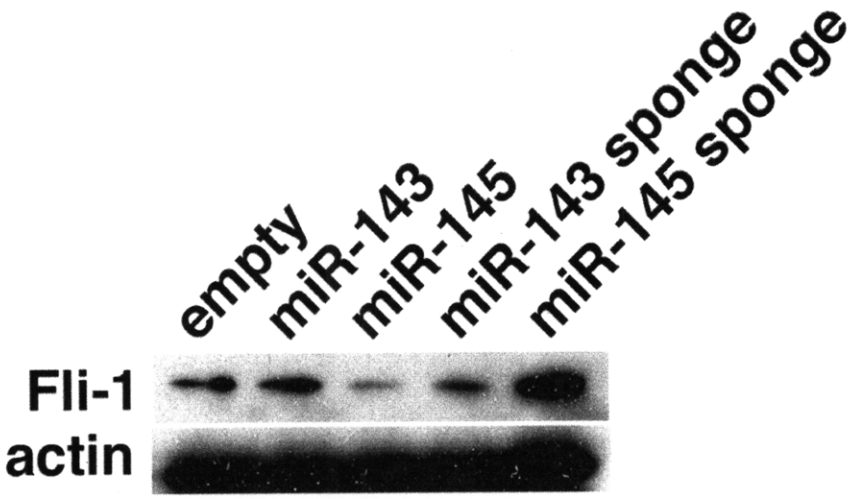

A
log2(Normalized Expression)
2
-3
-8
*
5q+ MDS
5q- MDS
B
log2(Normalized Expression)
-1.8
-2.4
-3.0
**
5q+ MDS
5q- MDS

Figure A2.3. Altered expression of miR-145 and Fli-1 in 5q- MDS. (A) Quantitative real-time polymerase chain reaction (Q-RT PCR) was performed for miR-145 in MDS patient samples wild type for 5q (5q+ MDS) and in samples from patients with 5q- syndrome (5q- MDS). Expression levels were normalized to snoRNA-142. Values are mean (n=4) with propagated error. *: $p<0.05$. (B) Quantitative real-time polymerase chain reaction (Q-RT PCR) was performed for Fli-1 in MDS patient samples wild type for 5q (5q+ MDS) and in samples from patients with 5q- syndrome (5q- MDS). Expression levels were normalized to GAPDH. Values are mean (n=4) with propagated error. **: $p<0.0005$.

Figure A2.4

A



B

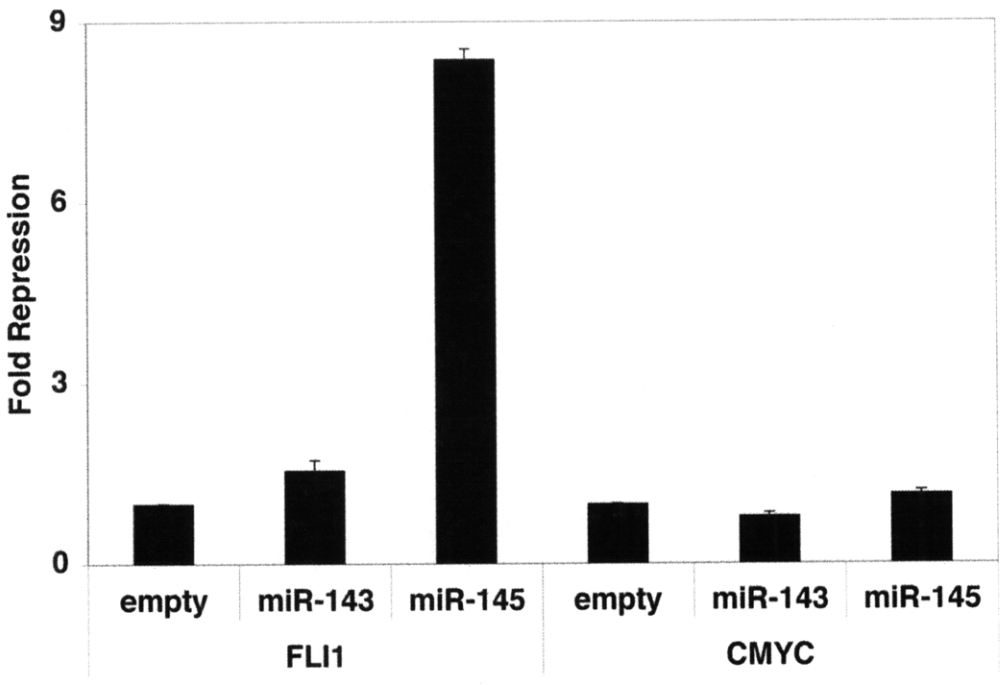
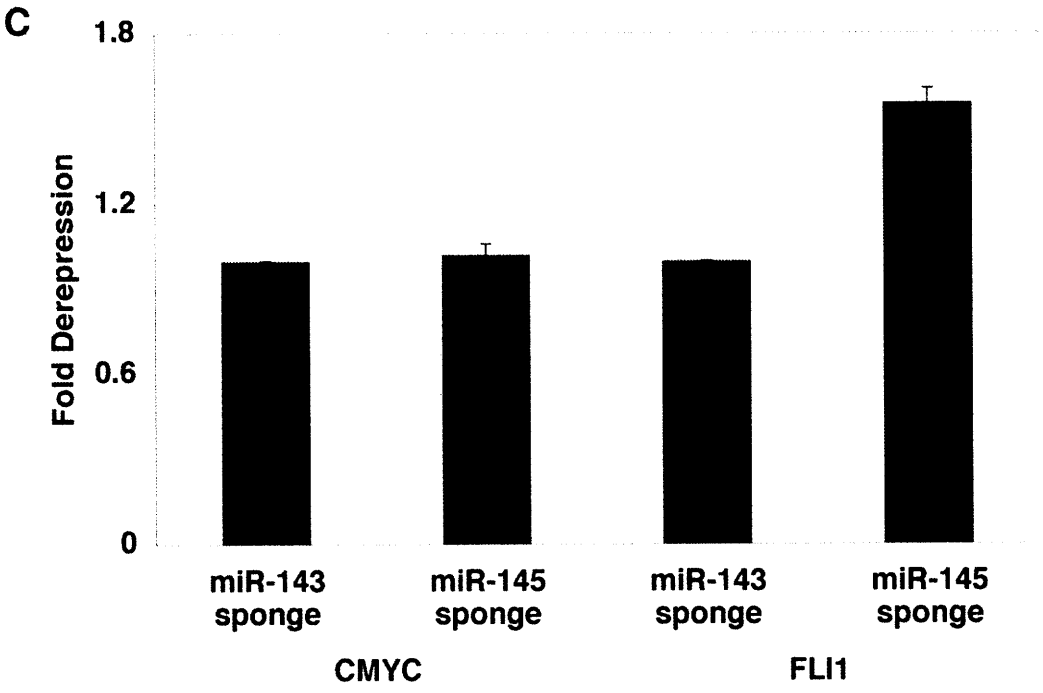


Figure A2.4 continued



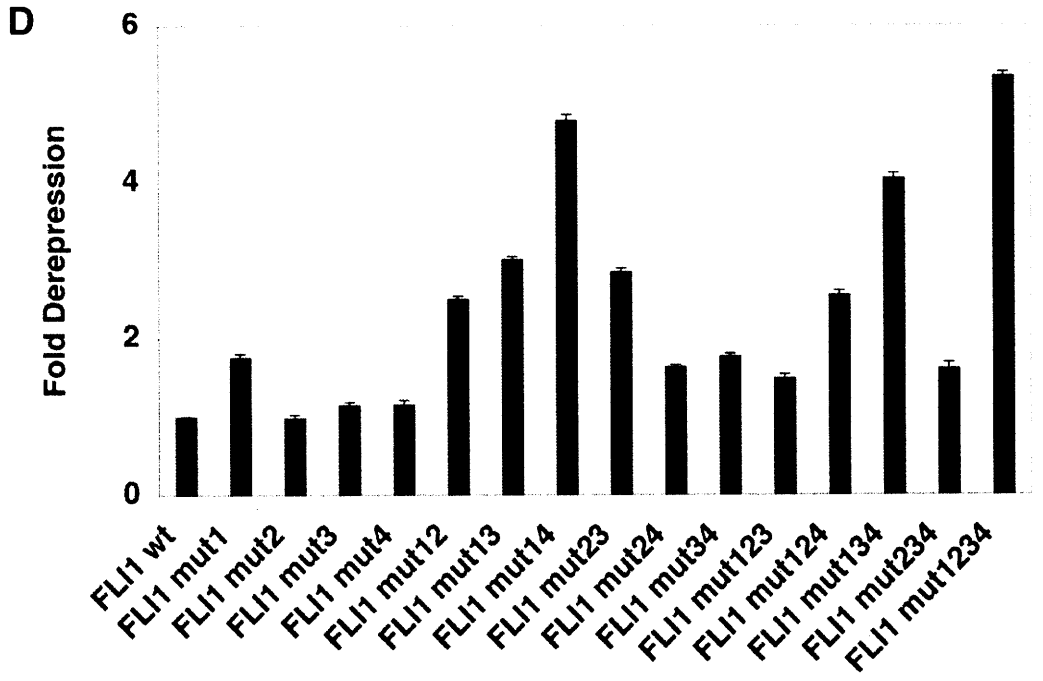


Figure A2.4. MiR-145 regulates Fli-1. (A) HEL cells were infected with control lentiviruses, lentiviruses overexpressing miR-143 or miR-145, or with lentiviruses expressing miRNA sponges target miR-143 and miR-145. Western blotting was performed for Fli-1 and actin. (B) Luciferase assays were performed in 293T cells co-transfected with control lentiviruses or lentiviruses over-expressing miR-143 or miR-145 and pRL-TK containing bulged siCXCR4 binding sites or murine Fli-1 and c-Myc 3' UTRs. Firefly luciferase (pGL3) was used as a transfection control. Expression was normalized to pGL3 levels and subsequently normalized relative to pRL-CXCR4 expression. Values are mean +/- s.d. (n=6) with propagated error. (C) Luciferase assays were performed in 293T cells co-transfected with lentiviruses expressing sponges targeting miR-143 or miR-145 and pRL-TK and pGL3 vectors as described above. Normalization was performed as described above. Values are mean +/- s.d. (n=6) with propagated error. (D) Luciferase assays were performed in 293T cells co-transfected with lentiviruses over-expressing miR-145 and pRL-TK containing either the wild type murine Fli-1 3' UTR (FLI1 wt) or combinations of point mutations in miR-145 binding sites (FLI1 mut1-mut4). Firefly luciferase (pGL3) was used as a transfection control. Expression was first normalized to pGL3 levels and subsequently normalized relative to FLI1 wt expression. Values are mean +/- s.d. (n=6) with propagated error.

repression of the Fli-1 reporter (Figure A2.4B). Conversely, inhibition of miR-145 by a miR-145 sponge caused a modest but significant derepression of the Fli-1 3' UTR (Figure A2.4C). Notably, neither overexpression nor inhibition of miR-145 affected expression of a similar reporter targeting the c-Myc 3' UTR, even though miR-145 has recently been suggested to target c-Myc (Sachdeva et al., 2009). Furthermore, we believe that the repression of Fli-1 by miR-145 is direct, as mutation of the predicted miR-145 binding sites in the Fli-1 3' UTR led to progressively greater derepression, with mutation of all four sites achieving a near complete restoration of reporter expression (Figure A2.4D). Overall, these results indicate that miR-145 regulates Fli-1 through its 3' UTR.

To characterize the functional consequence of Fli-1 derepression via loss of miR-145, we sought to overexpress Fli-1 by lentivirus. In particular, we expressed the Fli-1 open reading frame without its 3' UTR, so that it cannot be regulated by miR-145. When the Fli-1 lentivirus was transduced into CD34+ HSCs, there was a significant increase in the ratio of megakaryocyte to erythroid cells, similar to that seen with the miR-145 sponge (Figure A2.5). Furthermore, when we compared 5q+ MDS samples with 5q- MDS samples, we observed a significant increase in Fli-1 mRNA in 5q- MDS (Figure A2.3B). As it has been shown that targets strongly regulated by miRNAs at the protein level overwhelmingly exhibit changes at the mRNA level, this change in Fli-1 transcript levels likely reflects differences in miRNA-mediated repression (Baek et al., 2008). Taken together, these results indicate that Fli-1, which is overexpressed via loss of miR-145, regulates megakaryocyte and erythroid differentiation as expected in 5q- syndrome.

While the above results suggest a role for miR-145, and its regulation of Fli-1, in suppression of 5q- syndrome, it has been shown that at least one protein-coding gene, RPS14,

Figure A2.5

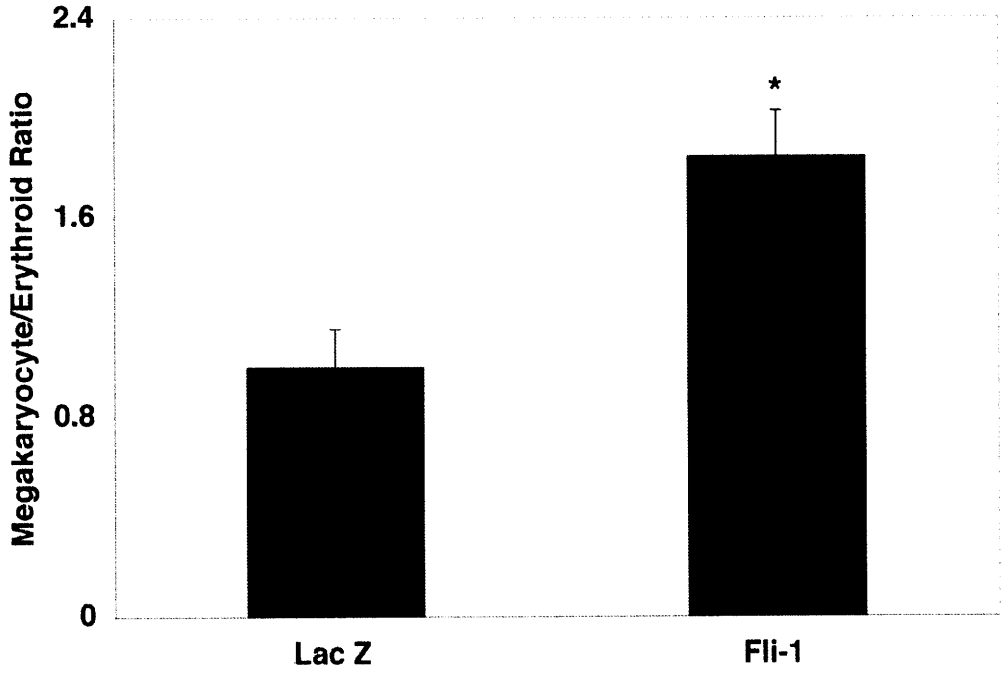


Figure A2.5. Fli-1 overexpression phenocopies miR-145 inhibition. Relative to a control cDNA (LacZ), overexpression of Fli-1 promotes megakaryocytic relative to erythroid differentiation in CD34+ HSCs as assessed above. Values are mean +/- s.e.m. with propagated error. *: $p<0.05$.

contributes to the disease state (Ebert et al., 2008). Thus, we wanted to combine miR-145 inhibition with loss of RPS14. When we combined the miR-145 sponge with three short hairpin RNAs (shRNAs) targeting RPS14 in CD34+ HSCs, we noted a significant increase in the ratio of megakaryocyte to erythroid cells compared to loss of RPS14 alone (Figure A2.6). Thus, loss of miR-145 appears to cooperate with loss of RPS14 in the megakaryocyte-erythroid differentiation effects seen in 5q- syndrome.

Taken together, these results provide substantial evidence that loss of miR-145 promotes 5q- syndrome via up-regulation of Fli-1, a known transcriptional regulator of megakaryocyte development. Therefore, it is likely that this up-regulation of Fli-1 specifically contributes to the thrombocytosis observed in 5q- syndrome. It also appears that loss of miR-145 cooperates with loss of RPS14 to promote development of 5q- syndrome, making it a coordinate gene deletion syndrome. This is an important finding, as it is the first example of a miRNA undergoing coordinate loss with a protein-coding gene in a chromosome deletion syndrome. Moreover, it suggests that other chromosome deletions, many of which occur in cancer, might involve the combined loss of protein-coding genes and non-coding RNAs. Thus, future genomic analyses in human disease should examine the effects of deletions on coding and non-coding genes. Finally, an intriguing feature of 5q- syndrome is its exquisite sensitivity to lenalidomide, which potently induces erythroid differentiation in this context (List et al., 2006). However, the mechanism of action of lenalidomide in this context is largely unknown. The results presented here provide for the intriguing possibility that lenalidomide alters the expression of miR-143 and miR-145, thus promoting erythroid differentiation only within the context of the disease.

Figure A2.6

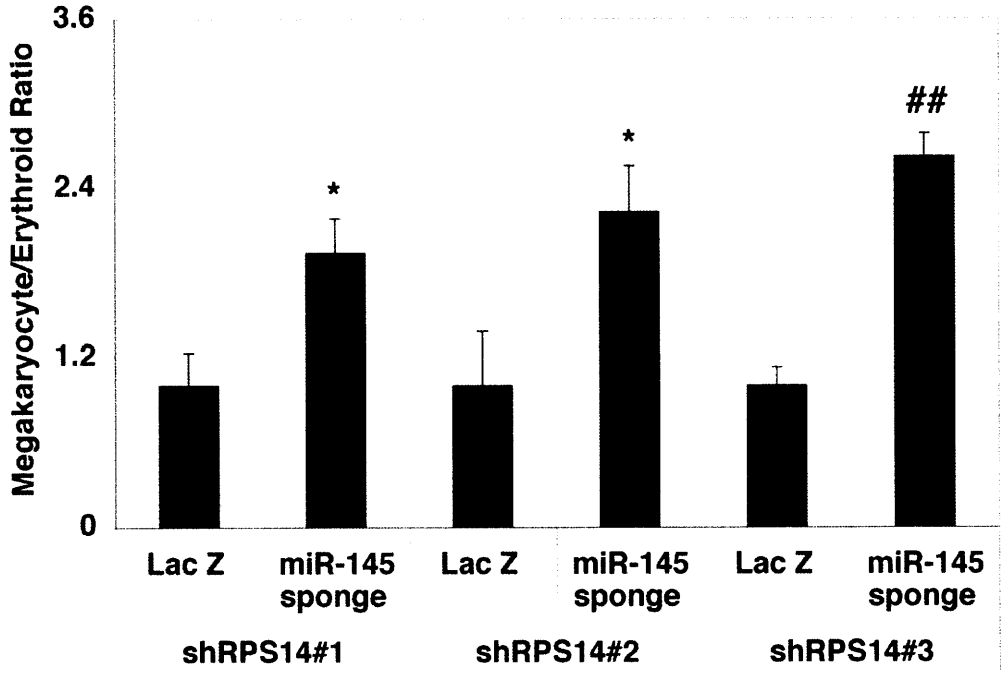

Megakaryocyte/Erythroid Ratio
3.6
2.4
1.2
0
*
*
##
Lac Z
miR-145 sponge
Lac Z
miR-145 sponge
Lac Z
miR-145 sponge
shRPS14#1
shRPS14#2
shRPS14#3

Figure A2.6. MiR-145 inhibition cooperates with loss of RPS14 in the megakaryocyte-erythroid lineage decision. Relative to a control lentivirus (LacZ) co-infected with a series of shRNAs against RPS14 (shRPS14 #1-3), expression of a miRNA sponge targeting miR-145 promotes megakaryocytic relative to erythroid differentiation in CD34+ HSCs as assessed above. Values are mean +/- s.e.m. with propagated error. *: $p<0.05$; ##: $p<0.005$.

Materials and Methods

Cell culture. HEL cells were acquired from ATCC and were cultured under standard conditions. CD34+ cells were acquired and grown as described previously (Ebert et al., 2008).

Construction of miRNA overexpression vectors. pLVX.miR-143.puro was initially generated by amplification of miR-143 (plus approximately 250 bp flanking sequence) from wild type mouse tail DNA with 5'-GAACTCGAGGCATAAAGAGACGGATGGGGGGC-3' and 5'-GCCAGATCTGTTGAGAAGGGTTCCGAGGGTG-3', digestion with XhoI and BglII and cloning into pLVX.puro to generate pLVX.miR-143.puro. pLVX.miR-145.puro was initially generated by amplification of miR-145 (plus approximately 250 bp flanking sequence) from wild type mouse tail DNA with 5'-TAACTCGAGCTGGAGGACCGCAGCGAGAAG-3' and 5'-GCCAGATCTCAAGGTCTACAGGGAAGGCATG-3', digestion with XhoI and BglII and cloning into pLVX.puro to generate pLVX.miR-145.puro.

Construction of miRNA sponge vectors. MiRNA sponges were generated against miR-143 and miR-145 as described previously with some modifications (Ebert et al., 2007). In particular, synthetic miRNA binding sites were generated via minigene synthesis (Integrated DNA Technologies). To generate pLenti6.miR-143 sponge.blast, the miR-143 sponge was amplified from the minigene with 5'-CACCGAGCTACAGACGATCATCTCACCGG-3' and 5'-TGAGATGATTGCCTGTAGCTCTTTGTGAGATGAC-3' and cloned into pLenti6.D-TOPO-V5.blast per manufacturer's instructions (Invitrogen) to generate pLenti6.miR-143 sponge.blast. To generate pLenti6.miR-145 sponge.blast, the miR-145 sponge was amplified from the minigene with 5'-CACCAGGGATTCCTGCCTTAACTGGACCCGG-3' and 5'-GTCCAGTTGAAGCAGGAATCCCTTTTGGTCCAGTTG-3' and cloned into pLenti6.D-

TOPO-V5.blast per manufacturer's instructions (Invitrogen) to generate pLenti6.miR-145 sponge.blast.

Construction of Fli-1 overexpression vector. pLenti6.Fli-1.blast was generated by amplification of human Fli-1 from the pOTB7.Fli-1 cDNA with 5'-CACCATGGACGGGACTATTAAGGAGGCTC-3' and 5'-CTAGTAGTAGCTGCCTAAGTGTGAAGGCACGTG-3' and cloned into pLenti6.D-TOPO-V5.blast as described above to generated pLenti6.Fli-1.blast.

Construction of Fli-1 3' UTR reporter. pRL.Fli-1 3' UTR.TK was generated by amplification of the murine Fli-1 3' UTR from wild type mouse tail DNA with 5'-GAACTCGAGAACTAACACCAGTTGGCCTTCTGG-3' and 5'-GACGCGGCCGCGCACAATTTATTAAGGTCAAATTATTTTAC-3', digestion with XhoI and NotI and cloning into pRL.TK to generate pRL.Fli-1 3' UTR.TK.

Mutation of miR-145 binding sites in Fli-1 3' UTR. Site-directed mutagenesis of the predicted miR-145 binding sites in the Fli-1 3' UTR was performed per manufacturers' instructions (Stratagene). Mutagenesis oligonucleotides were as follows:

Mut1 forward:

GGCACAGTAGCCTTGAAGAGATAAGAAAACACCTTGTTCTTTCTTTTGGATAGAACC TTTG

Mut1 reverse:

CAAAGGTTCTATCCAAAAGAAAGAACAAGGTGTTTTCTTATCTCTTCAAGGCTACTG TGCC

Mut2 forward:

CATCGAATGAGTTAAATATTTAGGTTACACCTACGGTTTATACCATGATTCTGAGAA

AGGAG

Mut2 reverse:

CTCCTTTCTCAGAATCATGGTATAAACCGTAGGTGTAACCTAAATATTTAACTCATTC

GATG

Mut3 forward: GGACTCAATTCAGTGGATGGCAACACCTACACTGGCTCTGAGGCCAG

Mut3 reverse: CTGGCCTCAGAGCCAGTGTAGGTGTTGCCATCCACTGAATTGAGTCC

Mut4 forward:

GCTCTGAGGCCAGTGAAGTTTTTTGCCCAACACCTATTTAAAAGATGTGTGTC

Mut4 reverse:

GACACACATCTTTTAAATAGGTGTTGGGCAAAAAACTTCACTGGCCTCAGAGC

Lentivirus production and infection. Lentivirus production was performed as described (Rubinson et al., 2003).

Luciferase assays. Luciferase assays were performed as described previously (Doench et al., 2003).

Quantitative reverse transcription (Q-RT) PCR. Q-RT PCR of miR-143, miR-145, and snoRNA-142 was performed using the corresponding Taqman miRNA expression assays. Q-RT PCR of human Fli-1 and GAPDH was performed using Taqman assays Hs00231107_m1 and 4333764F. Synthesis of cDNA and Taqman qRT-PCR of Fli-1 and GAPDH were performed as described previously (Sweet-Cordero et al., 2005).

Western blotting. Western blotting was performed as described previously using antibodies against Fli-1 (ab53077 from AbCam) and actin (A2066 from Sigma) (Kumar et al., 2008; Kumar et al., 2007).

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APPENDIX 3: Development of lentiviral vectors to combine Cre-loxP technology and RNA interference in the mouse

I contributed figure A3.1. Etienne Meylan contributed figure A3.2. Nathan Young contributed figure A3.3.

Introduction

Genetically engineered mouse models provide an important tool in the study of tumorigenesis in the context of a whole organism (Frese and Tuveson, 2007; Van Dyke and Jacks, 2002). In particular, the development and application of conditional genetic strategies based upon, among others, the Cre-loxP site-specific recombination system has been invaluable for several reasons. First, this technology allows the examination of cancer genes whose germline loss of function is either lethal or compensated for during embryonic development, both of which obfuscate relevant results for tumorigenesis. Second, such systems provide temporal control of recombination, so that the Cre-mediated mutation can be induced at a specific time point as in the adult, which is when the preponderance of cancers is believed to initiate. Third, Cre-loxP regulation can be controlled in specific cells and tissues or in response to specific inducible signals, such as delivery of small molecules like pIpC or tamoxifen. Overall, conditional genetic systems generated via Cre-loxP have provided a novel set of reagents for examining tumor development.

While conditional genetics in the mouse has allowed for an unprecedented ability to engineer specific mutations in particular cells at particular times, it faces some practical limitations. First, generation of specific conditional alleles, though largely standardized in recent years, is still a laborious and time-consuming process. Second, once conditional alleles of interest are generated, combining individual alleles together is at best a long-term experimental approach and at worst impossible because of the lack of secondary conditional alleles available. Finally, the above time and labor constraints, based upon crossing pairs of conditional alleles, make systematic reverse genetic screens, in which one activates or inactivates a set of genes of

interest, essentially impossible. Thus, the development of methods that could combine Cre-loxP with other approaches to gain or loss of function genetics would be useful both in terms of experimental tractability and the capacity for reverse genetics.

RNA interference (RNAi), besides representing a novel method of gene regulation, has provided a new experimental reagent for engaging in loss of function of a given mRNA (Dykxhoorn et al., 2003). Moreover, since this method, which relies upon perfect base pairing of a small RNA with its target mRNA and subsequent cleavage and degradation of the transcript, regulates directly via the mRNA sequence, it has been possible to generate RNAi libraries against all known transcripts within a genome (Berns et al., 2004; Kolfschoten et al., 2005; Paddison et al., 2004; Westbrook et al., 2005). These libraries have provided a powerful method for probing a wide collection of transcripts *in vitro*, allowing for the discovery of new regulators of signal transduction pathways. In addition, the use of both retroviruses and lentiviruses has allowed for the development of transgenic mice in which RNAi can be regulated either by doxycycline-regulated transcription or Cre-loxP—mediated recombination (Dickins et al., 2005; Dickins et al., 2007; Kolfschoten et al., 2005; Stegmeier et al., 2005; Tiscornia et al., 2004; Ventura et al., 2004). In total, this data allows for the exciting possibility that RNAi screening can be performed *in vivo*.

While the above technologies (Cre-loxP and RNAi) have been demonstrated to be powerful tools for examining tumor biology, they have yet to be effectively combined so that one can coordinately engage in Cre-loxP recombination and RNAi *in vivo*. Here we describe the development of a bifunctional lentiviral vector for the combined delivery of Cre recombinase and short hairpin RNAs (shRNAs) to the adult mouse. We demonstrate that for several distinct targets knockdown occurs within mouse models of lung cancer and soft-tissue sarcoma.

Furthermore, we show that this knockdown can, in fact, functionally recapitulate results previously found using traditional genetics. Taken together, these results open the possibility of developing RNAi screens in Cre-loxP—regulated mouse models of cancer.

Results and Discussion

To combine Cre-loxP with RNAi, we generated a lentiviral vector, hereafter referred to as U6.Cre, composed of two independent transcriptional units (Figure A3.1A). The first is a U6-driven shRNA cassette, which is transcribed via RNA polymerase III (Ventura et al., 2004). The second is the Cre recombinase gene expressed via the phosphoglycerate kinase (PGK) promoter, which we have shown previously to allow for effective expression of Cre in target tissues upon infection (Kumar et al., 2008). To test the efficacy of this vector in achieving knockdown *in vitro*, we developed a series of shRNAs targeting *Mapk14*, whose product, p38alpha, was frequently shown to be a tumor suppressor in a K-Ras—driven model of lung cancer (Ventura et al., 2007). When U6.Cre viruses targeting p38alpha were transduced into mouse embryonic fibroblasts, we observed potent suppression of the target with all three shRNAs, with near complete loss of target protein for the last two (Figure A3.1B).

In order to see if these U6.Cre shRNAs would work *in vivo*, we intratracheally infected $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ mice, which have been shown to experience a severe lung adenocarcinoma in response to Cre, with these lentiviral vectors (Jackson et al., 2005). Twelve weeks after infection, we collected tumors from these animals and examined p38alpha expression. Though not as robust as *in vitro*, we observed substantial repression of p38alpha in the tumors of mice

Figure A3.1

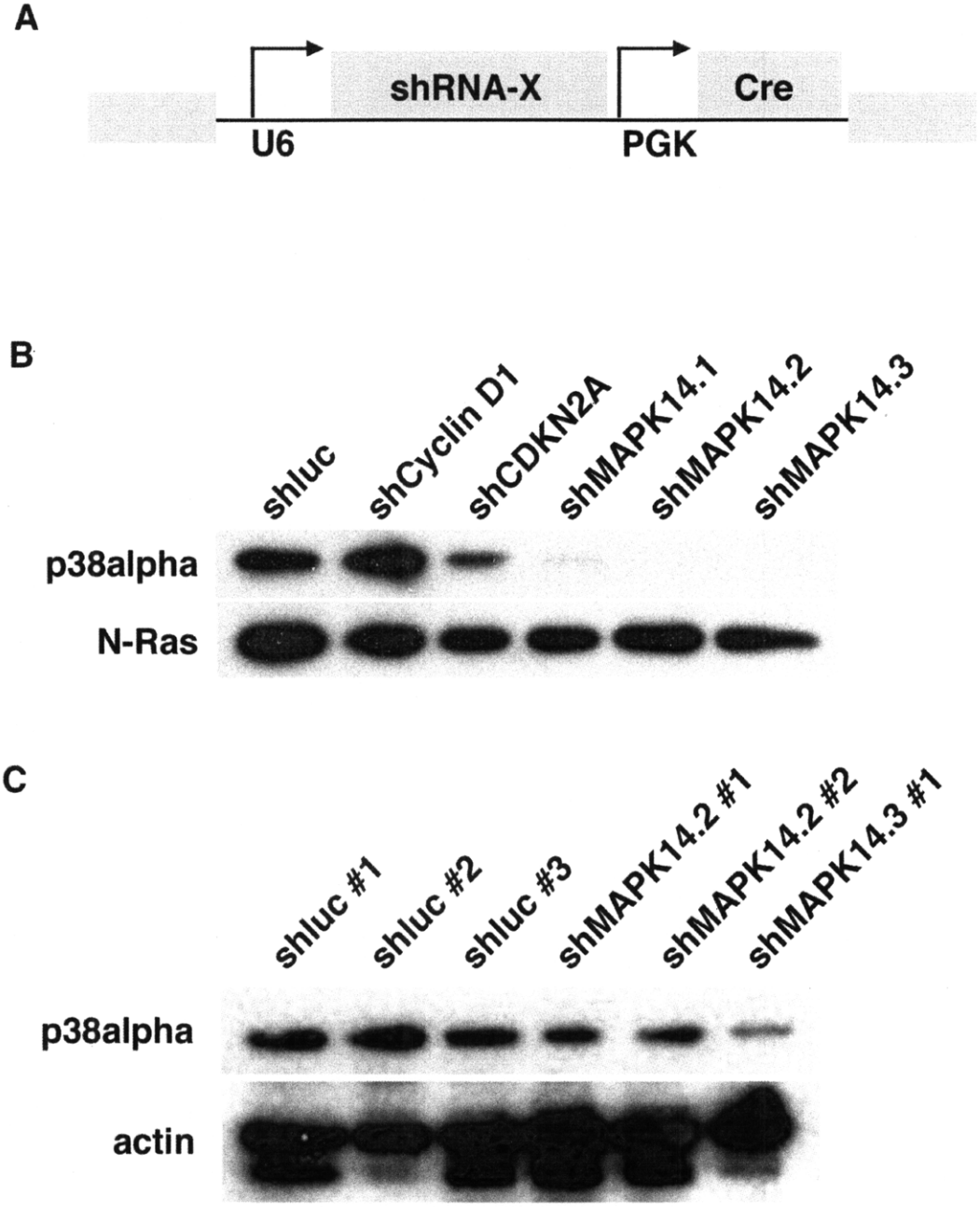
A
shRNA-X
Cre
U6
PGK
B
shluc
shCyclin D1
shCDKN2A
shMAPK14.1
shMAPK14.2
shMAPK14.3
p38alpha
N-Ras
C
shluc #1
shluc #2
shluc #3
shMAPK14.2 #1
shMAPK14.2 #2
shMAPK14.3 #1
p38alpha
actin

Figure A3.1 Development of a lentiviral vector for combined expression of an shRNA and Cre. (A) Diagram of the bifunctional lentiviral vector. (B) *Kras*$^{LSL\text{-}G12D}$ MEFs were infected with U6.Cre vectors containing shRNAs against *Renilla* luciferase (shluc), Cyclin D1 (shCyclin D1), CDKN2A (shCDKN2A), and against p38alpha (shMAPK14.1-3). Western blotting was performed against p38alpha, with N-Ras as a loading control. (C) *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{f/f}$ mice were intratracheally infected with lentiviruses expressing Cre and either shluc, shMAPK14.2, or shMAPK14.3. Twelve weeks after infection, tumors were collected and Western blotting was performed against p38alpha, with actin as a loading control.

infected with the shRNAs targeting p38alpha (Figure A3.1C). These differences could further be explained by normal tissue contamination within the tumors, as the lung adenocarcinomas in this model frequently are infiltrated by both stromal fibroblasts and macrophages. In total, this data suggests that a bifunctional lentiviral system should allow for combined expression of an shRNA and Cre *in vivo*.

To ensure that this vector is applicable to additional targets, we designed an shRNA targeting NEMO, an important regulator of the canonical NF-kappaB signaling pathway (Karin, 2006). When infected into a murine lung cancer cell line, we observed significant repression of NEMO (Figure A3.2A). Similarly, when infected into $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ mice, we observed significant repression of NEMO within a tumor from a mouse infected with the NEMO shRNA (Figure A3.2B). In sum, this data further supports the use of U6.Cre in knockdown of target genes in combination with Cre.

While the above studies suggest that it is possible to knockdown target mRNAs *in vivo* with U6.Cre, it is possible that such knockdown will be insufficient to affect tumor biology. This has been demonstrated categorically for p53, in which greater than 90% loss of p53 protein is insufficient to abrogate its ability to respond to DNA damage (Hemann et al., 2003). Thus, we applied U6.Cre to a mouse model of soft-tissue sarcoma, in which Cre expression in the hindlimb of $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ or $Kras^{LSL\text{-}G12D}$; $Cdkn2d^{f/f}$ causes the development of an extremity rhabdomyosarcoma (Kirsch et al., 2007). In this model, complete loss of the p19Arf-p53 pathway is required at the onset of tumorigenesis; animals that are heterozygous mutant for either of these components are unable to form tumors after hindlimb injection. We speculated that an shRNA targeting the mRNAs produced from *Cdkn2a* might be able to recapitulate the effect of complete of p19Arf. Importantly, for this to be true, tumors from animals that are

Figure A3.2

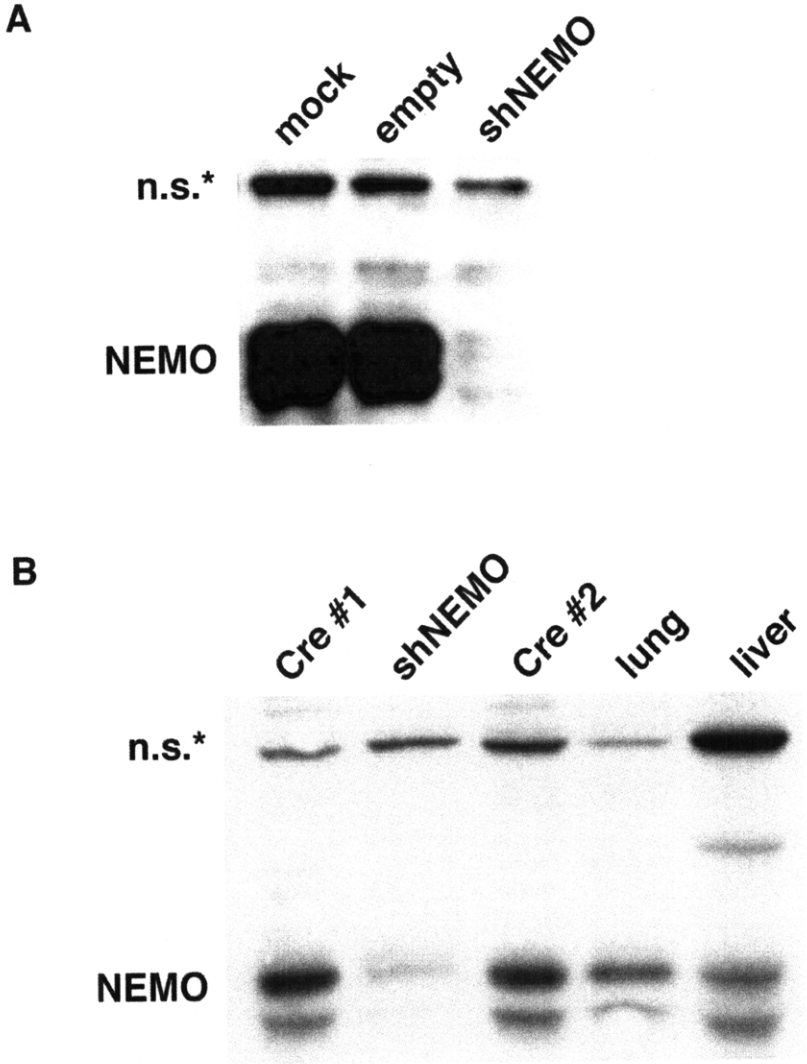
A
mock
empty
shNEMO
n.s.*
NEMO
B
Cre #1
shNEMO
Cre #2
lung
liver
n.s.*
NEMO

Figure A3.2 Knockdown of NEMO *in vitro* and *in vivo* with U6.Cre. (A) Mouse lung cancer cells were not infected with an empty U6.Cre virus or infected with U6.Cre virus expressing an shRNA targeting NEMO. Western blotting was performed against NEMO. A higher molecular weight, non-specific band (n.s.*) was used as a loading control. (B) $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ mice were intratracheally infected with lentiviruses expressing Cre alone or with a U6.Cre virus expressing an shRNA against NEMO. Twelve weeks after infection, tumors, normal lung, and normal liver were collected and Western blotting was performed against NEMO, with the non-specific band used as a loading control.

heterozygous mutant at this locus should retain the wild type allele, as the target is being depleted by RNAi and does not require deletion.

When we intramuscularly infected *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/GFP}$ animals with U6.Cre vectors targeting luciferase, E2F1, or CDKN2A, we generated tumors that failed to express p19Arf, as expected (Figure A3.3A). Surprisingly, when we infected *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ animals with U6.Cre vectors target CDKN2A, we were able to generate sarcomas, which does not occur normally with Cre delivery alone. Moreover, these tumors have sustained knockdown of both mRNAs produced from the *Cdkn2a* locus, p19Arf and p16Ink4a. Furthermore, these tumors do not lose the wild type allele of *Arf*, suggesting that RNAi-mediated knockdown is sufficient to suppress p19Arf and allow for soft-tissue sarcoma development (Figure A3.3B). In addition, sarcoma-free survival of *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ animals was comparable to that of *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/GFP}$ animals when both were infected with the shRNA to CDKN2A (Figure A3.3C). In contrast, while *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/GFP}$ animals infected with U6.Cre shRNAs against luciferase formed tumors readily, *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ animals infected with the same virus never formed tumors (data not shown). Taken together, this data suggests that U6.Cre-mediated RNAi allows for recapitulation of genetic loss of function in a mouse model of sarcoma.

Overall, these results indicate that the U6.Cre vector is an effective means of combining Cre-loxP technology with RNA interference *in vivo*. In particular, this system allows for infection of several cell types, as we were able to achieve viral delivery and knockdown in both the lung epithelium and hindlimb skeletal muscle. Moreover, this vector allows for delivery of Cre and an shRNA in the adult, providing the capacity to model tumorigenesis in adult animals.

Figure A3.3

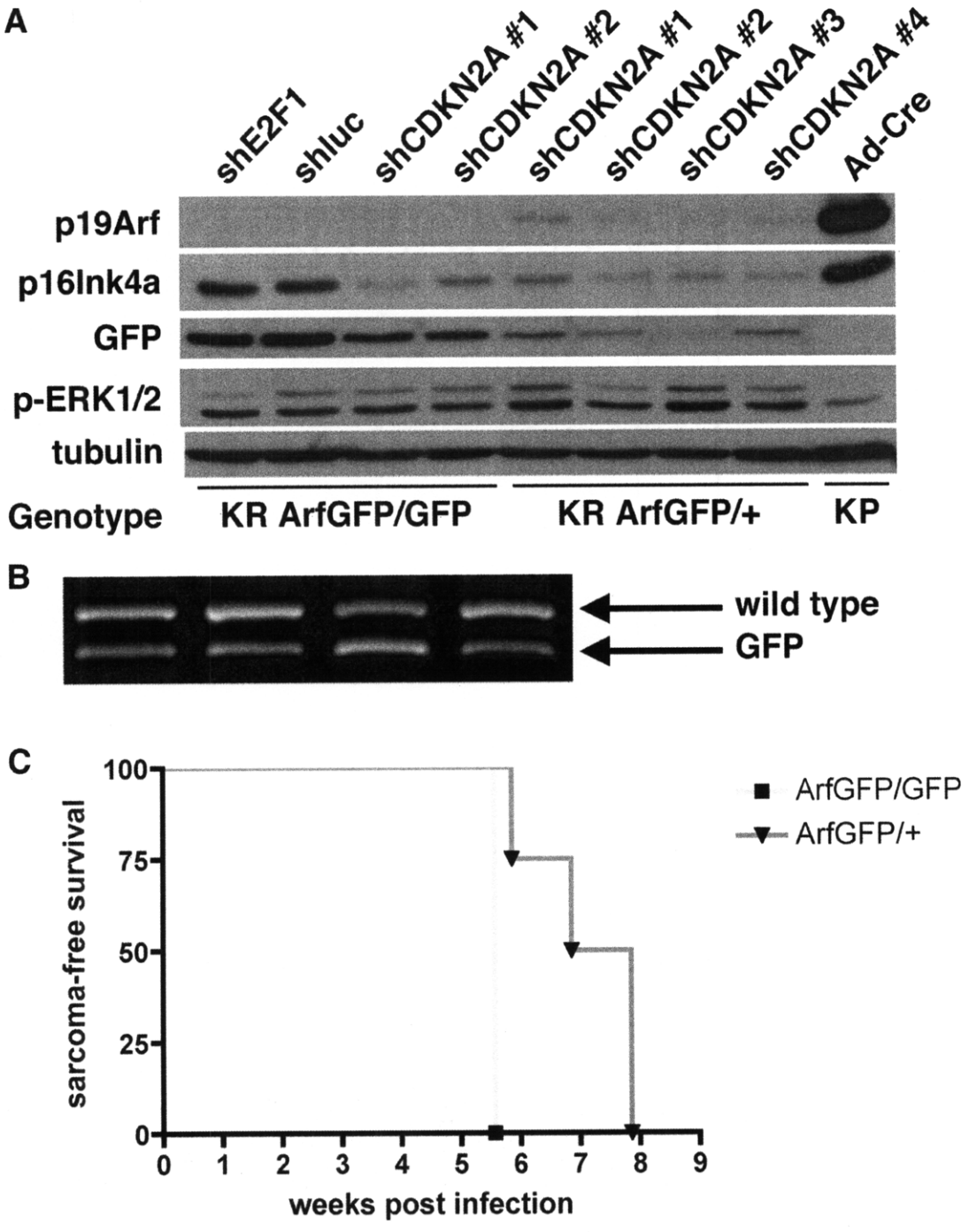
A
shE2F1
shluc
shCDKN2A #1
shCDKN2A #2
shCDKN2A #1
shCDKN2A #2
shCDKN2A #3
shCDKN2A #4
Ad-Cre
p19Arf
p16Ink4a
GFP
p-ERK1/2
tubulin
Genotype
KR ArfGFP/GFP
KR ArfGFP/+
KP
B
wild type
GFP
C
sarcoma-free survival
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9
weeks post infection
ArfGFP/GFP
ArfGFP/+

Figure A3.2 Knockdown of p19Arf by U6.Cre recapitulates p19Arf loss in a mouse model of soft-tissue sarcoma (A) *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/GFP}$ mice (referred to as KR ArfGFP/GFP) and *Kras*$^{LSL\text{-}G12D}$; *Rag2*$^{-/-}$; *Arf*$^{GFP/+}$ mice (referred to as KR ArfGFP/+) mice were intramuscularly infected with lentiviruses expressing Cre and either shluc, shE2F1, or shCDKN2A. *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{f/f}$ mice were intramuscularly injected with adenovirus expressing Cre as a positive control. Sarcomas were collected and Western blotting was performed for p19Arf, p16Ink4a, GFP, p-ERK1/2, and tubulin. (B) DNA was prepared from the KR ArfGFP/+ shCDKN2A tumors described above and PCR was performed at the *Arf* locus. (C) Post-infection sarcoma development was measured in KR ArfGFP/GFP and KR ArfGFP/+ mice infected with shRNAs against CDKN2A.

Finally, these findings provide the potential for reverse genetic screens by RNAi in conditional genetically engineered mouse models of cancer.

Materials and Methods

Cell culture. KP lung cancer cells were generated by trypsin digestion and propagation of a lung tumor from a *Kras*$^{LSL\text{-}G12D}$; *Trp53*$^{f/f}$ mouse (Jackson et al., 2005). *Kras*$^{LSL\text{-}G12D}$ MEFs were generated as described previously (Tuveson et al., 2004). Cells were grown under standard conditions.

Construction of U6.Cre. The U6 cassette was XbaI and XhoI digested from the pSico^{R} vector described previously and cloned into the XbaI- and XhoI-digested UbiC.luciferase.PGK.Cre vector (a kind gift of M. Dupage) (Ventura et al., 2004).

Design and cloning of shRNAs. ShRNAs were designed using pSicoOLIGOMAKER and cloning was performed as described (Ventura et al., 2004). The oligonucleotides used for shluc were described previously (Kumar et al., 2007). The additional shRNA oligonucleotides were as follows:

shMAPK14.1:

TGGCATCGTGTGGCAGTTAATTCAAGAGATTAACTGCCACACGATGCCTTTTTTC

TCGAGAAAAAAGGCATCGTGTGGCAGTTAATCTCTTGAATTAACTGCCACACGATGC

CA

shMAPK14.2:

TGCAGGGACCTTCTCATAGATTCAAGAGATCTATGAGAAGGTCCCTGCTTTTTTC

TCGAGAAAAAAGCAGGGACCTTCTCATAGATCTCTTGAATCTATGAGAAGGTCCCTG

CA

shMAPK14.3:

TGGAAGAGCCTGACCTATGATTCAAGAGATCATAGGTCAGGCTCTTCCTTTTTTC

TCGAGAAAAAAGGAAGAGCCTGACCTATGATCTCTTGAATCATAGGTCAGGCTCTTC

CA

shNEMO:

TGGACATGCTGGGTGAAGAATTCAAGAGATTCTTCACCCAGCATGTCCTTTTTTC

TCGAGAAAAAAGGACATGCTGGGTGAAGAATCTCTTGAATTCTTCACCCAGCATGTC

CA

shCyclinD1:

TGCATGTTCGTGGCCTCTAATTCAAGAGATTAGAGGCCACGAACATGCTTTTTTC

TCGAGAAAAAAGCATGTTCGTGGCCTCTAATCTCTTGAATTAGAGGCCACGAACATG

CA

shCDKN2A:

TGCTGGGTGGTCTTTGTGTATTCAAGAGATACACAAAGACCACCCAGCTTTTTTC

TCGAGAAAAAAGCTGGGTGGTCTTTGTGTATCTCTTGAATACACAAAGACCACCCAG

CA

shE2F1:

TGGAGAAGTCACGCTATGAATTCAAGAGATTCATAGCGTGACTTCTCCTTTTTTC

TCGAGAAAAAAGGAGAAGTCACGCTATGAATCTCTTGAATTCATAGCGTGACTTCTC

CA

Lentivirus production and infection. Lentivirus production was performed as described (Rubinson et al., 2003).

Intranasal, intratracheal and intramuscular infection. Mice were infected intratracheally and intramuscularly as described (Kirsch et al., 2007; Kumar et al., 2008).

Western blotting. Western blotting was performed using standard methods. Antibodies were as follows: p38alpha (9212 from Cell Signaling Technology); N-Ras (sc-31 from Santa Cruz Biotechnology); actin (A5441 from Sigma); NEMO (sc-8330 from Santa Cruz Biotechnology); p16Ink4a (sc-1207 from Santa Cruz Biotechnology); p19Arf (sc-32748 from Santa Cruz Biotechnology); p-ERK1/2 (9101 from Cell Signaling Technology); tubulin (2128 from Cell Signaling Technology); GFP (NB600-597 from Novus Biologicals).

Deletion analysis of *Arf* locus. Deletion at the *Arf* locus was performed by PCR as described previously (Zindy et al., 2003).

Mice. $Kras^{LSL\text{-}G12D}$; $Trp53^{f/f}$ animals were generated as described previously (Jackson et al., 2005). $Kras^{LSL\text{-}G12D}$ animals were crossed to $Rag2^{-/-}$ animals to generate $Kras^{LSL\text{-}G12D}$; $Rag2^{+/-}$, which were interbred to produce $Kras^{LSL\text{-}G12D}$; $Rag2^{-/-}$ animals. These animals were bred to $Arf^{GFP/GFP}$ animals to produce $Kras^{LSL\text{-}G12D}$; $Rag2^{+/-}$; $Arf^{GFP/+}$ animals (Zindy et al., 2003). These animals were backcrossed to $Kras^{LSL\text{-}G12D}$; $Rag2^{-/-}$ animals to produce $Kras^{LSL\text{-}G12D}$; $Rag2^{-/-}$; $Arf^{GFP/+}$ animals, which were intercrossed to produce the experimental cohort.

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Madhu S. Kumar

EDUCATION

- Massachusetts Institute of Technology, Ph.D. Student, Biology Department (Fall 2004–present)
- University of Pennsylvania, Undergraduate, B.A. *summa cum laude* in Biochemistry and Chemistry, GPA 3.95/4.00 (Fall 2000–Spring 2004)

RESEARCH

Laboratory of Dr. Tyler Jacks, Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology (Spring 2005–present)

- Examined the role of microRNA (miRNA) processing in cellular transformation and tumorigenesis. Established a functional role for global downregulation of miRNAs in tumor development.
- Characterized the functional effect of the *let-7* miRNA family on non-small cell lung cancer (NSCLC) development. Demonstrated that let-7 induction suppresses tumorigenesis *in vivo* through regulation of the Ras family and HMGA2 and described putative resistance to let-7 therapeutic delivery.
- Developed a lentiviral vector for co-expression of small RNAs and the Cre recombinase. Applied this system to expression of both miRNAs and short hairpin RNAs in a mouse model of NSCLC.
- Examined coordinate loss of protein-coding genes and miRNAs in human cancer predisposition syndromes.

Laboratories of Drs. Barbara Weber and Marcia Brose, Department of Medicine, University of Pennsylvania (Fall 2001–Spring 2004)

- Performed mutation screen of *BRAF*, *NRAS,* and *KRAS* genes in a panel of melanoma cell lines and lung cancers to characterize novel mutations.
- Performed mutation search of *BRAF* and *NRAS* genes in a panel of papillary thyroid cancers of various histopathologies to determine mutations rates of histological variants.
- Applied array comparative genomic hybridization to the study of head and neck squamous cell carcinoma through the analysis of 20 paired tumor-adjacent normal samples and a panel of head and neck and lung cancer cell lines.
- Characterized the role of mutant B-Raf in a papillary thyroid cancer cell line through both RNA interference against B-Raf and treatment with pharmacological agents targeted to the B-Raf kinase signaling cascade.

Laboratory of Dr. Alice Haddy, Department of Chemistry, University of North Carolina Greensboro (Summer 2001)

Laboratory of Dr. Marjan van der Woude, Department of Microbiology, University of Pennsylvania (Fall 2000–Spring 2001)

TEACHING

Chemistry and Biology Tutor, Tech Tutors of America (Spring 2008–present)

Teaching Assistant, Spring 2006 and 2008, 7.17, Experimental Molecular Biology: Biotechnology

Chemistry Mentor, Mentoring Program, Department of Chemistry, University of Pennsylvania (Fall 2003–Spring 2004)

Chemistry Tutor, Tutoring Center, University of Pennsylvania (Fall 2002–Spring 2003)

AWARDS AND HONORS

Recipient, Human Frontier Science Program Long-Term Fellowship, Fall 2009

Mentor of Speaker, MIT Biology Undergraduate Research Symposium, Spring 2009

Recipient, MIT Ludwig Graduate Research Fellowship, Fall 2008

Mentor of Recipient, Salvador E. Luria Prize, MIT Department of Biology Undergraduate Awards Banquet, Spring 2008

Mentor of Recipient, MIT Biology Undergraduate Research Symposium Award, Spring 2007

Recipient, Teresa Keng Graduate Teaching Prize, MIT Department of Biology, Fall 2006

Recipient, National Science Foundation Graduate Research Fellowship Award, Spring 2005

Recipient, Priestley Prize, Department of Chemistry, University of Pennsylvania, Spring 2004

Recipient, College Alumni Society Travel Grant, University of Pennsylvania, Spring 2004

Recipient, University Scholars Funding, University of Pennsylvania, Summer 2003, Spring 2004

Recipient, Goldfeder Family Undergraduate Research Grant, University of Pennsylvania, Summer 2003

Phi Beta Kappa, University of Pennsylvania, Spring 2003

Dean's List, University of Pennsylvania, Fall 2000–Spring 2004

University Scholar, University of Pennsylvania, Fall 2001–Spring 2004

Vagelos Scholar of Molecular Life Sciences, University of Pennsylvania, Fall 2000–Spring 2004

Benjamin Franklin Scholar, University of Pennsylvania, Fall 2000–Spring 2001

PUBLICATIONS

Ma C, Quesnelle KM, Sparano A, Rao S, Park MS, Cohen MA, Wang Y, Samanta M, **Kumar MS**, Aziz MU, Naylor TL, Weber BL, Fakharzadeh SS, Weinstein GS, Vachani A, Feldman MD, Brose MS. Characterization CSMD1 in a large set of primary lung, head and neck, breast and skin cancer tissues. *Cancer Biology and Therapy*. 2009 May 9;8(10).

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PRESENTATIONS

Kumar MS, Chen CY, Jacks T. Induction of let-7g in an autochthonous model of non-small cell lung cancer. Beatson International Cancer Conference: Cell Growth, Metabolism, and Cancer. Cancer Research UK, June 15-18, 2008.

Erkeland SJ, **Kumar MS**, Pester RE, Chen CY, Sharp PA, Jacks T. The *let-7* microRNA family suppresses non-small cell lung tumorigenesis *in vivo*. The Role of Non-Coding RNAs in Cancer. American Association for Cancer Research, October 31-November 3, 2007.

Kumar MS, Erkeland SJ, Pester RE, Sharp PA, Jacks T. The *let-7* family of microRNAs functionally hinders non-small cell lung tumorigenesis. Mechanisms and Models of Cancer. The Salk Institute, August 8-12, 2007.

Kumar MS, Lu J, Ventura A, Golub TR, Jacks T. Defective microRNA maturation plays a functional role in tumorigenesis. LXXI Cold Spring Harbor Sympsoium on Quantitative Biology, May 31-June 5, 2006.

Sparano A, **Kumar MS**, Quesnelle KM, Brose MS. Gene Copy Number Alterations Involving CSMD1 in Oral Squamous Cell Carcinoma. 2005 Annual Meeting, American Academy of Otolaryngology-Head and Neck Surgery Foundation, September 25-28, 2005.

Brose MS, **Kumar MS**, Quesnelle KM, Volpe P, Sewell D, Feldman M, Weber BL. Characterization of head and neck squamous cell carcinoma using aCGH and expression profiling. 96th Annual Meeting, American Association for Cancer Research, April 16-20, 2005.

Baloch Z, **Kumar MS**, Lai M, Volpe P, LiVolsi VA, Mandel SJ, Brose MS. Rate of BRAF and NRAS mutations in thyroid nodules undergoing fine needle aspiration. 76th Annual Meeting, American Thyroid Association, September 29-October 3, 2004.

Kumar MS, Moore KE, Brose MS. Functional analysis of B-RAF in a papillary thyroid cancer cell line. 76th Annual Meeting, American Thyroid Association, September 29-October 3, 2004.

Brose MS, **Kumar MS**, Volpe P, O'Donnell R, Ziober B, Sewell D, Feldman M, Weber BL. Genome-wide characterization of oral cavity squamous cell carcinoma using aCGH and expression profiling. 95th Annual Meeting, American Association for Cancer Research, March 27-31, 2004.

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REFERENCES

Dr. Tyler Jacks, PhD
MIT Center for Cancer Research, E17-517
40 Ames Street
Cambridge, MA 02139
Tel: 617-253-0262
Email: tjacks@mit.edu

Dr. Phillip A. Sharp, PhD
MIT Center for Cancer Research, E17-529
40 Ames Street
Cambridge, MA 02139
Tel: 617-253-6421
Email: sharppa@mit.edu

Dr. Todd R. Golub, MD
Broad Institute of MIT and Harvard

7 Cambridge Center
Cambridge, MA 02142
Tel: 617-252-1927
Email: golub@broad.harvard.edu