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14. ABSTRACT To date, most cancer research has focused on alterations in the sequence, gene structure, copy number and expression of protein coding genes. However, there are increasing studies discovered that a diversity of non-coding RNAs decoded in the genome also plays an essential role in cancer. MicroRNAs (miRNAs), which are small, 21-24nt RNAs generated by the key enzyme Dicer, represent a prominent class of such non-coding RNAs. It has been reported that some miRNAs act as oncogenes to promote tumor formation in collaboration with protein-coding oncogenes. On the other hand, several miRNAs are found to function as tumor suppressors. Our previous study revealed a miRNA family, miR-34, as direct transcriptional target of p53, the master tumor suppressor gene. To address the role of miR-34 in cancer formation and maintenance, we generated cell lines over-expressing miR-34. We have demonstrated that ectopic expression of miR-34 in both primary and tumor cell lines can induce growth arrest through repression of cell cycle genes, and we have shown in animal models that tumor cells over expressing miR-34 have disadvantage in tumor initiation and maintenance. Our work placed miRNAs as one of the central mediators of p53 tumor suppressor network, which plays an important role in many cancer types, including breast cancer.					
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Introduction

Cancer arises from genetic lesions that result in uncontrolled proliferation, cell survival, loss of differentiation and invasive growth. So far, cancer studies have focused on genetic alterations in protein coding genes. It is only recently that non-coding RNAs, in particular, microRNAs (miRNAs) have been shown to play important roles in cancer. Since then, a number of studies further support the idea that miRNAs can be components of oncogenic and tumor suppressor networks.

MiRNAs are small, non-coding RNAs which regulate gene expression through post-transcriptional repression. Nascent miRNA transcripts (pri-miRNAs) are first transcribed from the genome, and then processed sequentially by two key ribonuclease III enzymes, Drosha and Dicer, to generate mature miRNAs duplexes which are 21nt to 24nt in length. Usually, one strand from the miRNA duplex is incorporated into the effector complex, the RNA-induced silencing complex (RISC). RISC recognizes specific target mRNAs through imperfect base-pairing, and down-regulates their expression by post-transcriptional gene silencing.

MiRNAs recognize their target genes by binding to their complementary base-pairing sites on the target mRNA. A series of mutational analyses indicated that the most critical interactions between the miRNA and its targets occur within the 5' region of the miRNA. Therefore, the eight nucleotides at the 5' end of a miRNA are designated as the "seed" sequence, whose complementarity to the target mRNA has been employed to search for candidate targets. In a recent study by Lewis et al., more than 5300 human genes are predicted as conserved miRNA targets, representing 30% of human genome.

Increasing evidence has suggested that miRNAs are components of oncogene and tumor suppressor pathways. Inappropriate expression and structural alterations of miRNA genes have been found in a variety of tumor types, and several functional studies have shown the oncogenic or tumor-suppressive potential of specific miRNA families. Our study uncovered the miR-34 family of miRNAs in the p53 tumor suppressor network. The functional study of miR-34 has shown that miR-34 possess anti-proliferative potential by repressing cell cycle genes. Deletion of miRNAs of the miR-34 family has been reported in several human tumours and cancer cell lines. And our animal data has indicated miR-34 may act as tumor suppressor which may afford new opportunities for diagnosis and treatment of human cancer. Furthermore, the generation of miR-34 knockout animals provides a platform to characterize miR-34's role in various mouse cancer models, including breast cancer.

Body

Creation of Dicer deficient cells and animals

Dicer is a ribonuclease enzyme that is involved in the biogenesis of miRNAs and other small RNAs. To elucidate the role of Dicer and miRNAs in mammals, and particularly in cancer, we created embryonic stem (ES) cells with a conditional targeted allele of Dicer. We found that once induced to delete Dicer, these cells were deficient in proliferation. Indeed, most Dicer deficient ES cell clones were unable to outgrow.

To further investigate this phenotype, we used the ES cells to establish a mouse line harboring the conditional Dicer allele. This system was used to delete Dicer in various adult and developmental compartments, including oocytes, skin and brain. Interestingly, we found that there is a requirement for Dicer for the correct assembly and function of the meiotic spindle in oocytes.

Conditional expression of miR-34 in tumorigenesis

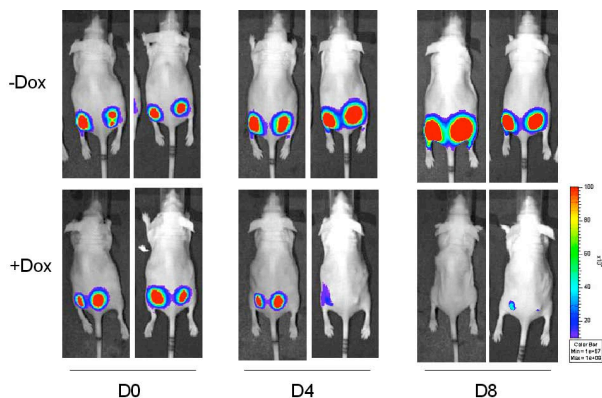


Fig 1. Acute induction of mir34-a results in complete tumor regression. (clone 1)

In some clones, miR-34a induction leads to complete remission of S.C. tumors.

clones, some undergo apoptosis after Dox treatment while the other showed a senescence phenotype. Several cell clones were tested *in vivo*. Clone 1 showed a possible apoptosis phenotype and tumors regressed after mir-34a activation (Fig 1). Clone 2 and clone 3 showed a cellular senescence phenotype upon Dox treatment, and it was soon apparent that all tumors halted growth as compared to the untreated tumors (data not shown), Overall, these results imply that miR-34 can act as tumor suppressors *in vivo*.

In vivo delivery of miR-34a siRNA in mouse model

Recent advances in *in vivo* delivery of synthetic miRNA or its antagonists suggest miRNA can be applied as cancer therapy. We next investigated the possibility of using tumor suppressor miR-34a small RNA as potential therapeutic tools.

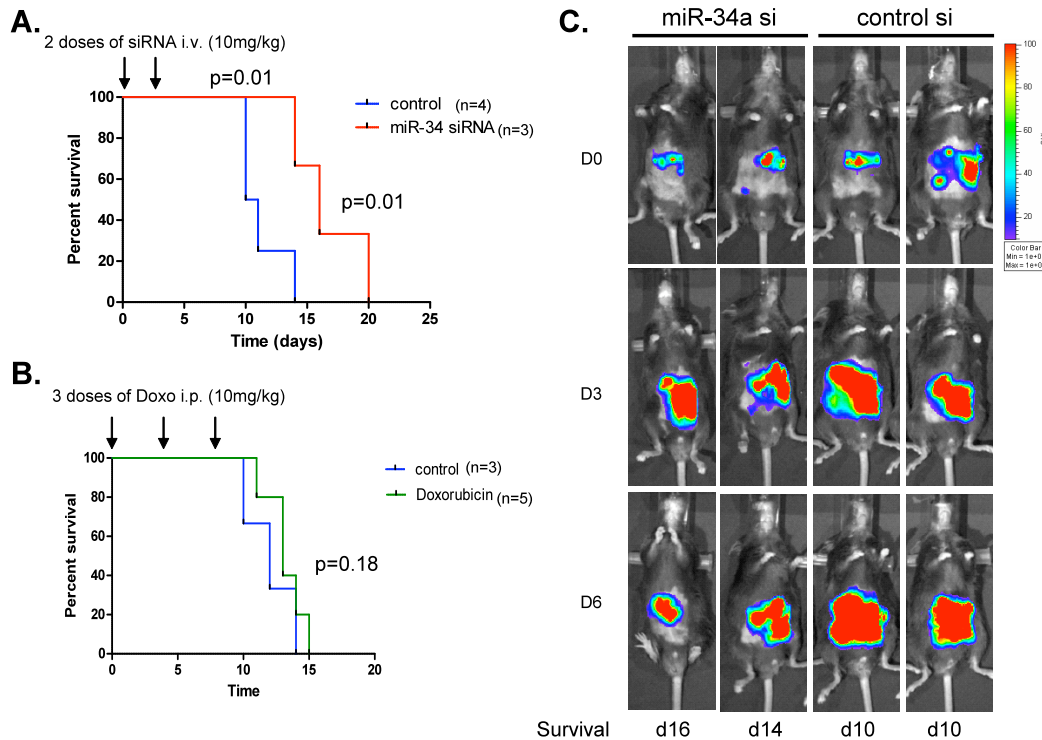


Figure 2. Testing *in vivo* delivery of miR-34a siRNA in a mouse model of liver cancer. (A) C57/B6 mice were transplanted with p53^{-/-};Ras liver tumor cells into the liver. 21 days post transplantation, the mice were treated with miR-34a siRNA or control siRNA in invivolectamine reagents. Kaplan-Meier curves shows survival of the mice. The first treatment is set as D0. (B) B6 mice receiving transplantation of p53^{-/-}; Ras liver tumor cells were treated with chemotherapy drug Doxorubicin or vector (control). Kaplan-Meier curves shows survival of the mice as in (A). (C) Luciferase imaging of the mice treated with miR-34a or control siRNA indicates the delayed tumor progression in mice treated with miR-34a siRNA..

Intravenous injections (i.v.) of chemically modified small RNA duplex can efficiently deliver siRNA to organs such as lung, kidney, liver and spleen. This technique features high stability against nucleases, minimal induction of the interferon response and easy tracking of administered RNAi duplexes using fluorescently labeled siRNA duplex.

As shown in Fig 2A, we treated a subset of our tumor harboring mice with miR-34a siRNA by two doses of i.v. injections and the other mice with control siRNA. The control mice survived 11.25±1.89 days post treatment and the mice receiving miR-34a injection significantly improved survival (16.67±3.06 days). In contrast, a commonly used chemotherapy drug Doxorubicin, failed to provide survival advantage in the same experiment setting (Fig 2B). Using *in vivo* bioluminescence imaging, we confirmed that miR-34a siRNA delays the progression of tumor growth (Fig 2C). These results suggest the potential of using miR-34a siRNA as a tool in cancer therapy.

Development of miR-34a knockout animals

Our previous analysis of *mir-34's* function reveals its role in mediating cell cycle arrest and suppressing a family of cell cycle related genes. To further explore *mir-34's* function in p53 mediated tumor suppression network, we created constitutive loss-of-function alleles for

We transplanted p53^{-/-}; Ras-IRES-Luciferase tumor cells into the livers of recipient mice (Fig 2). The luciferase tag allowed us imaging and quantifying tumor burden in mice. We used invivolectamine[®] reagents for *in vivo* delivery of miR-34a siRNA. Invivolectamine[®] is a liposome based RNAi delivery technique (InvitrogenTM).

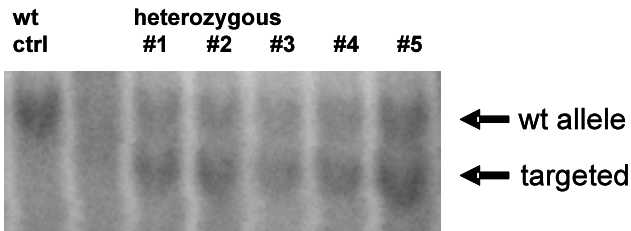


Figure 3: Crossing miR-34a heterozygous mice to make null mice. Southblot to genotype heterozygous miR34^{+/-} mice.

mir-34s in mice and tried to use these animals as a platform to characterize *mir-34*'s role in various mouse cancer models.

We constructed the constitutive knock-out alleles of *mir-34s* in which the primary miRNA transcript is replaced by LacZ (the gene encoding β -galactosidase). Targeted ES cells were microinjected into blastocysts by Dr. Sang Yong Kim in the CSHL animal facility. The chimeric mice were

born and were bred for germline transmission. Heterozygous *mir-34a* deficient animals were obtained through successful germline transmission (Fig 3), and we set up heterozygous to heterozygous cross to obtain *mir-34a* null mice.

Generation of miR-34a deficient cell lines

Mir-34a null mice were born from heterozygous to heterozygous cross and showed no obvious prenatal or postnatal developmental defects. We've generated MEF (mouse embryonic fibroblast) from *mir-34a* null and paired wild type embryos. Southern blotting shows successful deletion of *mir-34a* in the nulls compared to wildtype (Fig 4 up-left). Similar results were obtained by genomic PCR using allelic specific primers (Fig 4 lower-left). QPCR analysis shows there is undetectable (ND) *mir-34a* mature miRNA in the knockout MEF population (Fig 4 right) where as *mir-34b,c* are expressed at normal level.

We are in the process of characterizing the phenotype of *mir-34a* knockout MEF. As *mir-34a* is induced by p53 and its overexpression resulted in cell cycle arrest or apoptosis, we hypothesized that loss *mir-34a* allele may lead to increased cell proliferation and protection against irradiation induced apoptosis. Preliminary data showed that *mir34^{-/-}* MEF, like the *p53^{-/-}*, grow faster than wildtype MEF in the population doubling assay. BrdU incorporation assay showed there are more S phase cells in *mir-34a* null MEF (data not shown).

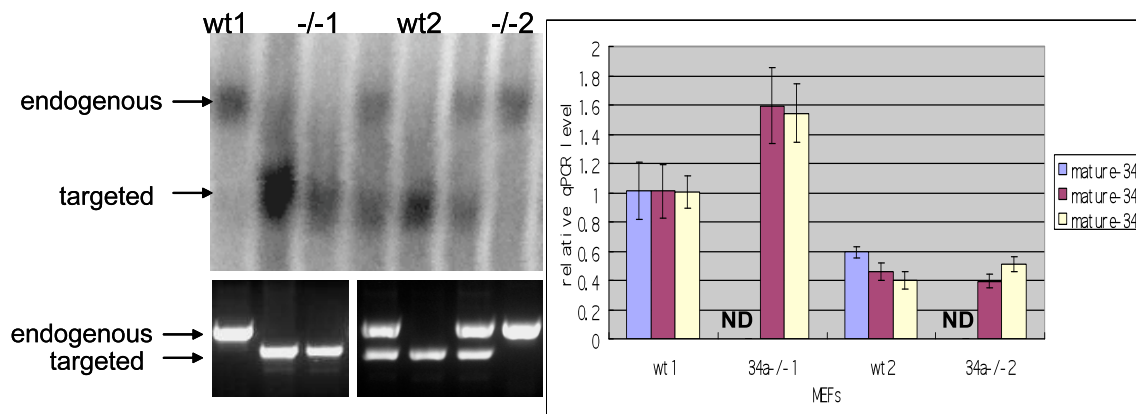


Figure 4 (A) Southern blot of paired wildtype and *miR-34a^{-/-}* MEF. (B) PCR from genomic DNA. (C) QPCR using primers detecting mature *miR-34a,b*, or *c*. ND, not detectable.

Key Research Accomplishments

- The generation of Dicer deficient cell lines and mice
- The identification of a miRNA family, miR-34, as direct transcriptional target of p53 tumor suppressor, mediating cell cycle arrest and apoptosis.
- Conditional expression of miR-34a in tumor cells result in delayed tumor growth or even tumor regression
- In vivo delivery of miR-34a as potential tool of cancer therapy
- The development of miR-34a knockout animals and cell lines

Reportable outcomes

Manuscripts

He X, He L, Hannon GJ. The guardian's little helper: microRNAs in the p53 tumor suppressor network. *Cancer Res.* 2007 Dec 1;67(23):11099-101.

He L, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nat Rev Cancer.* 2007 Nov;7(11):819-22.

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Murchison EP and Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery. *Curr. Opin. Cell Biol.* Jun;16(3):223-9

Conclusion

Dicer is a key enzyme in the biogenesis of miRNAs and other small RNAs. We generated genetic tools for studying the role of Dicer in mammals. In particular, we found that Dicer is required for the survival and proliferation of embryonic stem cells, and for spindle integrity in mouse oocytes. Dicer deficient cells and mice are an important resource for the community, and provide an interesting opportunity to study the global role of miRNAs in cancer.

P53 responds to DNA damage or deregulation of mitogenic oncogenes through the induction of cell cycle checkpoints, apoptosis, or cellular senescence. Mutations in *p53* are often associated with aggressive tumor behavior and poor patient prognosis. The p53 tumor suppressor network has been intensively studied; however, genetic analyses long hinted at the existence of components that remained elusive. For example, although p53 is clearly a transcriptional activator, numerous reports indicated that p53 also represses the expression of specific genes either directly or indirectly. The manner in which this occurred was obscure, with both transcriptional and posttranscriptional suppression as possible mechanisms. In the latter case, the discovery of extensive networks of miRNAs, offered the possibility that p53-mediated control of miRNA expression could allow it to act indirectly to repress target gene expression at the posttranscriptional level.

Our studies have identified miR-34 as a miRNA component of the p53 network, for the first time revealing interplay between proteins and non-coding RNAs in this pivotal tumor-suppressor pathway. Ectopic expression of miR-34 recapitulates the biological effects of p53, including growth arrest in our study and apoptosis by several recent studies, through its ability to dampen the expression of pro-proliferation and anti-apoptotic genes. Along with the report that deletion of miR-34 family miRNAs has been found in many cancer types, our animal work implies miR-34 as potential tumor suppressor and cancer therapy tool. These findings suggest that miRNAs, and in a broader sense non-coding RNAs, may be previously unrecognized but integral components of established oncogene and tumor-suppressor networks.

Thus, it is critical to explore miRNA's value as novel therapeutical targets and/or diagnosis markers. Since the *in vivo* delivery of sequence-specific miRNA mimics and antagonists has been gaining great appreciation, it's technically possible to express or inhibit certain miRNAs in tumors and test their role in tumor maintenance. In addition, large-scale expression studies of miRNA profiles in multiple human tumor types have revealed that miRNA signatures are correlated with the developmental lineage and differentiation status of various tumors. Moreover, miRNA signatures can be used to identify certain poorly differentiated tumors, many of which were difficult to be classified based on mRNA profiles. Such findings suggest an unexpected potential of miRNAs as diagnostic tools, and possibly therapeutic targets.

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