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RNAi, microRNAs, and human disease

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Abstract MicroRNAs (miRNAs) are short, noncoding RNAs that posttranscriptionally regulate gene expression. Over 300 miRNA genes have been identified in the human genome. We have undertaken the study of miRNA function in mammals. Using a custom microarray platform, we investigated miRNA expression patterns in mammalian development and in cancer. We found that many miRNAs are downregulated in cancer. On the other hand, several miRNA genes are overexpressed in tumor cell lines and primary tumors. Seven of these cancer-associated miRNAs are clustered in a single primary transcript termed chr13orf 25 or OncomiR-1. This cluster is located in a region amplified in lymphoma and several solid malignancies. Ectopic expression of these miRNAs in a mouse model of lymphoma accelerated disease progression. In addition, the lymphomas had reduced apoptosis and were more disseminated into secondary regions. This work establishes noncoding RNAs, and specifically miRNAs, as oncogenes in human cancers.

Keywords miRNA · MicroRNA · OncomiR · Lymphoma

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Introduction

As mammalian genome sequences have been completed, it has become increasingly clear that large regions of intergenic sequence are present. Only 2% of the human genome consists of coding sequence for "known" genes. Recent expression data, however, suggest that as much as 50% of the genome is transcribed into RNA [3, 4, 13]. Many of these transcripts do not contain an open reading frame and thus are not likely translated into protein. Rather, they might encode biologically active RNA, or noncoding RNA. One such group of noncoding RNAs is microRNAs (miRNAs). miRNAs are endogenous triggers of the RNA interference (RNAi) pathway. This gene-silencing pathway was discovered by Andrew Fire, Craig Mello and colleagues in 1998 [6]. They found that injection of double-stranded (ds)RNA into adult *Caenorhabditis elegans* leads to silencing of genes that are homologous to the dsRNA trigger. This silencing was observed in the injected adult and in F1 progeny. Early work by the groups of Fire and Mello revealed that gene silencing by RNAi is a novel genetic pathway and not a result of antisense inhibition [6, 21]. Furthermore, their work demonstrated that gene silencing is a result of the destruction of mRNAs homologous to the dsRNA trigger.

Work by a large number of biochemists and geneticists led to a working model of the RNAi pathway (see [27] for a review). In this pathway, dsRNA is processed by the ribonuclease III enzyme Dicer to generate approximately 21-nucleotide short interfering RNAs (siRNAs). Dicer is assisted by the dsRNA binding protein TRBP. In a concerted reaction, siRNA is loaded into the RNA-induced silencing complex (RISC). The guide strand of the siRNA locates homologous

mRNAs, which are cleaved by the endonuclease Argonaute [7]. Subsequently, the mRNA fragments are degraded and gene silencing occurs.

The RNAi pathway has acquired popularity due to its value as an experimental tool. This is most apparent in work on cultured mammalian cells. It is now possible to silence the expression of a gene of interest by transfection of homologous siRNAs. Such an approach has been extended to whole genomes [12, 20]. In *C. elegans*, plants, and mammals libraries of dsRNAs or siRNAs are being used as genetic screening tools. It should be emphasized, however, that the RNAi pathway is an endogenous gene regulatory pathway.

While dsRNA is the most common trigger of RNAi, any single-stranded RNA that can fold into a stem-loop structure is a potential trigger due to the double-stranded nature of the stem. Such RNA structures are encoded in many genomes and are termed miRNAs. They function as endogenous triggers of RNAi and serve as regulators of gene expression.

The founding miRNA gene, *lin-4*, was identified by Gary Ruvkun, Victor Ambros and colleagues in *C. elegans* [16, 28]. This gene is essential for proper timing of events during larval development. The discovery of a second *C. elegans* miRNA, *let-7*, implied that these noncoding RNAs belong to a larger group of undiscovered genetic regulators [23]. Both *let-7* and *lin-4* are conserved throughout evolution. *Let-7*, in particular, is 100% conserved from *C. elegans* to human.

Recently, a large number of miRNA genes have been identified. Over 350 miRNAs have been verified in humans, and the final number is predicted to reach 1,000. In addition, hundreds of miRNA genes have been identified in other organisms ranging from *C. elegans* to *Drosophila* to plants. To understand the function of miRNAs, it is essential to characterize the mRNA targets. Several computational algorithms have been developed for this purpose. For example, an algorithm developed by Chris Burge, David Bartel and colleagues predicts that each miRNA targets several hundred mRNAs [17]. Therefore virtually all human genes are potentially regulated by miRNAs. While many genes are predicted as targets, the degree of gene silencing may be small. In that case, many miRNAs may function as a "fine tuning" mechanism to prevent inappropriate expression of a large set of genes. This is in contrast to the function of *C. elegans lin-4* and *let-7*, which likely perform a "master switch" function by regulating a small number of targets to a large degree.

miRNA biogenesis

Figure 1 shows an overview of the miRNA biogenesis pathway (see [14] for a review). miRNAs are transcribed by RNA polymerase II. The primary transcript, termed the pri-miRNA, is capped and polyadenylated and typically *cis*-spliced. The miRNA stem loop can be present in an intron or an exon. Approximately 25% of human miRNAs are located in introns of protein-coding genes [24]. An example of this is the miRNA cluster comprising miR-106b, miR-93, and miR-25 located in an intron of the protein-coding gene *Mcm7*. The timing of splicing versus miRNA processing is not known.

After the pri-miRNA has been transcribed it is sequentially processed to liberate active miRNA species. The first step is removal of the stem loop by the ribonuclease Drosha (see [14] for a review). This generates the precursor, or pre-miRNA. This species is approximately 75 nucleotides long and contains the active, mature miRNA component. The precursor is exported to the cytoplasm by Exportin-5. It is further processed by the ribonuclease Dicer to generate an approximately 22-nucleotide dsRNA that resembles

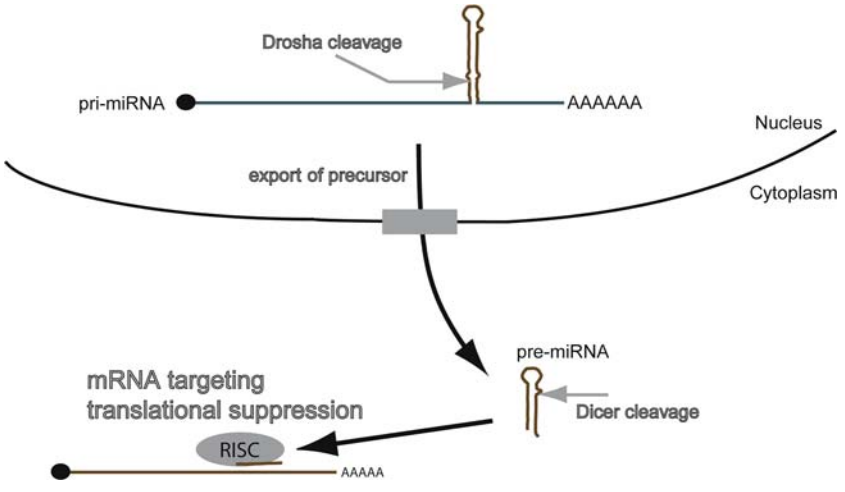

Fig. 1 Overview of the miRNA biogenesis pathway. miRNAs are generated as primary transcripts termed pri-miRNAs. After two ribonuclease cleavage steps the mature, approximately 22-nucleotide miRNA species is produced. Mature miRNA is incorporated into the RNAi effector complex RISC where it directs translational suppression of targeted mRNAs

siRNA. The active strand, termed the guide strand, is incorporated into the RNAi effector machine RISC. This RNA is referred to as "mature" miRNA. It guides RISC to homologous mRNAs where it targets them for translational suppression and mRNA degradation [27].

While 350 miRNAs have been identified in humans, little is known about the cellular pathways that they regulate. miRNAs have been linked to a number of developmental processes. Several reports have also correlated miRNA expression with tumorigenesis (see [8] for a review). Such reports certainly represent only a small fraction of the cellular functions of miRNAs.

Technologies to study miRNA biology

This author's laboratory has embarked on a program to develop technologies for the study of miRNA function. Our first project was the development of a microarray platform for miRNA expression analysis. The goal of this was to identify miRNA genes that correlate with specific biological processes. This was followed by the development of retroviral vector systems for overexpression of miRNAs. These vectors are used in cellular- and animal-based phenotypic assays for functional confirmation of miRNAs identified by expression profiling. Recently, we have been developing a functional screen for identifying targets of miRNAs.

Our miRNA microarray platform is a spotted oligonucleotide array [26]. The probes correspond to oligonucleotides antisense to all mature human and mouse miRNA sequences. Our labeling method has been optimized for miRNAs. Using T4 RNA ligase we ligate a Cy3-conjugated dinucleotide to the miRNA pool in a total cellular RNA fraction. This labeled RNA is hybridized to the microarray. Standard data analysis methods are used to generate clustered heat maps.

Our initial studies have demonstrated that these arrays are specific for mature miRNA species. RNAs were gel-isolated from 22-, 35-, 65-, and 90-nucleotide size ranges. Only RNAs from the 22-nucleotide size range had significant hybridization to the array. Mismatched probes were included on the array to determine specificity; those with a single mismatch yielded minimal hybridization, demonstrating specificity.

Our initial expression profiling efforts were against standard mouse tissues and embryonic stages. As previously reported, we observed limited miRNA expression in early development. Many miRNAs commence expression mid-gestation, with a large number expressed in adult tissues. We have noted tissue-restricted expression of several miRNAs [26].

miRNA expression and cancer

Our next goal was to analyze miRNA expression in cancer. We compared expression levels in tumor cell lines versus in normal tissues. To identify miRNAs that have a general role in cancer we profiled cell lines from a wide range of tissue origins. The cell lines included carcinomas, sarcomas, neuroblastomas, and teratocarcinomas. The results were striking: tumor cell lines had a similar expression pattern regardless of tissue of origin (Fig. 2). For example, the *Let-7* family of miRNAs is highly expressed in all differentiated tissues; in cancer cell lines, however, this expression was much reduced (Fig. 2a). This miRNA family has recently been shown to target the oncogene Ras [10]. This provides a model whereby cells increase *let-7* during differentiation resulting in decreased Ras expression and therefore increased proliferation. A second cluster of miRNA genes exhibited reduced expression in tumor lines. These genes are shown in Fig. 2b. Previous reports have linked reduced expression of these miRNAs with cancer [2, 19]. In particular, *miR-15*/*miR-16* is deleted in a large fraction of chronic lymphocytic leukemia patients [2]. One validated target of these miRNAs is the anti-apoptotic gene *Bcl-2* [5]. Cells that harbor the deletion of *miR-15*/*miR-16* have elevated levels of Bcl-2, which may account for the tumor suppressor function of these miRNAs.

The reduced expression of miRNAs is similar to previously published data on expression in primary tumors [18, 25]. Many miRNAs that are highly expressed in differentiated tissues are reduced in cancer. Reduction of miRNA expression presents an interesting parallel between cancer and development. As in cancer, early developmental stages have reduced levels of many miRNAs that are abundant in differentiated tissue. This may reflect a loss of differentiation that is a hallmark of cancer.

Not all miRNAs were reduced in cell lines, however. A small group of miRNAs was highly expressed in most cell lines compared with normal tissues (Fig. 2c). These miRNAs included miR-17, miR-18, miR-19a, miR-20, miR-19b, and miR-92 [9]. All six miRNA stem loops are encoded in a single primary transcript named chr13ORF25 [22]. These six stem-loops generate seven mature miRNAs, all of which are highly expressed in most cell lines. We hypothesize that these miRNAs promote cellular events that are hallmarks of cancer including increased proliferation, decreased apoptosis, etc. Therefore we named the primary transcript for these miRNAs "OncomiR-1," for oncogenic miRNA-1 [9].

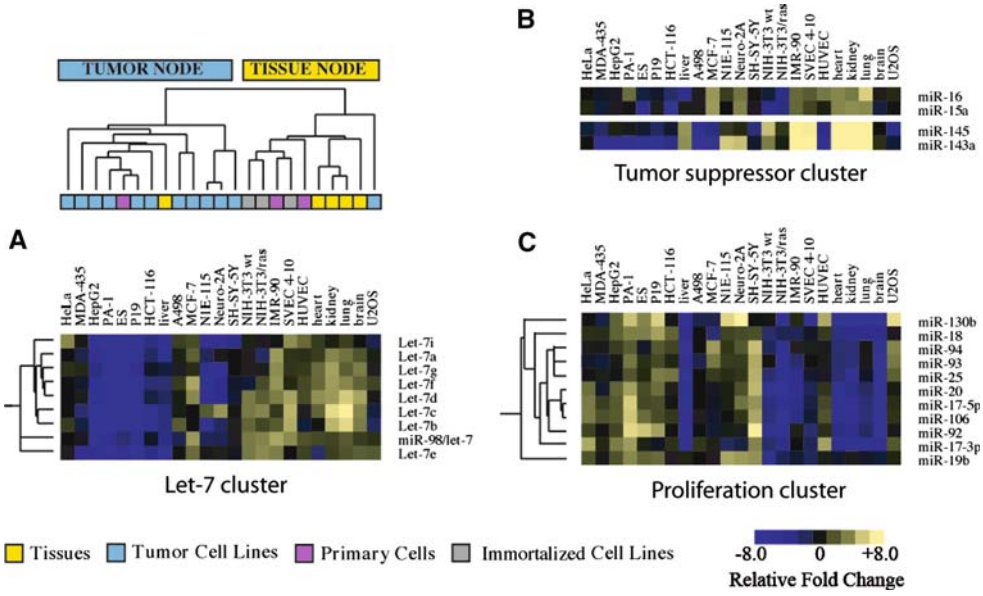

Fig. 2 miRNA expression in tumor cell lines. Normalized, log-ratio expression measurements for 124 human and mouse miRNAs were hierarchically clustered in both dimensions according to the method of Thomson et al. [26]. *Yellow* indicates high expression relative to the mean, and *blue* indicates low expression. Three clusters are shown

If these miRNAs contribute to human cancer we would expect to find them within cancer-associated amplicons. In this regard, at chr13q31 there is a region that is amplified in solid tumors and lymphomas [11, 22]. The only annotated gene in this amplicon is GPC-5, a cell surface protein with no known link to cancer. OncomiR-1 is also located within this amplicon, raising the possibility that it is the relevant cancer-susceptibility gene at this locus. An analysis of mature miRNAs from OncomiR-1 demonstrated that their expression correlated with the copy number of the chr13q31 amplicon [9].

To test the role of OncomiR-1 in tumorgenesis we turned to a mouse model for Burkitt's lymphoma [1]. Transgenic animals expressing the *c-Myc* oncogene from immunoglobulin heavy chain enhancer develop B-cell lymphomas due to *c-Myc* expression in early B cells. Similarly, hematopoietic stem cells (HSCs) derived from these mice generate B-cell lymphomas with similar latency when introduced into recipient animals. Using this system it is possible to test the functional role of a gene of interest in lymphomagenesis by ectopic expression of that gene in derived HSCs. We constructed a retroviral expression vector driving constitutive expression of OncomiR-1. This vector was introduced into HSCs and injected into recipient animals. Expression of OncomiR-1 dramatically accelerated development of lymphoma [9]. For example, among control animals that expressed *c-Myc* only, 70% were lymphoma-free after 90 days. In contrast, animals expressing OncomiR-1 developed lymphoma beginning at 45 days, and by 90 days all animals had developed lymphoma.

The expression cassette additionally directed expression of a GFP marker, allowing whole-animal imaging of tumors. Control animals expressing *c-Myc* alone had tumors that were limited to the lymphoid compartments. In contrast, animals additionally expressing OncomiR-1 had tumors that were highly disseminated. These data demonstrate that OncomiR-1 accelerates lymphoma and promotes a more disseminated disease.

Histologic examination was performed on tumors from control animals and animals expressing OncomiR-1. Control tumors had a high apoptotic index, a hallmark of Myc-expressing lymphomas. Tumors expressing OncomiR-1, in contrast, exhibited little apoptosis. This suggests that OncomiR-1 functions by limiting the apoptotic response to oncogene expression. The mechanism of apoptosis inhibition is not known at present.

Conclusion

Our data and data from other laboratories have outlined a hypothesis between miRNAs and cancer. Tumors exhibit reduced expression of many miRNA genes including the *Let-7* family. This promotes loss of differentiation, which is a common feature of cancer. In contrast, several oncogenic miRNA genes are expressed at high levels in cancer. These miRNAs may promote stem properties of tumors since they are also highly expressed in stem cells. Several questions remain in the field. What is the mechanism for the widespread alteration of gene expression in cancer? Is it transcriptional, or is miRNA biogenesis altered? Are

miRNAs viable therapeutic targets? Recent work by Markus Stoffel's group has demonstrated miRNA inhibition in the live mouse [15]. This raises the possibility of anticancer therapeutics based on miRNA inhibitors.

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