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MicroRNAs as oncogenes

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MicroRNAs (miRNAs) are a class of non-coding RNAs that function as endogenous triggers of the RNA interference pathway. Originally discovered in *Caenorhabditis elegans*, this group of tiny RNAs has moved to the forefront of biology. With over 300 miRNA genes identified in the human genome, and a plethora of predicted mRNA targets, it is believed that these small RNAs have a central role in diverse cellular and developmental processes. Concordant with this, aberrant expression of miRNA genes could lead to human disease, including cancer. Although the connection of miRNAs with cancer has been suspected for several years, four recent studies have confirmed the suspicion that miRNAs regulate cell proliferation and apoptosis, and play a role in cancer.

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Introduction

Non-coding RNAs as oncogenes

Efforts over the past 40 years have uncovered a large number of genes that contribute to human cancer [1]. The sequencing of the human and mouse genomes, combined with genomics approaches such as microarray analysis, has accelerated the discovery of cancer susceptibility genes. In spite of the large number of identified genes, many more might have been overlooked. This is because most of the genomics efforts have been directed at annotated, protein-coding genes. The use of tiling genomic microarrays (see Glossary), in which the design of the probe ensures that all nucleotides are covered across every chromosome, rather than just across known exons, has indicated that an unexpectedly large fraction of the genome is transcribed [2•]. Recent estimates suggest that as many as 50% of all nucleotides are transcribed [3•,4•]. This is amazing, because only ~2% of nucleotides reside in known exons. Interestingly, more than half of such transcripts are non-polyadenylated [2•]. These RNAs would be invisible to many analyses because the first step is often reverse transcription from the 'canonical' polyA tail. These findings raise the possibility that many oncogenes and tumor suppressor genes remain to be discovered.

Many of these hidden genes represent non-coding RNAs (ncRNAs) — that is, RNAs that do not contain an open reading frame (ORF) or code for protein. Few ncRNAs have a well-defined function. One group that is well characterized — at the biochemical level — is miRNAs. These are ~22-nucleotide RNAs that post-transcriptionally regulate gene expression through the RNA interference (RNAi) pathway (see [5] for a review). MiRNAs are generated as a primary transcript (pri-miRNA) by RNA polymerase II. These transcripts are spliced and polyadenylated and resemble mRNAs, although they may or may not contain an ORF (see Figure 1). The functional component is a stem–loop structure that can be located in an exon or an intron. For example, the miRNA genes *mir-106b*, *mir-93* and *mir-25* are located within an intron of the protein-coding gene *MCM7* [6]. The stem–loop structure is sequentially processed by the ribonucleases Drosha and Dicer to yield the mature miRNA. This RNA is loaded into the RNAi effector complex RISC (RNA-induced silencing complex), where it directs post-transcriptional silencing of complementary mRNAs. Given that the mature sequence is short, and exact complementarity is not required for silencing, a large number of different mRNAs can be engaged by a single species of miRNA. This is supported by computational methods that have been designed to predict targets [7–10]. Predicted targets have ranged from dozens to hundreds of mRNAs per miRNA.

The founding miRNA, *lin-4*, was discovered in *C. elegans* by the groundbreaking work of Ruvkun and Ambros [11,12]. Subsequently, thousands of miRNAs have been identified in a wide range of plant and animal genomes. There are currently 326 confirmed miRNAs in humans, and computational searches suggest that the total count might approach one thousand [13,14]. The large number of genes, combined with the regulatory nature of miRNAs, suggests that they are essential regulators of a wide range of cellular processes.

miRNA genes are associated with genomic alterations in cancer

Shortly after the initial cohort of human miRNAs was identified, it was appreciated that many were located near genomic breakpoints [15]. For example, the deletion at chromosome 13q14 had been studied for years, owing to

Glossary

Amplicon: Region of a human chromosome that is present at more than one copy per haploid genome. This occurs in cancer cells, by a poorly understood process. Amplicons often contain oncogenes such as *c-Myc* and provide a growth advantage to the cancer cell.

Copy number aberration: Alterations in chromosomal region copy number. Three general classes of aberration exist: amplification (amplicon; see above), deletion and translocation. All are commonly found in human cancer.

Latency: Time-frame between oncogenic events and appearance of cancer.

Mitotic index: Fraction of cells that are in the mitosis phase of the cell cycle. This number provides information about the proliferation rate of the cell population.

Multicolor flow-sorter (FACS): Instrument that quantifies and separates particles on the basis of fluorescent intensity. The most common use is separation of cells after fluorescent labeling of surface proteins. The method described in this review refers to measurement of polymer beads that are hybridized to miRNA species, for the purpose of quantification of expression levels.

Tiling genomic microarrays: Conventional oligonucleotide microarrays have probes designed against exons of known genes. These arrays can quantify the expression of known genes but do not detect unknown genes. Tiling arrays cover essentially all nucleotides on a chromosome, and thus can be used for the identification of novel genes. The disadvantage of this array platform is the large number of probes that are required. The most recent Affymetrix tiling array is based on 25-nucleotide probes that overlap at 5-nucleotide intervals. For this format, approximately ten high-density arrays are needed to cover a single human chromosome.

its high frequency in chronic lymphocytic lymphoma and several solid tumors [16]. The most likely candidate for the cancer susceptibility gene at this locus is the miRNA cluster *mir-15a–16*, containing *mir-15a* and *mir-16-1* [16]. How might deletion of these miRNAs promote tumorigenesis? Recent data suggest that both miR-15a and miR-16 potentiate the normal apoptotic response by targeting the anti-apoptotic gene *BCL-2* [17]. In this manner, these miRNAs function as tumor suppressors, and re-expression of miR-15a–16 in lymphoma cells that harbor the 13q14 deletion was shown to promote apoptosis. This finding raises the intriguing possibility of miR-based therapeutics.

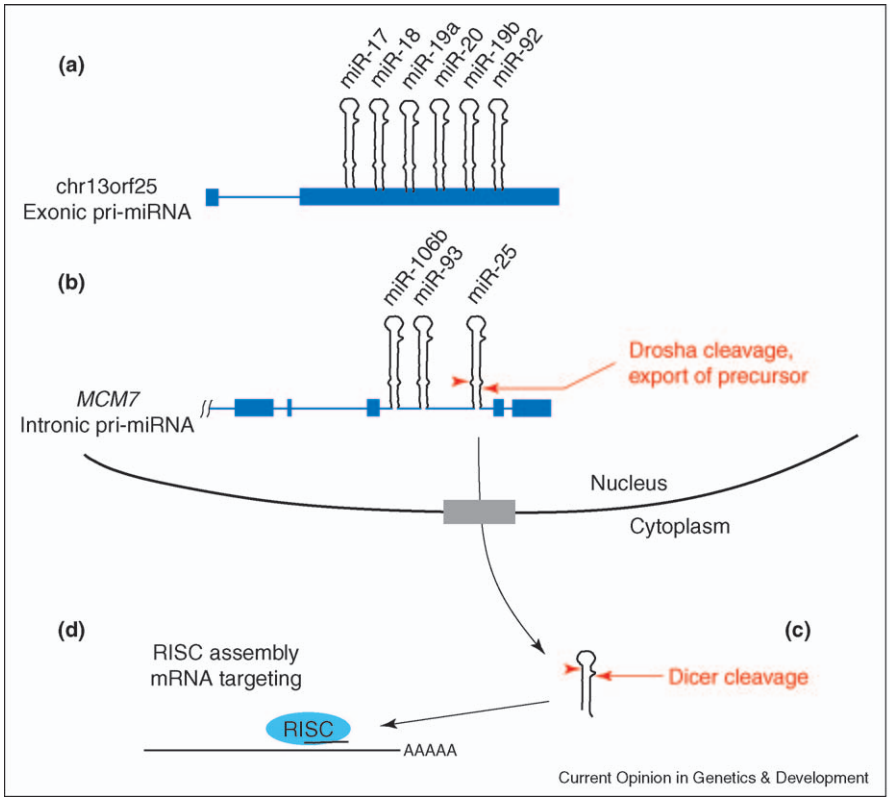
In addition to deletions, miRNA loci have also been mapped to chromosomal amplicons (see Glossary). An example of this is the well-studied chromosome 13q31 region, which is amplified in follicular lymphoma, B-cell lymphoma and several lung, head and neck carcinomas [18]. The gene whose expression is most strongly correlated with disease is chr13orf25 (chromosome 13, ORF 25) [19]. This gene has a very small ORF that probably does not generate a translated protein. Within this transcript, however, are the precursors for the miRNAs miR-17, miR-18, miR-19a, miR-20, miR-19b and miR-92. Elevated expression of the mature miRNAs from this cluster, collectively denoted as *mir-17–92*, has been verified in primary lymphoma and a wide range of tumor-derived cell lines (JM Thomson, S Goodson and SM Hammond, unpublished) [20,21••]. Direct evidence for a role of these miRNAs in tumorigenesis has been demonstrated in a mouse model for Burkitt's lymphoma (see below) [21••].

Widespread reduction of miRNA gene expression in cancer

Gene expression analysis is a proven method for identification of cancer-associated genes. Similar approaches have been undertaken with miRNA genes. Initial work used northern blot analysis of candidate miRNA genes, linking reduced expression of *mir-143*, *mir-145* and *let-7* in tumors [22,23,24••]. More recent tumor expression analyses have taken advantage of microarrays (see [25] for review). A novel approach was taken by Todd Golub and colleagues [26••]. The short nature of mature miRNAs (~22nt) raises concerns about probe specificity. This can be improved by performing hybridization in solution. Golub's group developed an elegant strategy to quantify these solution hybrids using multicolor flow-sorting (FACS; see Glossary). They used this method to examine the expression levels of 217 miRNA genes across 334 primary tumors, normal tissues and tumor-derived cell lines [26••]. Their data are striking: tumors display an expression profile reminiscent of that in the tissues from which they were derived. In fact, miRNA expression profiles were a better indicator of tissue lineage than are conventional mRNA profiles. Although the relative patterns of miRNA expression were partially maintained, the absolute expression level of many miRNAs was significantly reduced in tumors. Our understanding of miRNA function in mammals suggests that these molecules play a role in determination and/or maintenance of lineage during development. For example, most miRNA genes are not expressed in embryonic stem cells but are induced during development in tissue-restricted patterns [27,28]. Functional data support this. miR-1 promotes differentiation into the muscle lineage, and miR-181 directs B-cell differentiation [29,30]. The widespread reduction in miRNA levels might indicate the reduced differentiation that is a property of cancer. Indeed, histology demonstrated that tumors displaying the poorest degree of differentiation had the lowest levels of miRNA expression.

As with all expression analysis, there is no discrimination between cause and effect. That is, does a reduction in lineage-specific miRNAs promote tumorigenesis or is this reduction merely a downstream effect? The most convincing data come from studies on *C. elegans let-7*. This miRNA is essential for the transition of the fourth larval stage into the adult. Frank Slack and colleagues [24••] have shown that *let-60*, the *C. elegans* ortholog of the oncogene *Ras*, is a direct target of *let-7*. Induction of *let-7* in adult worms promotes differentiation, in part, by suppression of cell proliferation. In mammals, *let-7* expression is low in embryonic stem cells and increases during development, reaching a maximum in differen-

Figure 1



Overview of miRNA biogenesis. MiRNAs are transcribed by RNA polymerase II. The primary transcript (pri-miRNA) is typically several kilobases long and can be a protein-coding RNA or a ncRNA. **(a,b)** An exonic, ncRNA pri-miRNA is shown (encoded from chr13orf25), in addition to an intronic pri-miRNA (*MCM7*). Exons are indicated by blue boxes, and introns by blue lines. (b) The precursor (pre-miRNA) is excised by the RNase III enzyme Drosha at the indicated red arrow points. **(c)** The pre-miRNA is exported from the nucleus and is processed by the RNase III enzyme Dicer at the indicated red arrows. **(d)** The guide strand, which is antisense to the target mRNA, is loaded into the effector complex RISC, where it directs the RISC to target mRNAs. Gene expression of targets is achieved by translational inhibition and mRNA degradation. Nucleic acid regions are not shown to scale.

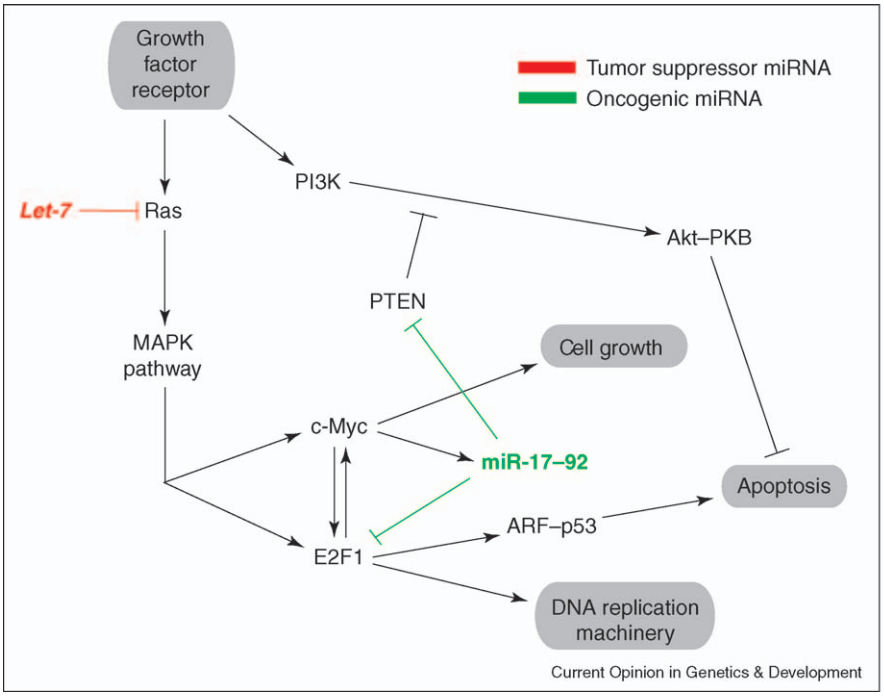
tiated adult tissues [27,28]. The functional consequence of this induction has not been defined; however, all three mammalian Ras genes have *let-7* target sites. This supports the idea that *let-7* suppresses cell proliferation in differentiated cells, whereas tissue-restricted miRNAs, such as miR-1, promote differentiation along specific lineages. As discussed above, inappropriate suppression of these miRNAs might be a necessary step in oncogenic loss of differentiation.

miRNAs as oncogenes

Although most miRNA genes display reduced expression in cancer, there are notable exemptions. The best-characterized example is the *mir-17–92* cluster. These miRNAs were introduced above as being the likely susceptibility gene at the chr13q31 amplicon. We demonstrated the causal role of these miRNAs in cancer in a mouse model for lymphoma. In this model, expression of the oncogene *c-Myc* is directed from the immunoglobulin heavy chain enhancer (*Eμ-Myc*). This results in ectopic expression in B-lymphocyte progenitor cells, thus promoting B-cell lymphoma. The contribution of additional genetic factors to tumorigenesis can be determined by the increase, or decrease, in latency (see Glossary) of lymphoma development. In this manner, co-expression of miR-17–19b, a truncated portion of miR-17–92, strongly accelerated lymphomagenesis. This was the first functional evidence of a miRNA, or any noncoding RNA, acting as a mammalian oncogene. For this reason we refer to the host transcript of *mir-17–92* as OncomiR-1.

Although the cellular function of miR-17–92 is not known, the pathology of tumors indicated low rates of apoptosis. In this lymphoma model, tumors derived from *c-Myc* expression alone have extensive apoptosis. The key function of miR-17–92 thus appears to abrogate Myc-induced apoptosis. A confirmed target of miR-17 and miR-20 is the transcript of the cell cycle transcription factor gene *E2F1* [31••]. Although this protein can promote cell proliferation by transactivation of S-phase

Figure 2



Impact of oncogenic and tumor suppressor miRNAs on cell proliferation pathways. *let*-7 represses translation of all three Ras GTPase genes. Loss of *let-7* in tumors promotes flux, although the Ras-MAPK pathway thus promotes the cell cycle. This effect is augmented by overexpression of miR-17–92, which reduces oncogene-induced apoptosis. This is a result of miR-17 and miR-20 suppression of E2F1, and miR-19a/b suppression of PTEN. All pathways are simplified due to large numbers of uncharacterized targets of miRNAs. Abbreviations: ARF, alternative reading frame protein of p16INK4a locus; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; PtdIns-3K, phosphoinositide-3 kinase; PTEN, phosphatase and tensin homolog.

genes, it also has the ability to promote apoptosis through the ARF–p53 pathway. Does this explain the anti-apoptotic mechanism of miR-17–92? Not fully, because targeted deletion of *E2F1* does not accelerate Eμ-Myc lymphomagenesis [32]. miR-19 has been demonstrated to downregulate the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) [33]. This would increase flux through the PI3K–Akt pathway, a known survival-promoting signal. Given that several hundred target mRNAs are predicted for each miRNA, there are likely to be many uncharacterized targets of the *mir-17–92* cluster. It is probable that suppression of many target mRNAs combines to promote cell survival. Figure 2 depicts an overview of miRNA regulation of the cell proliferation and apoptotic pathways.

To further complicate the story, c-Myc, which is a transcription factor, directly transactivates the *mir-17–92* host gene [31••]. In our mouse lymphomagenesis model, we are ectopically expressing c-Myc in B-progenitor cells. Given that c-Myc should directly transactivate these miRNAs, why does additional, ectopic expression of *mir-17–92b* accelerate disease? Presumably, there are other transcriptional regulators of these miRNAs. Such regulatory circuits may play a role in human cancer.

An unrelated miRNA gene, *mir-155*, might also function as an oncogene in lymphoma. High expression of *mir-155* and its host gene, *BIC*, has been reported in several types of B-cell lymphoma [34]. Interestingly, this miRNA promotes cell proliferation and cooperates with c-Myc in an avian lymphoma model system [35]. It is not known if there is a biological connection between miR-17–92 and miR-155. They do no share sequence similarity; however, that does not preclude the possibility that they regulate shared sets of mRNA targets. A more complete understanding of the role of oncogenic miRNAs awaits a comprehensive set of validated targets.

Stem cells, miRNAs and cancer

One model for tumorigenesis proposes that a small fraction of cells in a tumor have properties of stem cells [36]. These cancer stem cells are responsible for initiating and maintaining the tumor. By contrast, the bulk cells in the tumor have some degree of differentiation and are not tumorigenic. What is the connection with miRNAs? As described above, tumors display a miRNA profile that is reminiscent of that in stem cells. They have reduced expression of many miRNAs but retain expression of stem cell miRNAs, including miR-17–92. There is also a biochemical link between the RNAi pathway and the

maintenance of stem cells. Homologs of the essential RISC component Argonaute are required for stem cell maintenance in *Drosophila* and plants (see [37] for a review). A more direct connection has been established by collaborative work between the Carthew and Ruohola-Baker laboratories [38••]. Loss of miRNA function, by mutation of Dicer-1, reduced proliferation of germline stem cells in *Drosophila*. The cell cycle block was between G_1 and S phase, consistent with an increase in expression of the cyclin-dependent kinase-inhibitor Dacapo. Indeed, predicted miRNA target sites are present in the 3′UTR (untranslated region) of Dacapo. Importantly, the sites correspond to miRNAs that are expressed in stem cells. The human homolog of Dacapo is p27-Kip1, a primary regulator of S-phase induction. Whether this gene is a miRNA target in mammals is not know; if it was, it would provide a powerful intervention point for an oncogenic miRNA to promote cell proliferation.

miRNAs as therapeutic targets

If miRNAs represent key contributors to human cancer, they must be considered as potential therapeutic targets. The most logical therapeutic molecule would be one in which a nucleic acid is modified for stability in serum and is capable of cellular uptake. In this manner, miRNA therapeutics would borrow techniques from the antisense research community, which has been developing therapeutic RNAs for more than a decade. For example, two research groups have shown that 2′-O-methyl-modified RNAs can act as antisense inhibitors of miRNA function in cultured cells [39,40]. These molecules could be used to target oncogenic miRNAs, such as miR-17–92 (see also Update). In the case of tumor suppressor miRNAs, the desired therapeutic strategy would be to increase their function in cells. Introduction of serum-stabilized pre-miRNAs might achieve this. In this example, introduction of pre-*let-7* would suppress Ras expression, leading to tumor regression. Given that the expression of many miRNAs is reduced in cancer, there are a large number of candidate therapeutic targets.

Conclusions

The identification of oncogenic miRNAs within the *mir-17–92* cluster, and tumor suppressor miRNAs of the *let-7* family has ushered in a new era of cancer research. Clearly, miRNA genes must be considered along with classic oncogenes and tumor suppressors as viable contributors to the oncogenic phenotype. To begin with, copy number aberration (see Glossary) and conventional microarray data need to be re-evaluated in the context of miRNA genes. Novel experimental approaches will need to be developed to study these non-coding RNAs. For example, a revealing experiment would be the targeted deletion of *let-7* genes from the mouse. In addition to developmental disorders, one would expect this mouse to be highly tumor-prone. The difficulty lies in targeting the eight loci for this highly redundant gene family.

A second consideration is the large number of ncRNAs that are not miRNAs. These present additional difficulties because there are no common sequence signatures, or biochemical actions, for the many potential gene products. In summation, our understanding of the molecular basis of cancer might be at an earlier stage than we realize. MiRNAs point to a potential regulation of development and disease, and open a new avenue of investigation into ncRNAs as effectors of diverse cellular processes.

Update

Recent work has demonstrated that modified antisense RNAs can inhibit miRNA function in the adult mouse [41••]. Three daily intravenous injections lead to robust suppression of miRNA in most tissues for a period of weeks. The notable exception was brain. These data support the idea of 'antagomirs', or therapeutic inhibitors of miRNAs [41••].

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This report describes miRNA inhibition in the adult mouse by injection with modified antisense RNAs. This provides exciting therapeutic possibilities, as well as a knockdown strategy for studying the biology of miRNAs.