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CANCER MECHANISMS

The Role of MicroRNAs in Cancer

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Cancer is a complex and dynamic disease, involving a variety of changes in gene expression and structure. Traditionally, the study of cancer has focused on protein-coding genes, considering these as the principal effectors and regulators of tumorigenesis. Recent advances, however, have brought non-protein-coding RNA into the spotlight. MicroRNAs (miRNAs)†, one such class of non-coding RNAs, have been implicated in the regulation of cell growth, differentiation, and apoptosis [1]. While their study is still at an early stage, and their mechanism of action along with their importance in cancer is not yet fully understood, they may provide an important layer of genetic regulation in tumorigenesis, and ultimately become valuable therapeutic tools.

INTRODUCTION

MicroRNAs are small, non-coding RNAs of 18 to 24 nucleotides, discovered in 1993 in the nematode *Caenorhabditis elegans*. Lee et al. [2] found that a gene crucial for *C. elegans* post-embryonic development, *lin-4*, does not code for a protein, but rather is transcribed into a 22-nucleotide RNA molecule. This molecule could repress the expression of the *lin-14* mRNA by directly interacting with its 3' untranslated region (UTR) [3]. While this was recognized as a new method of gene regulation, it was initially considered as an oddity peculiar to *C. elegans*. The discovery of *let-7*, another small RNA involved in developmental timing in *C. elegans* [4], and the finding that both its sequence and temporal expression pattern were largely conserved in a variety of organisms [5] immediately suggested these small RNAs might in fact play important and conserved roles in gene regulation, and the identification of hundreds of miRNAs in the worm, fly, and mammalian genomes followed [1].

To date, more than 300 miRNAs have been discovered in humans, and computational analyses predict that up to 1,000 miRNAs exist in the genome [6,7]. Since miRNAs can regulate more than one target, estimates indicate they may be able to regulate up to 30 percent of the protein-coding genes in the human genome [8], highlighting their importance as global regulators of gene expression.

miRNA BIOGENESIS AND METHOD OF ACTION

MiRNAs are transcribed by RNA Polymerase II into large precursor RNAs, often several kilobases in length, called pri-

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†Abbreviations: miRNAs, MicroRNAs; mRNA, Messenger Ribonucleic Acid.

miRNAs. Notably, the majority of human miRNAs are transcribed from regions found within introns of either protein-coding or non-protein-coding transcripts, but a minority is found in isolated regions of the genome, within the exons of noncoding mRNA genes, or within the 3'UTRs of mRNA genes.

In the nucleus, these pri-miRNAs are capped and polyadenylated prior to being processed by Drosha, a member of the RNase III enzyme family, in conjunction with the double-stranded RNA-binding protein DGCR8/Pasha. This processing step produces segments ~70 nucleotides in length, which fold into stem-loop structures known as pre-miRNAs [9]. These are exported from the nucleus in a GTP-dependent fashion by exportin 5 and are subject to an additional processing step by another RNase III enzyme, Dicer. This step releases a double-stranded RNA duplex, ~22 nucleotides in length, which in turn is incorporated into the miRISC complex, in analogous fashion to that observed in RNA interference (RNAi). In this complex, the mature miRNA strand is retained, and the complex is now capable of regulating its target genes.

The identification of miRNA target transcripts remains one of the greatest challenges in the field today. MiRNAs can be organized into families based on sequence homology, which is particularly strong at the 5' end of the mature miRNA, suggesting this section of the mature transcript has been preserved through evolution and thus plays an important role in the process of target recognition. Indeed, studies have shown this 5' region, often called the "miRNA seed," to be crucial for both the stability of the mature miRNA and its incorporation into the miRISC complex [10-14]. Bioinformatic approaches have taken advantage of this "miRNA seed" to predict miRNA targets across the genome. It has been predicted that a single miRNA can bind over 200 different target transcripts, and, notably, these targets are highly diverse, from transcription factors to transporters [15-24].

There is nevertheless evidence supporting a role for the regions not encompassed within the "miRNA seed." Perhaps the clearest example of this is found in *let-7*, whose complete mature miRNA sequence is conserved across species [25], suggesting an important role for the 3' region of the miRNA in target transcript binding. Further insights into the miRNA-target recognition mechanism are needed, as they will make target prediction much more accurate and efficient.

A POTENTIAL ROLE FOR miRNAS IN CANCER

Cancers of all types share a number of characteristics, such as the loss of cellular identity, an increased ability to grow and proliferate, and alterations in the systems controlling cell death. Studies performed in a variety of organisms have revealed that miRNAs have the ability to regulate these cellular processes, suggesting that they could be involved in cancer. For instance, *lin-4* and *let-7* control the timing of developmental events in *C. elegans*. Mutations in these miRNAs resulted in abnormalities in cell-cycle exit as well as in the execution of a terminal differentiation program, preventing cells from reaching their fully differentiated state [2].

In *Drosophila*, it has been found that over-expression of the Bantam miRNA causes excessive growth of wing and eye tissue by blocking the pro-apoptotic action of the gene *hid* [26, 27]. It was also found that another *Drosophila* miRNA, miR-14, can act as a strong suppressor of apoptosis. Deletion of this miRNA caused increased expression of the apoptotic effector caspase, *Drice*, suggesting a direct regulation of this factor by miR-14 [28]. Likewise, the miR-2/6/11/13/308 family of *Drosophila* miRNAs has been reported to induce widespread apoptosis in embryos through the regulation of the proapoptotic factors *hid*, *grim*, *reaper*, and *sickle*, which act through the inhibition the caspase inhibitor Diap1 in response to a number of natural and toxic conditions [29].

Another aspect in which miRNAs appear to have a role is the proper differentia-

tion of cells into different tissues. For example, miR-273 and the miRNA encoded by *lys-6* have been shown to play important roles in the patterning of the *C. elegans* nervous system [30,31], and miR-430 has been implicated in brain development [3]. The importance of miRNA in development has been shown also in mammalian systems, examples of which are miR-181 in the differentiation of hematopoietic cells toward the B-cell lineage [33], miR-374 in pancreatic islet-cell development [34], miR-143 in adipocyte differentiation [35], miR-196 in limb patterning by SHH [36], and miR-1 in heart development [37].

miRNAS AS TUMOR SUPPRESSORS

miR-15a and miR-16-1 in CLL

The first study directly suggesting that dysregulation of miRNA played an important role for miRNAs in tumorigenesis came from Calin et al. [38] It was known that loss of chromosomal region 13q14 was strongly associated with B cell chronic lymphocytic leukemia (CLL), the most common form of leukemia in the Western hemisphere; however, no gene or genes had been definitely directly linked with CLL. After refining the critical region within 13q14 to about 30kb, Calin et al. failed to identify any protein coding genes but recognized that two miRNAs, miR-15a and miR-16-1, were clustered within this chromosomal locus. Indeed, expression of these miRNAs was found to be diminished or completely ablated in > 65 percent of CLL cases examined. Further studies revealed a germline C-to-T mutation seven base pairs downstream of the miR-16-1 hairpin in two out of 75 CLL patients. This mutation was not found in any of 160 control individuals. Because the mechanisms for miRNA biogenesis are not yet fully understood, it is unclear what the precise effect of this mutation is; however, it was found that the mutation correlated with diminished expression of this miRNA in patient-derived CLL cells, while preventing the processing of the miRNA into its mature form when expressed in heterologous cell systems [39].

The common loss of miR-15a and miR-16-1 in CLL, as well as the loss of 13q14 in mantle cell lymphoma (50 percent of cases), multiple myeloma (16 to 40 percent) and prostate cancer (60 percent) [38,40], strongly suggests that these two miRNAs act as tumor suppressor genes. While their full target complement is unknown, they appear to mediate their effects largely by down-regulating the anti-apoptotic protein BCL2. This protein is often found expressed at high levels in CLL and is thought to be important for the survival of the malignant cells [41]. Consistent with a role for these miRNAs in the down-regulation of BCL2, loss of miR-15a and miR-16-1 correlates with elevated levels of BCL2, while heterologous expression of these miRNAs results in decreased levels of the endogenous proteins [42]. The 3'UTR of the *Bcl2* transcript has been found to contain binding sites for miR-15a and miR-16-1, and reporter constructs containing the *Bcl2* 3'UTR are down-regulated upon co-expression with miR-15a and miR-16-1. Moreover, expression of these miRNAs is capable of inducing apoptosis in a leukemia cell line. Further research is necessary to better evaluate whether miR-15a and miR-16-1 exert their effects mainly through BCL2 or target additional transcripts in CLL. Nonetheless, the evidence available supports an important role for miR-15a and miR-16-1 in the prevention of lymphomagenesis and leukaemogenesis.

The let-7 family can negatively regulate Ras

As noted earlier, the miRNAs of the *let-7* family were among the first to be described, and experiments performed in *C. elegans* provided the first indication that they may be involved in cancer. Early studies showed that loss-of-function *let-7* mutants were defective in the transition from late larval to adult stage. Upregulation of *let-7* in adult seam cells, on the other hand, is necessary to induce cell-cycle exit and terminal differentiation. If this miRNA is absent, these cells fail to differentiate, undergoing additional rounds of division, a phenomenon often observed in cancer cells [43].

There are 12 *let-7* homologs in the human genome, organized in eight clusters. These clusters map to fragile sites associated with lung, breast, urothelial, and cervical cancers, suggesting that *let-7* family members may act as tumor suppressors. At least four of these clusters have been confirmed to be commonly lost in malignancies [44]. Studies by Takamizawa et al. and Yanaihara et al. have presented evidence that transcripts of certain *let-7* homologs are significantly downregulated in human lung cancer and that low levels of *let-7* correlate with poor prognosis [45,46]. Moreover, it has been shown that transient expression of miRNAs of the *let-7* family in cell lines derived from lung adenocarcinomas inhibited colony formation, suggesting that these miRNAs could have growth-suppressing properties and opening the possibility that *let-7* might be used as a therapeutic tool.

Important insights into the mechanism through which *let-7* is capable of controlling cellular proliferation came from our laboratory, derived from the observation that the *let-7* family member miR-84 negatively regulates *let-60*, the ortholog of human *HRAS*, *KRAS*, and *NRAS* in *C. elegans* [47]. We have shown that certain members of the *let-7* family are capable of genetically interacting with RAS, and *let-7* and RAS are reciprocally expressed in lung tumor samples. We also found that *let-7* is capable of negatively regulating RAS in human cells, and overexpression of *let-7* in human cancer cell lines results in reduced levels of RAS protein, compared to untreated cells. As expected, if *let-7* levels are reduced in cancer cell lines, levels of RAS protein increase significantly. Moreover, through the use of reporter constructs, it was confirmed that the regulation of RAS by *let-7* takes place through the direct interaction of the miRNA with the 3'UTR of Ras mRNA.

The human RAS family of proteins is one of the most important components of a myriad of signaling cascades, has been shown to possess oncogenic activity, and is often mutated in tumors [48]. As such, these studies position *let-7* as a promising therapeutic tool for the treatment of lung cancer, as well as other malignancies resulting from the overexpression of RAS. It will be important to more carefully analyze the specific contributions of the individual members of this miRNA family, in order to better understand the *let-7*-mediated regulation of cell growth.

Other miRNAs acting as tumor suppressors

Studies have implicated other miRNAs in tumorigenesis. MiR-143 and miR-145, for instance, have been shown to be constantly down-regulated in colorectal tumors [49], and recent studies by Croce et al. also have shown that the downregulation of these miRNAs is a common occurrence in breast carcinomas and breast cancer lines [50]. Moreover, the location of these miRNAs is known to be in a genomic locus frequently deleted in myelodysplastic syndrome [44]. While some studies have ascribed the down-regulation of miR-143 and miR-145 in colon cancer to a block in Dicer processing [49], further studies are required to determine whether the expression levels of these miRNAs are specifically altered.

miRNAS AS ONCOGENES

Several microRNAs may regulate MYC in a dynamic manner

MicroRNAs can also act as oncogenes, either directly by down-regulating tumor suppressors or indirectly by down-regulating genes that can act to restrict the activity of known oncogenes. An example of this is found in the dynamics of the interaction between miR-155 and the *MYC* oncogene, a transcription factor with the ability to regulate cell growth through the induction of both cell proliferation and apoptosis [51]. Notably, *MYC* is often mutated or amplified in human cancers. The miRNA miR-155 has been linked with *MYC* overexpression and B-cell cancers, suggesting it may be playing a role in the regulation of this oncogene.

miR-155 is encoded by nucleotides 241-262 of *BIC*, which was originally identified as a transcript derived from an integration site for the avian leukosis virus and

found to be overexpressed in B-cell lymphomas [52,53]. The understanding of how *BIC* might mediate its effects was limited for several years, as it remained unclear how a presumably non-protein-coding RNA with little overall conservation among avian, murine, and human genomes could consistently produce lymphomas and induce the overexpression of *MYC*. Metzler et al. analyzed the phylogenetically conserved regions in *BIC* and found that the highest homology was located in a 138 nucleotide section of the gene, which in fact encoded the pri-miRNA for miR-155 [54]. Subsequent studies showed that miR-155 expression is upregulated 100-fold in pediatric Burkitt lymphoma, as well as in Hodgkin's lymphoma and primary mediastinal and certain subsets of large-B cell lymphomas [55, 56,56]. The role of miR-155 is not restricted to B cell lymphomas, however. Recent studies have reported that miR-155 is upregulated in breast, lung, colon, and thyroid cancers [57-59]. It has also been reported that mice overexpressing miR-155 under the control of the Eμ enhancer rapidly develop B-cell malignancy, underscoring the ability of miR-155 to induce lymphomagenesis without the requirement for other major genetic changes, strongly suggesting miR-155 can act as a bona fide oncogene [60]. Nevertheless, the mechanism of action of miR-155 is unknown, stressing the need for further study of this miRNA.

The interaction with *MYC* is not exclusive to miR-155. Recent studies have described the relationship between *MYC*, cancer, and the 13q31 locus [61,62]. This genomic locus is amplified in a number of cancers, such as diffuse large-B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and primary cutaneous B-cell lymphoma. However, the only gene overexpressed in this amplicon is a non-protein-coding RNA, *C13orf25* [63], which has been shown to encode the *miR-17-92* cluster, containing seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1. He et al. have shown that cell lines carrying an amplification of 13q31 were analyzed, and it was found that six miRNAs had increased expression, five of which belonged to the *miR-17-92* cluster [61]. Furthermore, it was also shown that the pri-miRNA for the *miR-17-92* cluster was upregulated in 65 percent of the tested B cell lymphoma samples. These data suggested a direct involvement of this miRNA cluster in tumorigenesis.

Further indication of the involvement of the *miR-17-92* cluster in cancer was found in subsequent experiments, in which the *miR-17-19b-1* cluster (the vertebrate-specific segment of the *miR-17-92* cluster) was overexpressed using a retroviral system in hematopoietic stem cells (HSCs) from mice carrying the *Myc* transgene. Lethally irradiated animals which received HSCs expressing both *miR-17-19b-1* cluster and *Myc* developed malignant lymphomas more rapidly than those receiving HSCs expressing *Myc* alone. The lymphomas from mice expressing both *Myc* and *miR-17-19b-1* were characterized by increased cell proliferation and decreased apoptosis levels, when compared to the malignancies arising from the mice receiving HSCs with *Myc* alone. Complementary experiments showed that this effect is exclusive to the miRNAs of the *miR-17-19b-1* cluster and any one individual miRNA from this cluster is unable to cause accelerated lymphomagenesis. Together, these results suggest that the miRNAs composing the *miR-17-19b-1* act cooperatively as oncogenes, presumably by targeting proapoptotic and anti-proliferative factors.

Independently, O'Donnell et al. identified the *miR-17-92* cluster as a group of potentially cancer-related genes through microarray analysis of a set of human, mouse, and rat miRNAs to identify miRNAs with altered levels in a human B-cell line that overexpresses MYC [62]. It was found that MYC induced the expression of the *miR-17-92* cluster. Moreover, chromatin immunoprecipitation experiments confirm this interaction by establishing that MYC binds within the first intronic region of *C13orf25*, suggesting that MYC has the ability to directly regulate the *miR-17-92* cluster.

An intriguing scenario was suggested by the bioinformatic prediction that the members of the *miR-17-92* cluster, *miR-17-*

5p and *miR-20a*, target the transcription factor E2F1. E2F1 regulates the transition from G1 to S phase of the cell cycle by regulating genes that are involved in DNA replication, cell division, and apoptosis [64]. Notably, E2F1 is known to function in a reciprocal positive-feedback loop with MYC. Consistent with this, it was found that inhibition of *miR-17-5* and *miR-20a* in a cervical cancer-derived cell line resulted in a concomitant increase in the expression level of E2F1. Likewise, mutations of the sites complementary to *miR-17-5p* and *miR-20a* in the 3'UTR of E2F1 resulted in increased reporter activity, compared with that of constructs harboring the wild-type sequence of E2F1's 3'UTR. These results suggest a model in which MYC can induce the transcription of both the *miR-17-92* cluster and E2F1; in turn, the miRNAs of the cluster negatively regulate the transcription of E2F1. One consequence of this model is that miRNAs of the *miR-17-92* cluster directly regulate the effector of MYC-mediated cell growth.

An apparent paradox, however, also emerges from these results. In the presence of MYC, as noted above, the *miR-17-92* cluster would function as a tumor suppressor, curbing the cellular growth mediated by E2F1, which is in contradiction with the results of He et al. that implicated *miR-17-92* as an oncogene. These results can perhaps be reconciled by considering that while E2F1 can promote cellular proliferation, it has been reported that its presence in excess levels can induce apoptosis [64]. As such, the negative regulation of E2F1 by the miRNAs of the *miR-17-92* cluster might in fact prevent E2F1 levels from reaching the threshold at which apoptosis is triggered, thus acting as an oncogene by supporting MYC-mediated proliferation.

It is clear that the interaction of miRNAs with their targets is not as straightforward as initial studies might have suggested and intricate regulatory networks are in place for any one cellular system. There exists the possibility that the precise role of *miR-17-92* in a given cell depends on what other miRNAs are present, which in turn may be affected by spatial and temporal considerations.

miRNA SIGNATURES AND DETECTION METHODS

The multiple lines of evidence indicating that microRNAs are differentially expressed in normal and in tumor samples suggest that miRNAs could serve as useful tumor profiling tools. Indeed, profiling studies have shown that each cancer type possesses a distinct miRNA expression signature, and this signature can provide useful information about the malignancy. The adaptation of high-throughput technologies have facilitated the study of the expression of multiple miRNAs in a given sample, making it possible to profile substantial sample numbers with relative ease. Among the most important methods for miRNA profiling is the use of oligonucleotide miRNA microarray analysis, which allows the researcher to simultaneously determine the expression levels of hundreds of miRNAs in a given sample [65-67]. Multiple variations of this technique have been developed, underscoring its position as the central miRNA profiling tool [68-71]. Other important approaches to the problem are also available, such as quantitative PCR for precursor miRNAs [72] or mature miRNAs [73,74]; genome-wide approaches with serial analysis of gene expression (SAGE), such as miRAGE [75]; and bead-based flow cytrometric techniques [76].

Taking advantage of these tools, several groups have studied the miRNA expression of several types of cancer. Michael et al. [49] and Cummins et al. [75] have studied the expression profile of the known miRNAs in colorectal cancer samples. These studies independently found a general down-regulation of miRNA levels in tumor cells compared to normal colonic epithelium, while also observing a significant decrease in the levels of miR-143 and miR-145 beyond this general effect. Cummins et al. found a signature of 50 differentially expressed miRNAs in malignant vs. normal colonic epithelium, of which 32 miRNAs had reduced levels in tumor cells. Yanaihara et al. studied over 100 patient-matched pairs of primary malignant and normal adjacent lung tissue and found that the expression of

43 miRNAs was significantly different in the tumor tissues compared to the normal adjacent tissues. Of these miRNAs, 28 were down-regulated and 15 were up-regulated in the malignant tissue [46]. Similar studies have been carried out in CLL [77], breast cancer [50], glioblastoma [78], pancreatic cancer [79], hepatocellular carcinoma [80], and thyroid papillary carcinoma [58].

The ability to profile the miRNA expression of a tumor with accuracy and reproducibility in a clinical setting would constitute a valuable medical tool. Indeed, miRNA profiles have been shown to be highly informative. Using a bead-based approach, Lu et al. attempted to classify 17 poorly differentiated tumors with non-diagnostic histological appearance based on their miRNA expression profiles. A clinical diagnosis on these tumors was established by anatomical context, whether directly (a primary tumor observed in a given organ) or indirectly (a metastasis of a previously identified primary tumor). They were able to correctly diagnose 12 of the 17 samples, against 1/17 correct diagnoses for an analysis using their mRNA profile [76]. While the number of transcripts analyzed was much smaller for miRNAs than for mRNAs (~200 against > 15,000), it is possible that the miRNA-based diagnoses are more accurate because of the regulatory role of these molecules, while most mRNAs will not be regulatory in nature. Further studies, however, are necessary to ascertain the value of miRNAs as diagnostic tools, and their potential value as prognostic markers, by correlating miRNA expression with type-specific parameters such as metastatic potential, proliferative index, and response to existing treatments.

CLOSING REMARKS: miRNAS AS TREATMENT TARGETS

While the field of miRNAs — and particularly the study of the roles of miRNAs in cancer — is at an early stage, their potential as targeted therapeutic tools has not gone unnoticed. A promising approach is to target oncogenic miRNAs with oligonucleotides complementary for either their mature or precursor sequence, deemed anti-miRNA oligonucleotides (AMOs). A number of chemical modifications for these oligonucleotides are available, with the purpose of making them more stable and less toxic. 2'-O-methylated AMOs are perhaps the most common form of modified RNA, conferring limited nuclease resistance, and enhancing the binding affinity to RNA. Inhibition of *let-7* function with 31-nucleotide 2'-O-Me AMOs has been demonstrated in HeLa cells and *C. elegans* larvae [81], and 2'-O-Me AMOs have been reported to abrogate miRNA function in cellular assays [82,83], providing proof-of-principle on the blockage of miRNA function by extraneous oligomers. Another common modification to the structure of AMOs is that of the locked nucleic acid (LNA)-modified oligomers. In these RNAs, the 2' oxygen atom is linked to the 4' position through a methylene bridge, forming a rigid bicyclic structure locked into a C3'-endo (RNA) sugar conformation [84]. This modification results in greatly enhanced affinity for RNA and the formation of exceedingly strong duplexes with excellent mismatch discrimination properties [85-87]. This makes them prime candidates for AMO-based therapy, and results in rodents have been encouraging [88].

While the use of AMOs could be useful to inhibit oncogenic miRNAs, another therapeutic possibility concerns the cases in which certain miRNAs are under-expressed, such as *let-7* in lung cancer, or miR-15a and mir-16-1 in CLL. In this case, synthetic miRNAs — likely modified as discussed above — in either their mature or precursor form could be delivered to the target cells to counteract the deficiency and halt malignant growth. While this approach has been attempted in cell culture [89], further testing in animal models is required.

A pressing challenge to the use of miRNAs as therapeutic tools concerns finding methods to deliver synthetic miRNAs or AMOs to the desired tissues in a targeted and effective manner. Given that these molecules cannot discriminate between healthy and malignant cells, side effects of treatment remain a concern. Likewise, since these

studies are in their infancy, little is known about the pharmacokinetics of AMOs or synthetic miRNAs, limiting the discussion of their promise as therapeutic tools into the speculative arena. Upon reaching a deeper understanding of the mechanism of miRNA biogenesis and action, and the development of new delivery technologies, these small RNAs might well fulfill their promise as valuable therapeutics.

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