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microRNA: an Emerging Therapeutic

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Antisense-based molecules, RNA aptamers, and ribozymes have shown promising results in lab-based attempts at developing RNA based therapeutics.[1] A new wave of RNA based therapeutics is now emerging, following the demonstration of effective gene silencing by small RNA molecules. Synthetic small interfering RNA can direct the cleavage of perfectly complimentary sites in target mRNAs and knock down the expression of an unwanted gene in cell culture based studies. However, in vivo application of small interfering RNA (siRNA) technology is limited by the unpredictability of its effect in some cell types and the possibility of inducing immune responses at high concentrations. Moreover, a natural analogue of siRNAs, microRNAs (miRNAs) can downregulate targets with incomplete complementarity raising the possibility that an siRNA intended against a target may act as an miRNA against unintended targets. miRNAs, on the other hand, can sequester targets in silencing complexes and may be capable of simultaneously shutting down many members of a biological pathway. Herein, we provide an overview of miRNA mediated gene regulation in biological systems and highlight the opportunities in developing effective therapeutics and diagnostics based on miRNAs.

The first miRNA to be discovered by Ambros and co-workers, lin4, is involved in regulation of developmental timing in the nematode, *Caenorhabditis elegans*.[2,3] The high degree of conservation in the next miRNA to be found, let7,[4] prompted the search for miRNAs in mammalian genomes. The recent discovery of a whole army of these small naturally occurring RNA molecules in the living cell has not only changed our understanding of gene expression and regulation radically but has offered novel targets for therapeutic intervention and diagnostic detection. These molecules arise from noncoding regions of the genome carrying imperfect inverted repeats. Large noncoding transcripts and noncoding regions of messenger RNA may harbour stem loop secondary structures formed by these imperfect inverted repeats. The RNAse III enzyme Drosha with its partner DGCR8 acts on the large primary miRNA (pri-miRNA) to precisely excise out the stem loop structures called pre-miRNA. The pre-miRNA are exported out of the nucleus by Exportin 5 and in the cytoplasm another RNase III enzyme, Dicer chops the pre-miRNA to generate imperfect dsRNA duplexes by removing the loop region. One of the mature strands in the duplex, associates with a multiprotein complex to form a ribonucleoprotein complex. The mature miRNA associates with a member of the argonaute family of proteins and acts as a factor that lends specificity within the RNA induced silencing complex (RISC) or microRNA ribonucleoprotein (miRNP), targeting it to complimentary regions in the 3′ untranslated regions of target messenger RNAs[5] in mammalian systems. The RNA binding protein within the RISC complex, Ago2, can mediate cleavage of the target transcript if the small RNA is perfectly complimentary to it. In most cases, however, the miRNP complex carrying a partially complimentary miRNA interferes with translation from the target by mechanisms not yet fully understood. Proposed mechanisms[6] include reversible sequestration into processing bodies (P-bodies) in the cell, reduced elongation rates during translation, and targeted deadenylation. Bhattacharyya et al. reported that targets of the liver-specific miR-122 are sequestered in P-bodies under repressed conditions.[7] This study also suggests that miRNA mediated translational suppression can be reversed by mobilisation of targeted messengers from P-bodies during specific conditions like stress.[7] A study in zebrafish where large numbers of maternal transcripts are rapidly cleared during early development shows that the miRNA miR-430 triggers deadenylation of the target.[8] It is interesting to note that perfectly complimentary siRNAs are destructive in their action whereas natural miRNAs act as transient and probably reversible blocks in gene expression. See Figure 1 for an overview of miRNA formation and action in biological systems.

Many of the currently known 400 odd human miRNAs[9] are highly conserved in different organisms and play fundamentally important roles in regulation of thousands of target genes[10] involved in critical cellular processes like patterning during early development and regulation of growth. Not surprisingly, mutations that abrogate their function or lead to their dysregulation are associated with a variety of disease conditions ranging from cancer[11] to inappropriate aging.[12] Novel applications like the downregulation of dominant disease causing alleles are also being proposed.[13]

A new dimension has been added to the relation between miRNAs and disease following recent reports of their potential antiviral role.[14–16] Viruses may carry RNA or DNA as their genetic material and their life-cycle necessitates the exposure of this material to the molecular environment in the host cell. Viruses

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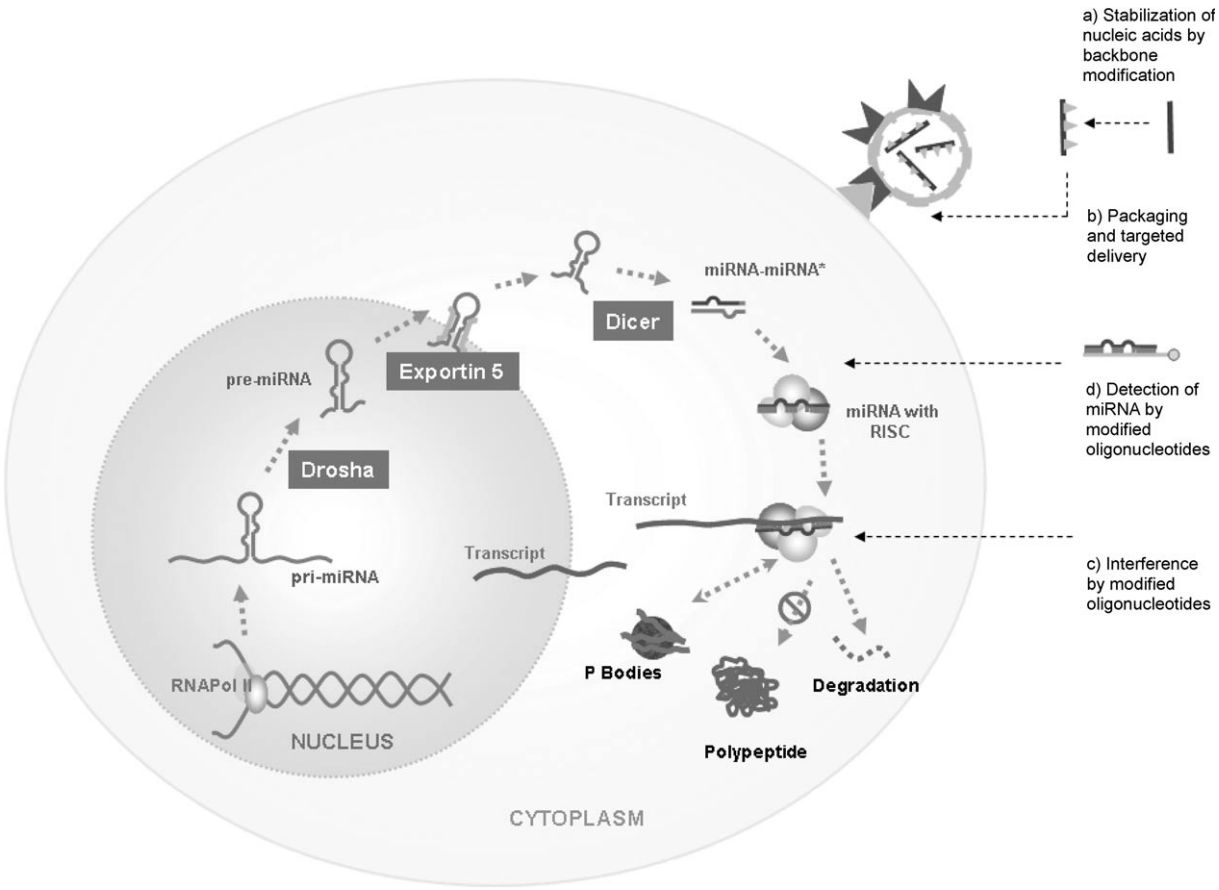

Figure 1. Schematic representation of miRNA formation and action in biological systems. a), b), and d) are important steps in development of therapeutics whereas c) is important in the development of diagnostics.

have proven to be elusive targets for the host immune system and therapeutics alike, because their rapid evolution into sequence variants allows them to evade the host surveillance and defence mechanisms and develop resistance to therapeutics. Recent advances have brought to light a number of instances where miRNAs play a critical role in 1) regulating a key viral gene, 2) modulating virus levels, and 3) altering susceptibility of the host cell to the virus. Primate foamy virus replication can be inhibited by miR-32 whereas binding of miR-122 to the hepatitis C virus seems to support its propagation in certain cell types.[14] Computational approaches have been used to identify human miRNA targets in critical genes of HIV-1.[15] Clearly, the array of antiviral miRNAs is growing. These antiviral miRNAs provide a hitherto unknown level of therapeutic intervention. Moreover, quantitative detection of miRNAs can provide diagnostic information about susceptibility to viral infections, similar to their proposed use as biomarkers in cancer.[17] miRNAs are also being explored as biomarkers for disease susceptibility and prognosis. For example, susceptibility to viral infection may be determined by the presence of specific miRNA molecules in a host cell. The presence of the miR-122 seems to be a critical difference between two types of liver cells, HepG2 and Huh7. Huh7 expresses the miRNA and allows replication of the hepatitis C virus, whereas the miR-122 negative HepG2 cells are nonpermissive for viral proliferation.[7] Mutations in the seed region of the miRNA prevent replication of the virus unless compensated for by a mutation in the virus. In contrast, PFV-1 virus replication seems to be restricted by miR-32.[18] Deletions at 13q14, a region of the 13th human chromosome, was known to be associated with chronic lymphocytic leukaemia (CLL). This region harbours miR-15 and miR-16, downregulation or deletion of which can account for a majority of CLL cases.[19] The repository of miRNAs in the cell can therefore communicate critical information about the susceptibility status of the cell to viral infections and oncogenesis. Accurate, quantitative detection of miRNA levels in biological samples is the next challenge in developing diagnostic markers based on miRNAs. However, the transition from fundamental knowledge to a successful therapeutic or diagnostic will depend on four key areas: 1) improved understanding of RNA–RNA and RNA–DNA interactions; 2) development of optimised stabilisation, packaging, and delivery agents; 3) innovations and improvements at the interface of chemistry and biology of nucleic acids, especially development of highly specific RNA binding agents; 4) development of accurate, high-throughput and cost-effective detection technologies.

miRNA-target interaction

Our understanding of RNA–RNA interactions has been drawn from the well established rules that govern DNA–DNA interactions. However, the range of permissible base–base interactions is far more in RNA–RNA hybrids than in their DNA–DNA counterparts. Interactions between biological molecules, present at low concentrations in the intracellular milieu are associated with small changes in entropy, a situation reminiscent of transcription factor–DNA interactions. miRNA action is expected to be dependent on intermolecular miRNA–mRNA (target) interaction,[20] intramolecular RNA–RNA interaction within miRNA precursors and the target as well as RNA–protein interaction. A crucial difference between miRNAs and siRNAs is the incomplete base-pairing in miRNA–target pairs. The role of base-stacking interactions, a critical factor in the prevalent DNA–DNA interaction rules[21] is likely to differ significantly because of the presence of gaps in the miRNA–target duplex. An integrated study involving biophysics, molecular biology, and bioinformatics is needed to develop tools based on the rules of miRNA–target interaction deciphered through extensive biophysical studies coupled with in vivo measures of efficacy.

The real challenge in deciphering miRNA biology is accurate prediction and experimental validation of miRNAs and their targets.[22] Novel computational methods for prediction of miRNAs have recently come up, including a handful of ab initio methods which would be of immense utility in discovering novel virus expressed miRNAs and nonconserved mammalian miRNAs. Similarly target prediction methods have also improved over time.[23] We have recently developed an improved algorithm for prediction of miRNA targets taking into account target site secondary structure and accessibility (unpublished results). Though there have been substantial improvements in the prediction accuracy of miRNA target prediction algorithms, they are far from perfect and still remain a major challenge for computational biologists.

Artificial miRNAs (amiRNAs) also offer huge potential as a therapeutic. These are engineered small RNAs which can specifically target one or more related transcripts and cause translational suppression. A recent study on artificial miRNAs has addressed their efficacy in plants[24] where the mechanism of interference is cleavage of a narrow set of targets compared to the wider range of animal miRNA targets. Our group has recently developed a novel algorithm to design highly specific miRNAs, for a transcript or group of transcripts (unpublished results). Understanding the complexity of miRNA regulation whereby miRNAs regulate biological processes at multiple steps would help us design amiRNAs specific for processes rather than particular transcripts. The immediate application of amiRNAs would probably be as an antiviral because the imperfect complementarity afforded by miRNAs may prove to be more advantageous than siRNAs given the rapid rate of mutations in viruses.

Anti-miRNAs, which are small oligonucleotides with perfect complementarity to the miRNA sequence, have been extensively used as a research tool to selectively knock down miRNA action. Anti-miRNAs would seem to be a good therapeutic candidate against virus expressed microRNAs as they share less homology with the host sequences and thus minimize the chances of off-target events.

Detection of miRNA

Besides adaptations of all the conventional gene expression detection methods a variety of novel approaches are being explored to detect miRNAs. Locked nucleic acid (LNA) modified oligonucleotides have been used as probes for improving sensitivity and specificity in in situ miRNA detection,[25–27] microarray based profiling,[28] northern hybridisation, and primer extension.[29] A study where FRET has been used in combination with RT-PCR showed that expression levels of miRNAs may range from 10 to 30,000 copies per cell, illustrating the challenge of developing detection methods with linear ranges that cross several orders of magnitude.[30] Another adaptation of FRET-based detection of miRNAs is their use with ribozymes where miRNA-induced structural changes trigger cleavage of a detector oligonucleotide releasing the fluorescent moiety from the quencher.[31]

RNA delivery

Highly selective RNA aptamers that bind to specific isoforms of vascular endothelial growth factor (VEGF) has been used in therapy of macular degeneration.[32] The RNA aptamers were stabilised by 2′-O Methyl modification of the backbone and pegylation was used to improve stability and solubility. As small RNAs are cleared from circulation rapidly by the kidneys,[33] a second purpose of packaging is to reduce renal filtration and increase effective concentrations. Another novel nanotechnology-based method has been demonstrated by the fusion of functional domains constructed from RNA: siRNA against the target, pRNA molecules of the phage phi9 for packaging, and receptor binding RNA aptamers to deliver the RNA assembly to cancer cells.[34] The nucleic acid binding properties of protamine have been used to develop antibody–protamine fusions to package and deliver RNA to targeted sites.[35] However, conjugating a nonspecific agent like cholesterol targeted to the ubiquitously expressed LDL receptor failed to show any tissue selectivity.[36] Liposome encapsulation of nucleic acids has been used successfully for in vivo delivery. Though not as specific and efficient as viral vectors in eliciting high expression, they are considered safe alternatives to viral vectors as a therapeutic delivery agent. Recent developments include conjugation with homing signals for tissue/cell-type specific delivery of nucleic acids.[36]

In their natural context, the mRNA target is affected by multiple miRNAs. In future, delivery systems may consider simultaneous delivery of multiple miRNAs, especially as such a multivalent approach would also prevent rapid emergence of resistant viral strains and cells within the cancer tissue.

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