Minireview

Additional stories of microRNAs

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Abstract

MicroRNAs (miRNAs) have been shown to be major regulators of eukaryotic gene expression. Traditionally, miRNAs were thought to control highly complex signal transduction and other biological pathways by targeting coding transcripts, accounting for their important role in cellular events. Traditional miRNA biogenesis and function focused on several key enzymes that functioned in miRNA maturation and miRNA inhibitory function upon binding to 3′-untranslated region of target transcripts. However, recent studies have revealed that miRNA biosynthesis and function is complicated, with many exceptions to conventional miRNA mechanisms. In addition to those noncanonical miRNA functions, this review introduces newly discovered biogenesis and regulatory mechanisms, as well as a new class of miRNA-sized small RNA and miRNA methylation. miRNA inhibition and intercellular miRNA signaling are also discussed. Taken together, these insights extend current understanding of miRNAs.

Keywords: microRNA, small RNA, miRNA-sized small RNA, RNA inhibition, exosome

***Experimental Biology and Medicine* 2014; 239: 1275–1279. DOI: 10.1177/1535370214544269**

Introduction

Current studies have shown diverse roles for RNA molecules, whether as intermediate molecules that generate enzymes, with catalytic activity like ribosomes, or with regulatory functions like microRNAs (miRNAs). Since their biogenesis and functions were first elucidated, miRNAs have become one of the most studied molecules in all biology. Traditionally, miRNAs were thought to control highly complex signal transduction and other biological pathways by targeting coding transcripts, accounting for their important role in cellular events.[1,2] Many miRNA research papers begin with ''miRNAs are ~22 nt, small non-coding RNAs that bind to 3′-untranslated region (UTR) of target mRNAs and have translational inhibitory function…'' This is likely because traditional studies on miRNA biogenesis and function focused on several key enzymes that control miRNA maturation and the mechanism of miRNA inhibition by binding to the 3′-UTR of target transcripts. These ideas must now be revised because various miRNAs have been shown to operate through noncanonical mechanisms. A previous review discussed certain noncanonical roles for miRNA studies and raised six issues that could not be addressed with miRNAs as originally characterized.[1] The issues concerned miRNAs binding other than the 3′-UTR, miRNAs existing elsewhere than the cytoplasm, alternative pathways for miRNA biogenesis, possible locations of miRNA genes, and miRNAs found in other than eukaryotic cells.[1] Very recently, even more discoveries regarding function and biogenesis of miRNAs have been made. Although many are still searching for missing pieces in the miRNA network, this mini review points to five other developments that will expand our knowledge of miRNAs and help researchers move beyond the conventional paradigm of miRNA studies.

miRNA-sized small RNA (msRNAs) in bacteria: microRNAs in microorganisms

The first viral miRNAs, reported in Epstein–Barr virus in 2004,[3] functioned in host and viral gene regulation. Many RNA viruses, including HIV, possess miRNAs that regulate target genes in the host.[4] However, many viral miRNAs may have also evolved to regulate their own virally encoded transcripts.[5] Even though the discovery of viral miRNA implied that miRNAs are evolutionarily well conserved, there remained a missing link between viral and eukaryotic miRNAs. What about bacteria?

Before miRNAs were found in eukaryotic cells, noncoding small RNAs (sRNAs) around 50–200 nt in length were discovered in bacteria.[6,7] Interestingly enough, the mechanisms utilized by sRNAs resemble those of miRNAs, which posttranscriptionally regulate targets by binding to target transcripts. RNAs even smaller than sRNAs and actually closer in size to eukaryotic miRNAs have also been recently identified in both Gram-positive and -negative bacteria.

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Unlike viruses that utilize their eukaryotic host enzyme system, not every bacterium is assisted by enzymes orthologous to DICER or Drosha/DGCR8, which are believed to be essential for miRNA maturation. This may account for the delay in finding bacterial miRNA. However, it is now believed that many miRNAs can be matured through a process that bypasses those essential enzymes,[1,8,9] making it possible to identify bacterial miRNAs.

msRNAs have been shown to exist in *Streptococcus mutans*[10] and *Escherichia coli*,[11] although the function of these RNAs remains unclear. Still, we cannot rule out the possibility that this small class of RNAs (~22 nt) may be functional in bacteria, in a manner similar to sRNAs or eukaryotic miRNAs. Note that like eukaryotic miRNAs, they are processed from hairpin-structured precursors and are abundant enough to be detected by northern blot.

The existence of msRNAs in bacteria implies that these kinds of small RNAs are evolutionarily well conserved from viruses and bacteria to mammals and that research into msRNA-mediated gene regulation may shed light on bacterial physiology.

Biogenesis and processing of miRNA: miRNA arm selection, loop-miRs, and tRNA-derived RNA fragments (tRFs)

Both canonical and noncanonical miRNA processing have been discussed in a previous review.[1] During typical miRNA biogenesis, one arm of the duplex is preferentially selected for entry into the RNA-induced silencing complex (RISC) in a process called miRNA arm selection (see review[2]). The precursor miRNA (pre-miRNA) is composed of 5 p and 3 p arms, referring to the direction of the mature miRNA strand. It is not clear how one arm of the miRNA duplex becomes dominant during biogenesis. Previously, models attributed selection to thermodynamic stability and energy-independent endonuclease-mediated selection with argonaute 2.[12,13] Recently, it was suggested that target abundance stabilizes the less dominant strand of miRNA, implying that target transcript regulation can affect the selection of mature miRNAs.[14] More recently, it was reported that 3 p-miRNA is successfully activated if the pre-miRNA is 5′-capped.[15] In addition to arm selection, the loop region of the pre-miRNA hairpin can also be used for so-called loop-miR, suggesting that double strands generated by typical miRNA processing are not absolutely needed for certain miRNAs.[16]

The exact mechanism of arm selection for each miRNA is still unclear, but the mechanisms described earlier may help in understanding miRNA maturation and the clinical application of small hairpin RNAs to patients.

miRNAs are processed from long, noncoding miRNA precursors (pre-miRNAs).[1,2] However, a significant number of the short (17–26 nt) RNAs are derived from mature or precursor tRNAs,[17] now called tRFs. Furthermore, one tRF, tRF-1001, was shown to have a cell proliferative function[17] and to respond to viral infection,[18] suggesting a biological function for these novel short RNAs. Endonuclease angiogenin-mediated tRF biogenesis indicates that tRF biogenesis is not due to random degradation of tRNAs.[18] Moreover, the existence of tRFs also suggests the existence of other RNA classes, such as ribosomal RNA (rRNA)-derived miRNAs, which have usually been ruled out during bioinformatics data processing.

Methylation and miRNAs

DNA methylation is a critical mechanism of epigenetic regulation. For RNA, eukaryotic rRNA methylation, found in the 1990s, is thought to be critical for ribosome biogenesis and function.[19,20]

Certain miRNA genes, like other genes, are regulated by methylation. In experiments using DNA methyltransferase knockout (KO) cell lines, it was shown that about 10% of miRNAs were downregulated, suggesting that DNA methylation may regulate miRNA expression in a direct or indirect manner.[21] In human cancer, CpG island methylation-associated silencing of miRNAs associated with antitumor features has also been identified.[22–24] For example, downregulated promoter methylation of the miR-34 b/c gene was found in malignant pleural mesothelioma cell lines.[25] Besides in cancer, methylation of DNA and miRNAs has been shown to be both cooperative and antagonistic in regulating gene expression related to autoimmune disease.[26]

Methylation is also crucial in plant miRNA biogenesis. The methyltransferase protein HEN1 can methylate miRNA duplexes directly in plants.[27] Methylated miRNAs may be protected from exonucleases and facilitate the recognition of RISC assembly.[27] Since there is no known HEN1 homolog in animals, it is not clear if animal miRNAs could also be modified. However, miR-675 was found to be processed from H19 long noncoding RNA (lncRNA), which belongs to a highly conserved imprinted gene cluster.[28] This suggests that even though they are not mature miRNAs, primary miRNAs can be modified, thereafter affecting miRNA processing. Interestingly, imprinted H19 lncRNA was reported to function as a natural miRNA sponge that antagonizes the let-7 miRNA (see ''miRNA inhibition: antagomir, miRNA sponges, circular RNA (circRNA), competing endogenous RNA (ceRNA), and KOs'' section).[29]

Previously, *de novo* RNA-directed DNA methylation using mRNA overexpression was proposed in plants.[30] Recently, some miRNAs have been shown to be involved in the DNA methylation pathway[31–33] and even in site-direct target gene methylation.[33]

In addition, the artificially modified 2′-O-methyl (2′-OMe) group of small RNAs, discussed below has long been used as siRNA or as an endogenous miRNA inhibitor due to its stability.[34–36]

Taking these findings together, we see that miRNAs have more functions in epigenetics than in their previously known posttranscriptional repressive role and that miRNA involvement in methylation is crucial for both target regulation and miRNA expression itself. Involvement of miRNAs in methylation will help us understand complicated regulation of miRNAs and their target genes.

miRNA inhibition: antagomir, miRNA sponges, circular RNA (circRNA), competing endogenous RNA (ceRNA), and KOs

Many approaches, including exogenous overexpression or inhibition, have been used in miRNA functional studies.

Silencing of miRNAs was first studied using miRNA antisense 2′-OMe oligonucleodites (oligos) *in vitro*.[34,37] Chemically modified 2′-OMe oligos and cholesterol-conjugated single-stranded RNA complementary to miRNAs, called *antagomirs*, were also shown to function *in vivo*.[36] They are thought to competitively inhibit miRNAs by binding to the mature miRNA strand.[38] Another class of competitive inhibitors, termed miRNA sponges, are transcripts expressed from strong promoters and contain several tandem binding sites to an miRNA of interest.[39] Interestingly, endogenous sponge-like noncoding RNAs can act as miRNA inhibitors. ciRS-7, a circRNA, harbors over 70 miR-7 binding sites and therefore reduces miR-7 activity.[40,41]

Another novel class of sponge-like RNAs is ceRNA, which includes certain lncRNAs. Loc285194, also called LSAMP antisense RNA 3, could inhibit tumor cell growth by suppressing miR-211 both *in vitro* and *in vivo*.[42] The muscle-specific lncRNA linc-MD1 also acts as a ceRNA in myoblasts by inhibiting miR-206/133 b.[43] In addition to lncRNAs, protein-coding transcripts can function as ceRNAs. For example, phosphatase and tensin homolog (PTEN), a key tumor suppressor, is regulated by many protein-coding RNAs (PTEN ceRNAs) through interaction with PTEN-regulating miRNAs.[44] This suggests that coding and noncoding transcripts, which regulate cellular processes by inhibiting miRNA functions, operate in ways more complex than we currently understand (for detailed information, see Cesana *et al.*[43] and Turner and Morris[45]).

To study loss of miRNA function *in vivo*, individual miRNA KOs were first introduced, with increasing numbers of KO mice continuously being reported.[46] Whereas one of the first miRNA KOs, that of muscle-specific miR-1, resulted in dysregulation of cardiogenesis,[47] some miRNA KOs show no obvious phenotype. This could be due to a functional redundancy among similar miRNAs or a family of miRNAs.[46] Nonetheless, the best way to understand the function of miRNAs is to generate individual miRNA KOs and analyze them in depth. Moreover, new genetic approach called clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system,[48] which in theory makes it easy to produce KO cells in every species, has been adopted for sequence-specific inhibition of miRNAs.[49] This new approach, also known as genome editing, is expected to become widespread in miRNA functional studies.

Intercellular signaling of miRNAs: exosomal miRNAs, salivary miRNAs, and circulating miRNAs

Cells in the body communicate with each other through secreted signaling molecules, including hormones, chemicals, peptides, lipids, or nucleic acids. It has been shown that miRNAs can regulate the secretion or expression of such molecules.[37,50,51] Recent evidence has shown that miRNAs themselves can be secreted, circulated, and taken up by other cells. Although ribonucleases are present in body fluids, RNAs, including miRNAs, are relatively stable because they are included within lipoprotein vesicles (or exosomes).[1,52] Exosomes are microvesicles or nanovesicles (30–90 nm) that carry proteins, lipids, and nucleic acids, including miRNAs, so miRNAs can circulate through the body.[53] Exosomal miRNAs are derived from multivesicular bodies that are protected from degradation and can be delivered over long distances within body fluids or the bloodstream (Figure 1).[54,55]

Possible gene exchange between cells through exosomally shuttled mRNA and miRNA was first reported by Valadi *et al*. from mouse and human mast cells.[56] A later study proposed that exosomal miRNAs were secreted in a ceramide-dependent manner.[52] Secreted miR-146 a, a tumor-suppressive miRNA, inhibited the expression of its target gene in the recipient cells, suggesting that miRNAs are transferable and functional in recipient cells.[52,57] Similar exosomally shuttled miRNAs are involved in communication between dendritic cells.[58] miRNA transfer may not be limited to individuals. Remarkably, miRNAs might move from mother to baby through the milk, like antibodies, and even between parasite microorganisms and their hosts. Salivary miRNAs have been characterized and can be easily used as biomarkers for oral diseases, including oral cancers, due to their fast and easy collection.[59,60]

Hormone-like functions of encapsulated miRNAs are intriguing and require further study. Circulating miRNAs and segments of DNA in all body fluids hold promise as markers for diseases, as the relative stability of body fluid miRNAs allows testing for noninvasive biomarkers for cancer and other diseases.

miRNA biomarker research is gaining traction, especially in conjunction with mature techniques such as miRNA microarrays, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and deep sequencing.[61,62] Many aspects of circulating miRNAs, such as their specificity, high stability, and easy detection, make them attractive targets of study in many diseases.

Information on extracellular circulating miRNAs can be found in the miRandola database.[63]

Conclusion and perspectives

This review has surveyed newly discovered biogenesis and regulatory mechanisms, including a new class of msRNAs and miRNA methylation. miRNA inhibition and miRNA as an intercellular signaling molecule were also discussed.

For a fuller understanding of miRNA functions, we must consider many aspects of miRNA biology and not be constrained by current dogma. The potential of miRNAs in facilitating the diagnosis, prognosis, and regulatory mechanisms of diseases has barely been realized. The studies described here suggest that miRNAs are in some way involved in regulating every biological event and will thus be essential in a range of future therapies. Specifically, various miRNA inhibitory systems and exosomal miRNA functions can be utilized in early diagnosis of

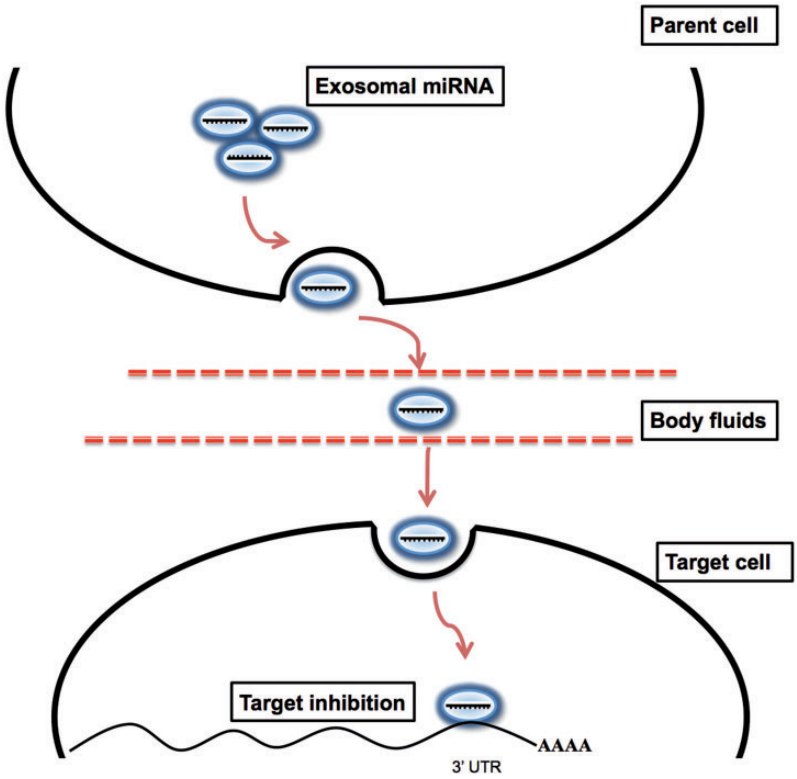

Figure 1 Schematic diagram of the exosomal, hormone-like function of secreted miRNA. Encapsulated miRNAs (miRNA-containing exosomes) are released from multivesicular bodies (MVBs) in the cell into blood or other body fluids. Released exosomes may deliver extracellular miRNAs to recipient cells over long distances. miRNAs within exosomes are transported into recipient cells (target cells) by membrane fusion and could inhibit translation of target genes in recipient cells. (A color version of this figure is available in the online journal)

diseases and in gene therapy for miRNA-related diseases. Although miRNA therapeutic approaches have some limitations, including off-target effects and functional redundancy, those limitations will eventually be overcome. Recently developed techniques, such as deep sequencing, direct-RNA sequencing, and digital polymerase chain reaction (PCR), will also enable rapid expression profiling of miRNAs and analysis of the novel class of small RNAs.

Taken together with a previous review,[1] the recent insights into miRNA presented here indicate directions for research in discovering miRNA-related mechanisms of gene regulation.

ACKNOWLEDGEMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Korean Government (MSIP, 2008-0062282).

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