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INVITED REVIEW

Defining Larger Roles for "Tiny" RNA Molecules: Role of miRNAs in Neurodegeneration Research

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Abstract Many facets of transcriptional and translational regulation contribute to the proper functioning of the nervous system. Dysfunctional control of mRNA and protein expression can lead to neurodegenerative conditions. Recently, a new regulatory control element—small noncoding RNAs—has been found to play a significant role in many physiologic systems. Here, we review the microRNA (miRNA) field as it pertains to discovery-based and mechanistic studies on the brain and specifically in neurodegenerative disorders. Understanding the role of miRNAs in the brain will aid to open new avenues to the field of neuroscience and, importantly, neurodegenerative disease research.

Keywords gene array · transcriptional regulation · translational regulation · neurodegeneration · HIV · neuron · mRNA · microRNA

To date (06/01/09), there are 9,539 microRNA (miRNA) entries in the online repository miRBase (version 13.0, http://miRNA.sanger.ac.uk) and many more new sequences are being added on a regular basis. The fundamental understanding of miRNA biogenesis and its function has tremendously increased over the past 5 years. Almost all fields in biology have been transformed with the discovery of small noncoding RNAs, leading to novel discoveries on how these molecules affect biological functions.

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miRNA biogenesis

As the name "micro" suggests, miRNAs are tiny ~20–22 nucleotide (nt) regulatory RNA molecules, which are encoded by the genome but are not translated into protein. Instead, they play a potential role to control the gene expression by associating with the 3′ untranslated region (UTR) of nascent messenger RNA (mRNA) molecules thereby repressing their expression. These miRNAs have been identified in a broad scale of genomes ranging from viruses to humans (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart and Ruvkun 2001; Bentwich et al. 2005; Berezikov et al. 2005).

The biogenesis of miRNAs has been extensively studied and reported. The early steps in biogenesis begin in the nucleus where the miRNA genes are predominantly transcribed by RNA polymerase II (with some by RNA polymerase III) into primary miRNA transcripts also called as pri-miRNAs (Cai et al. 2004; Lee et al. 2004; Borchert et al. 2006). The pri-miRNA is cleaved by a nuclear microprocessor complex, which includes an endonuclease RNA III enzyme named Drosha; and a protein component termed Di George critical region 8 (DGCR8), which also goes by the name Pasha in *Drosophila melanogaster* and *Caenorhabditis elegans* (Denli et al. 2004). This latter cofactor, DGCR8/Pasha, interacts with the ~33-bp stem of the pri-miRNA thereby aligning and anchoring it for subsequent Drosha cleavage (Han et al. 2006). The pri-miRNAs are then cleaved by Drosha in the nucleus to produce ~70-nt precursor miRNAs (pre-miRNAs) with a hairpin structure (Lee et al. 2003). The pre-miRNA is then shuttled to the cytoplasm where it is cleaved by another RNase III enzyme, Dicer, thereby generating a mature ~20–22-nt double-stranded RNA molecule (Hutvagner et al. 2001). The double-stranded molecule is unwound first, and the stable

strand is chosen to be recruited by a RNA-induced silencing complex (RISC) comprising of Argonaute and related proteins (Peters and Meister 2007). RISC binds to the 3′ UTR of the mRNA molecules with an imperfect complementary to the miRNA, leading to mRNA degradation and/or translational inhibition, resulting in decreased protein expression (Pillai 2005; Nilsen 2007). While sites of miRNA action in the 3′-UTR are the most common, the protein-coding region can also contain functional miRNA targets (Rigoutsos 2009).

Tools and techniques to isolate and identify miRNAs

For the past decade, substantial work has been done on identifying new miRNAs in the mammalian genome, and new techniques have been developed to monitor their function. The major challenge in miRNA biology is the enrichment of small species from the complex pool of abundant species that include rRNA, tRNA, and mRNA. Moreover, the miRNA itself exists in three forms: short mature form, hairpin pre-miRNA, and long pri-miRNA. The isolation of short mature forms (the functional form) is often a tricky process. Several techniques have been used to purify small RNA species from the pool and have made researchers' jobs more straightforward. Techniques such as forward genetics were extensively used in the past to study and identify miRNAs in *C. elegans* and *D. melanogaster*; and in fact, the first miRNAs, lin-4 and let-7, were discovered by this method (Lee et al. 1993; Reinhart et al. 2000).

However, due to several limitations (Berezikov et al. 2006) forward genetics has proven to be relatively inefficient. cDNA cloning and sequencing methods have proven to be very useful tools to identify many miRNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). This technique has been employed to samples expressing low amounts of RNA or to rare cell types (Berezikov et al. 2006). A variation in sequencing techniques called "deep sequencing" was introduced as a technological breakthrough using advanced sequencing platforms. Although this technique invariably helped to profile known and novel miRNAs at exceptional sensitivity, the main drawback was the need of good algorithms for eliminating the false-positive data. Recently, an ingenious publicly available software called "miRDeep" was developed to help estimate the false-positive rate and sensitivity of the runs (Friedlander et al. 2008).

Currently, the most widely used method for characterization of miRNAs expression has been the microarray method. The first probing was done as dot blotting on nylon membranes (Krichevsky et al. 2003), which proved to be very inexpensive but required larger amounts of RNA. Later, researchers developed a much more sensitive method to screen miRNAs from smaller samples by hybridizing the probes to glass slides (Liu et al. 2004). The microarray has a more preferable platform as it can conveniently screen a larger number of miRNAs and is flexible for probe design. The main limitation that is encountered in the microarray is the specificity of probe binding that is mainly caused by the short length of the probe. Several nucleic acid analogs have been introduced lately to the market such as the LNA (locked nucleic acid) and PNA (peptide nucleic acid) that result in more favorable hybridizations than the conventional array platforms. High-throughput microarray profiling is now used for detecting miRNAs and other small RNAs in complex tissues of higher mammals (Maroney et al. 2007). Arrays to assess miRNAs can be made by researchers and are also available from a number of commercial sources. However the true sensitivity, specificity, and reproducibility of these arrays remain to be determined.

Modified probes also have been designed to improve sensitivity and specificity for Northern blotting techniques, a technique which is also widely used for detection of miRNAs (Valoczi et al. 2004). Several other technologies have been developed to study miRNAs from diseased tissues and limited tissues, such as biopsy samples or brain sections. One of the widely used technologies for such small samples, as well as to validate array-based findings, is quantitative real-time polymerase chain reaction. Both the Taqman and LNA technology have been used to improve the sensitivity in detecting and quantifying low abundant miRNAs. Bead-based methods for analysis by flow cytometry, RNA-primed-array-based Klenow enzyme assay and nanotechnology are a few of the many technologies being developed for increasing the sensitivity of miRNAs (Kong et al. 2009).

miRNAs, the mammalian brain, and neurodegenerative disorders

The mammalian brain is the most complicated organ to study, not only from the gross functional, structural, and evolutionary distinctiveness but also due to its complexity at the molecular level. Though the human genome has been fully deciphered, the human brain transcriptome still remains to be solved. Progress is continuing on assessing mRNA expression patterns in the brain; for example, the Allen brain atlas (http://www.brain-map.org) is due to be completed in 2012. However, another level of complexity arises as one starts to screen for miRNAs that can regulate these genes in the human brain. The unprecedented role of miRNAs in regulating mRNA levels and protein has led to intense interest in studying miRNA expression and function in the central nervous system (CNS).

While discovery-based and profiling studies continue in addition to the crucial follow-up mechanistic studies, one aspect that has received less attention is the necessity to provide a regional and cell-type specific examination of miRNA expression in the normal and diseased brain. The brain not only is quite heterogeneous in structure and function, but also is made up of a number of cell types of which only a minority are neurons. While the effects of non-neuronal cell types on neurons and CNS function are profound, it is important to delineate whether changes in miRNAs that are found in the brain occur in neurons, glia, or other cell types. The only way to definitively show this is through in situ hybridization, which remains a difficult technique in miRNA assessment. While a number of new protocols have been described recently (Nuovo 2008; Nelson and Wilfred 2009; Nuovo et al. 2009a, b; Pena et al. 2009), optimized protocols, especially to include double labeling to allow cell-type identification, remain a distinct goal.

The importance of miRNAs in the nervous system was first described in *Danio rerio* (zebrafish) where a mutation in *dicer* led to failure to produce mature miRNAs and resulted in gross morphological defects in the nervous system (Wienholds et al. 2003). Effects of specific miRNAs on neurons have been found in a number of organisms including mammals. For example, during neurogenesis, the levels of both miR-124 and miR-9 are greatly increased, and in vitro experiments have linked both to neuronal differentiation (Conaco et al. 2006; Krichevsky et al. 2006). Deep sequencing of miRNAs derived from tissues and cell lines have revealed these and other miRNAs to be restricted to the CNS (Landgraf et al. 2007). Definitive proof of the role of miR-124 in neurogenesis has now been achieved in vivo, revealing its critical role in the differentiation of neurons from neural precursors (Cheng et al. 2009).

In addition to differentiation of neurons, miRNAs have been shown to affect crucial aspects of neurons. For example, neurite outgrowth is regulated by miR-132 (Vo et al. 2005). Furthermore, one crucial functional aspect of neurons, the synapse, is under miRNA control. In the hippocampus, miR-134 regulates the size of dendritic spines, sites of synaptic transmission (Schratt et al. 2006). Further linking of miRNAs to synaptic changes and the implications of such in brain development and plasticity was the recent demonstration that miR-138 controls dendritic spine morphogenesis (Siegel et al. 2009).

Recognizing the role of miRNA in neuronal development and control of neuronal functional elements, it is not surprising that many researchers have sought to link miRNAs to neurodegenerative diseases. Many studies have been done in Alzheimer's disease (AD), profiling miRNAs as well as linking miRNAs to expression of putative pathogenic molecules such as beta-site amyloid precursor protein-cleaving enzyme 1 (beta-secretase, BACE1). Two studies have linked different miRNAs (miR-29a/b-1 and miR-107) to modulation of expression of BACE1 in AD (Hebert et al. 2008; Wang et al. 2008); regulation of BACE1 by miR-298 and miR-328 has also been reported (Boissonneault et al. 2009). Notably, for miR-107, its expression was found decreased in the brains of those with AD even at the early stages of pathology (Wang et al. 2008). In addition to BACE1, studies have revealed that the related miRNAs miR-20a, miR-17-5p, and miR-106b can regulate the level of amyloid precursor protein in vitro, and it was found that the levels of miR-106b were decreased in the brains of AD patients (Hebert et al. 2009). Furthermore, miR-146a is increased in AD and linked to inflammatory and/or stress responses (Lukiw et al. 2008). Others have also reported differences in miRNA profiles in AD (Cogswell et al. 2008; Sethi and Lukiw 2009). Given the complexity of AD, it is likely that additional studies will add to our understanding of miRNA perturbations in this disorder.

Huntington's disease (HD), which results from polyglutamine- mutant huntingtin (Htt) protein, is characterized by neuronal mRNA dysregulation. One function of the Htt protein is to sequester the transcription factor REST in the cytoplasm. Mutant Htt is unable to perform this function and REST translocates to the nucleus, where it decreases neuronal gene expression. A number of miRNAs are targets for REST, and in a mouse model for HD (the R6/2 transgenic line), four of these: mir-29a, mir-124a, mir-132, and mir-135b show decreased expression in the cortex (Johnson et al. 2008). Examination of human HD brains revealed that mir-132 was also downregulated; however, in human samples, mir-29a and mir-330 (another REST-targeted miRNA) were upregulated (Johnson et al. 2008). The genes encoding miR-9/miR-9* are also targets for REST, and another study found that these miRNAs are also decreased in HD brain, and interestingly themselves regulate components of the REST complex (Packer et al. 2008). In keeping with such findings, there are a number of perturbations in miRNAs in both human and mouse models of HD (Johnson et al. 2008). Intriguingly, in addition to sequestering REST, wild-type Htt protein stabilizes interactions within the RISC, but the mutant Htt inhibits the formation of such complexes (Savas et al. 2008). Thus, in HD, the expression as well as function of miRNA may be altered, both leading to changes in neuronal protein composition.

Whereas HD results from polyglutamine-mutant Htt, spinocerebellar ataxia type I (SCAI) results from polyglutamine-mutant ataxin 1 (ATX1). The level of the mutant protein affects disease severity, and three miRNAs (miR-19, miR-101, and miR-130) can alter the level of

ATX1 (Lee et al. 2008). Interestingly, this study found that these miRNAs act cooperatively in reducing ATX1 levels, and are indeed expressed in infected neurons, and that in a cell culture model, their inhibition (leading to increased mutant ATX1) resulting in increased cytotoxicity, linking this miRNA regulation to disease mechanisms.

TDP-43-positive frontal temporal dementia with ubiquitin positive, tau-negative inclusions (FTLD-U) results from a number of different loss of function mutations in the progranulin (GRN) gene. Interestingly, a common genetic variant in the 3′-UTR of the GRN gene increases the risk for FTLD-U, and this variant leads to creation of an efficient binding site for miR-659, which was shown to reduce expression of GRN carrying this variant (Rademakers et al. 2008). Individuals with this variant had reduced levels of GRN in their brains, revealing another mechanism in FTLD-U through post-transcriptional decreased GRN expression.

Parkinson's disease (PD) results from the loss of dopaminergic neurons in the midbrain. Downregulation of miR-133b is found in the midbrain of humans with PD (Kim et al. 2007). Interestingly, this study demonstrated that miR-133b participates in a feedback loop with the transcription factor Pitx3 controlling the development and function of dopaminergic neurons. In autosomal-dominant PD as well as transgenic mouse models of PD, increased α-synuclein is causative for disease, and accumulation of α-synuclein is characteristic of spontaneous PD. α-synuclein has been found to be regulated by miR-7, and in the MPTP neurotoxin model of PD, miR-7 levels decrease (Junn et al. 2009).

While these disorders are sporadic and/or genetic in nature, neurodegeneration also occurs from infectious etiologies. Prion diseases (also known as transmissible spongiform encephalopathies) result from the abnormal conversion of a normal host protein into an infectious neuropathogenic form. In mice, dysregulation of miRNA expression was found in prion-infected brains, with both up- and downregulation of specific miRNAs (Saba et al. 2008). Human immunodeficiency virus (HIV)-associated neurocognitive disorder (HAND) develops in a subset of individuals infected with HIV-1 and results from an indirect neurotoxicity, since HIV only infects macrophages and microglia in the brain. While mRNA dysregulation has been demonstrated in humans with HAND as well as monkeys with simian immunodeficiency virus (SIV)-induced CNS disease (Roberts et al. 2003; Masliah et al. 2004; Roberts et al. 2006), miRNA alterations have not yet been reported. Given the findings in the other neurodegenerative disorders above, it will be of interest to assess whether the mRNA changes and the neuronal dysfunction are linked to miRNA alterations in people with HAND and in the monkey/SIV model.

Perspectives

There remains much needed discovery-based work on miRNA. New miRNAs are still being identified, and obtaining knowledge of the expression pattern of known and new miRNAs during neuronal development, plasticity, normal functioning, and disease is a significant challenge. This is compounded in the brain by the intermixture of different cell types within the brain, as well as the numerous different anatomic and functional regions within the brain. Although difficult with these small RNAs, there is no substitute for in situ hybridization studies to follow up various experimental findings in order to assess the regions and cell types (often necessitating combination with immunohistochemistry for cell type identification) responsible for miRNA expression patterns.

Regarding the cell types, it is notable that to date the focus of research has been on neuronal expressed miRNAs. While this is understandable, it ignores the very real possibility that glia or other cells within the CNS cells contribute to or are themselves responsible for the neuronal changes, as non-cell autonomous neurodegeneration is likely not uncommon (Lobsiger and Cleveland 2007). All cell types express miRNAs, and miRNA alterations likely have a role in tumors of the CNS, such as medulloblastomas and astrocytomas (Pang et al. 2009). In addition, neurons themselves have structurally and functionally distinct domains, and distinct miRNAs have been found in the dendrites and at the synapse (Schratt et al. 2006; Kye et al. 2007; Lugli et al. 2008); in concert mRNAs as well as protein synthetic machinery exists there as well (Schuman et al. 2006). Furthermore, astrocytes are integral components of the tripartite synapse, at the synpase, emphasizing the need to assess non-neuronal cell types that can affect neuronal function. Finally, while model organisms will continue to be of great utility, there is no substitute for studies performed on human brains, due to human specific functionality as well as true disease manifestations.

Many studies have identified an miRNA that is altered in expression, and then search for potential targets, using bioinformatic approaches followed by experimental validation. While this approach has been fruitful, there is a high chance we are losing important findings. First, the bioinformatic techniques to identify targets are imperfect and often have little overlap between different algorithms. Although false positives will be identified in the validation experiments, false negatives will be lost, and still other potential candidates will never be chosen for validation. Second, miRNAs each regulate multiple transcripts. While a focus on individual candidate genes is more accessible experimentally, the overall effect of altered miRNA expression is to change the level of hundreds of proteins (Baek et al. 2008; Selbach et al. 2008). Understanding how

these changes work in concert to affect function requires a broader approach in investigations. Third, as exemplified nicely in the SCAI study described above (Lee et al. 2008), a given miRNA species does not exist alone, and is expressed in the context of other miRNAs with which it may work cooperatively. A focus on a single miRNA, similar to focusing on a single target, may confound discovery of the true biological effect resulting from altered miRNA expression. In this regard, it has been recently shown that the common method of transfection to experimentally induce high levels of an miRNA under study can lead to competition with endogenous miRNA for the cellular processing machinery, leading to artifactual increases in their targets (Khan et al. 2009).

While there is no simple solution to these issues, investigation of miRNA function in neurodegenerative diseases has benefited from several approaches. For discovery-based approaches, transcripts for candidate disease-specific protein-coding genes can be examined bioinformatically for potential miRNA binding sites, and then tested for regulation by those miRNAs. Altered expression of identified miRNAs can be then assessed in models of disease or diseased tissue itself. A complementary strategy is to assess miRNA profiles in disease models or diseased tissue, verify altered expression of specific miRNAs, and perform bioinformatic analysis for transcripts that may be regulated by these miRNAs, followed by verification. Instead of bioinformatics, other approaches such as gene array or proteomic analysis of appropriate cell lines or primary cells engineered to over- or under-express these miRNAs, are likely to lead to novel avenues of research and understanding. (Baek et al. 2008; Selbach et al. 2008; Barbato et al. 2009). Investigation and understanding of the cell-type specific regulation is critical, as many of the discovery-based studies first examine miRNA in homogenates of mixed cell types from the brain. Studies in disease models, both in cell culture and in model organisms, will not only provide the needed proof of relevance of molecular studies to mechanistic systems, but enable the better understanding of the complex interactions that occur in cells, tissues, and organisms. In this regard, better computational tools will be essential, and a holistic systems biology would complement well the reductionist nature of study of single miRNAs and single target transcripts.

Conclusion

Brain research has taken a major turn towards translational medicine. The basic understanding of the nervous system in the fields of neuroanatomy, neurophysiology, and neurochemistry has been extensively studied; but, as of yet, the cure for neurodegenerative disorders is still a daunting task for neuroscientists. The technological breakthroughs in neuroscience research, such as genomics and proteomics, have already led to great understandings about the genes and proteins involved that cause several neurological diseases. However, a deeper understanding on the regulation of these genes and proteins is needed. The discovery of miRNAs has opened new avenues of research in neuroscience to understand neurodegenerative disorders. Contributions of miRNA study to the areas of brain development, neurogenesis, neural differentiation, and synaptogenesis have been striking. Recent work on miRNAs in neurodegenerative diseases is quite promising. The study on disease-associated miRNAs, their mRNA targets, and resulting changes in protein products will continue to be an exciting field of research, leading to a greater understanding of the regulatory effects of the miRNA, and how their dysregulation leads to neurodegenerative diseases.

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