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Short non-coding RNA biology and neurodegenerative disorders: novel disease targets and therapeutics

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Genomic studies in model organisms and in humans have shown that complexity in biological systems arises not from the absolute number of genes, but from the differential use of combinations of genetic programmes and the myriad ways in which these are regulated spatially and temporally during development, senescence and in disease. Nowhere is this lesson in biological complexity likely to be more apparent than in the human nervous system. Increasingly, the role of genomic non-protein coding small regulatory RNAs, in particular the microRNAs (miRNAs), in regulating cellular pathways controlling fundamental functions in the nervous system and in neurodegenerative disease is being appreciated. Not only might dysregulated expression of miRNAs serve as potential disease biomarkers but increasingly such short regulatory RNAs are being implicated directly in the pathogenesis of complex, sporadic neurodegenerative disease. Moreover, the targeting and exploitation of short RNA silencing pathways, commonly known as RNA interference, and the development of related tools, offers novel therapeutic approaches to target upstream disease components with the promise of providing future disease modifying therapies for neurodegenerative disorders.

INTRODUCTION

Progress in the development of neuroprotective and disease modifying treatments for neurodegenerative disease has been impeded by our still relatively poor knowledge of basic disease pathogenesis. Many current treatments target single cellular pathways downstream of disease initiation and which may be beyond an effective therapeutic window (1–3). Moreover, functional characterization of such diseases has often centred on the study of rare monogenic disease variants which are not necessarily informative of the commoner sporadic forms of neurodegenerative disease where combinations of multiple genetic loci and non-genetic determinants are thought to play crucial roles (4,5). Furthermore, recent genomic studies are revealing a multi-layered complexity to the organization of biological systems and gene regulatory networks and it is therefore likely that a deeper understanding of such networks will be necessary to fully appreciate the pathophysiological complexity underlying the common neurodegenerative disease phenotypes such as Alzheimer's disease (AD) and Parkinson's disease (PD). An important layer of biological complexity where new understanding is emerging relates to RNA biology and in particular to the roles of small non-coding RNAs and RNA-based gene regulatory networks. Non-coding RNAs are abundantly expressed in the central nervous system (CNS) and increasingly such RNAs, in particular the genome-encoded microRNAs (miRNAs), are being found to have important functions in nervous system development and function, as well as in neurodegenerative disease pathogenesis (6–9). miRNAs are able to negatively regulate gene targets via sequence-specific post-transcriptional gene silencing (PTGS), which is the principle mechanism behind the RNA interference (RNAi) pathway(s). With an increased understanding of such short RNA regulatory networks, the ability to target such networks or to

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utilize RNA-based methods will allow the development of a new class of disease modifying therapies to emerge (10,11).

GENE SILENCING BY SHORT NON-CODING RNA REGULATORY NETWORKS

In mammals, RNAi represents a set of highly conserved cellular pathways whereby double-stranded RNA (dsRNA) is processed into short RNAs of ~20–30 nt in length. These short RNAs associate with members of the Argonaute (Ago) family of proteins to regulate gene expression at the transcriptional and post-transcriptional level (recently reviewed in 12,13). RNAi has a myriad of roles in every fundamental aspect of mammalian cellular function and its discovery has led to a widened appreciation for the role of very small regulatory RNAs in eukaryote biology. One of the most exciting developments since the discovery of RNAi in 1998 has been the application of exogenous RNAi tools as artificial regulators of gene expression; with particular emphasis on the generation of a special class of drugs that are capable of inhibiting rogue gene elements.

The therapeutic development of RNAi has been made possible by usurping elements of the endogenous mammalian miRNA biogenesis pathway for PTGS (Fig. 1A). miRNAs consist of a class of short ~22 nt RNAs derived from longer processed dsRNA precursors. RNA Pol II transcripts with hairpin motifs or primary miRNAs (pri-miRNAs) are processed by the RNase III enzymes Drosha and Dicer into short miRNA duplexes. Single or multiple (polycistronic) pri-miRNA motifs can be found within exonic or intronic coding and non-coding mRNA, or within antisense orientation transcripts or transcripts that span intergenic regions (reviewed in 14,15). The mature miRNA associates with the Ago2-containing RNA-induced silencing complex (RISC) and RISC-loaded miRNAs are guided to the 3′-untranslated regions (UTRs) of target mRNAs by a 'seed region': a specific complementary region between nucleotides 2–7 of the guide strand (16). Seed region matches within the 3′-UTRs of mRNAs primarily induce translation repression by one or more of the following mechanisms: transcriptional cleavage, blocking of ribosomal function, deadenylation or shunting of mRNAs to transcriptionally inactive cytoplasmic P bodies (reviewed in 17,18). However, Ago2 is responsible for the post-transcriptional cleavage of the target RNA and is confined to miRNAs or short interfering RNAs (siRNAs) with near-perfect sequence complementarity with their cognate target. Interestingly, endogenous mammalian miRNAs are rarely found to bind their targets completely. Since seed region matches alone can suffice for translational suppression, this suggests that a single miRNA potentially regulates up to a 100 mRNA targets (19). RNAi guide sequences may additionally associate with Ago1 in a RNA-induced transcriptional silencing complex (RITS) to induce transcriptional gene silencing (TGS), which is characterized by the targeting of siRNAs or short antisense RNAs to promoter elements, resulting in transcriptional inhibition through silent-state epigenetic modifications of DNA and associated nucleosomes (20–23) (Fig. 1B). As siRNA-directed TGS elicits more permanent epigenetic modifications it suggests that, unlike PTGS, TGS may offer a more sustained inhibition of gene expression. This represents an exciting and important novel approach for the development of therapeutic gene silencing modalities.

miRNAs are not the only source of short RNA duplexes and it remains to be seen what role these newer RNAi pathways will have in the development of novel therapeutics. A number of short 24–31 nt RNAs in the germline are associated with Piwi-family proteins and are referred to as a Piwi-interacting RNAs (piRNAs) (reviewed in 13) (Fig. 1C). These short RNAs, which originate from repeat-rich regions of the genome, are processed through a distinct Dicer-independent mechanism (24) and transcriptionally silence transposons by establishing *de novo* DNA methylation in murine fetal testes (25–27). A more recent RNAi-related pathway was discovered in studies of mouse oocytes and embryonic stem (ES) cells. An abundant class of endogenous siRNAs or endo-siRNAs was found to be derived from transcripts with long inverted repeats or from convergent and divergent transcripts of pseudogenes or transposons (28–30) (Fig. 1C). Other than blocking retrotransposition, little is known about the function of endo-siRNAs; it is unlikely that these RNAi species are limited to embryonic/oocyte cells since complementary RNA hairpins or duplexes form ubiquitously between *cis*- and/or *trans*-associating RNAs.

SHORT NON-CODING RNA REGULATORY NETWORKS AND NEURODEGENERATIVE DISEASE

The idea that dysregulation of the PTGS pathway might be a manifestation of or that specific non-coding RNAs, in particular miRNAs, might be directly causative of neurological disease is gaining ground. That specific non-coding miRNAs play fundamental roles in mammalian development, ES cell differentiation and in CNS development and function is now increasingly appreciated. A diverse repertoire of miRNAs is abundantly expressed in the CNS in tightly regulated and highly specific spatial and temporal patterns, and numerous miRNAs have now been associated with fundamental roles in neurobiology including in neuron-specific gene regulation and neuron-specific pre-mRNA splicing, neural cell lineage specification, neurogenesis and synaptogenesis (31–37). Moreover, altered miRNA expression and function has been strongly implicated in cancer and cardiovascular disease and so the notion of non-coding RNA involvement in disease pathogenesis is not new; indeed, a comprehensive resource relating miRNAs to human disease, *miR2Disease*, has recently been made available to the scientific community (38). A putative role for specific miRNA involvement in the control of neuronal cell number and in neurodegenerative disease (see Table 1 for summary) was first hinted at from experiments in model organisms, including in mice, in which Dicer was inactivated (39–43). For example, mice homozygous for conditionally floxed Dicer allele and expressing Cre recombinase under control of the dopamine transporter regulatory elements, resulting in conditional Dicer knockout in midbrain dopamine neurons, show specific and progressive dopamine neuron loss (41). Evidence for disruption of specific miRNAs in PD is provided by the finding of miR-133b deficiency in the midbrains

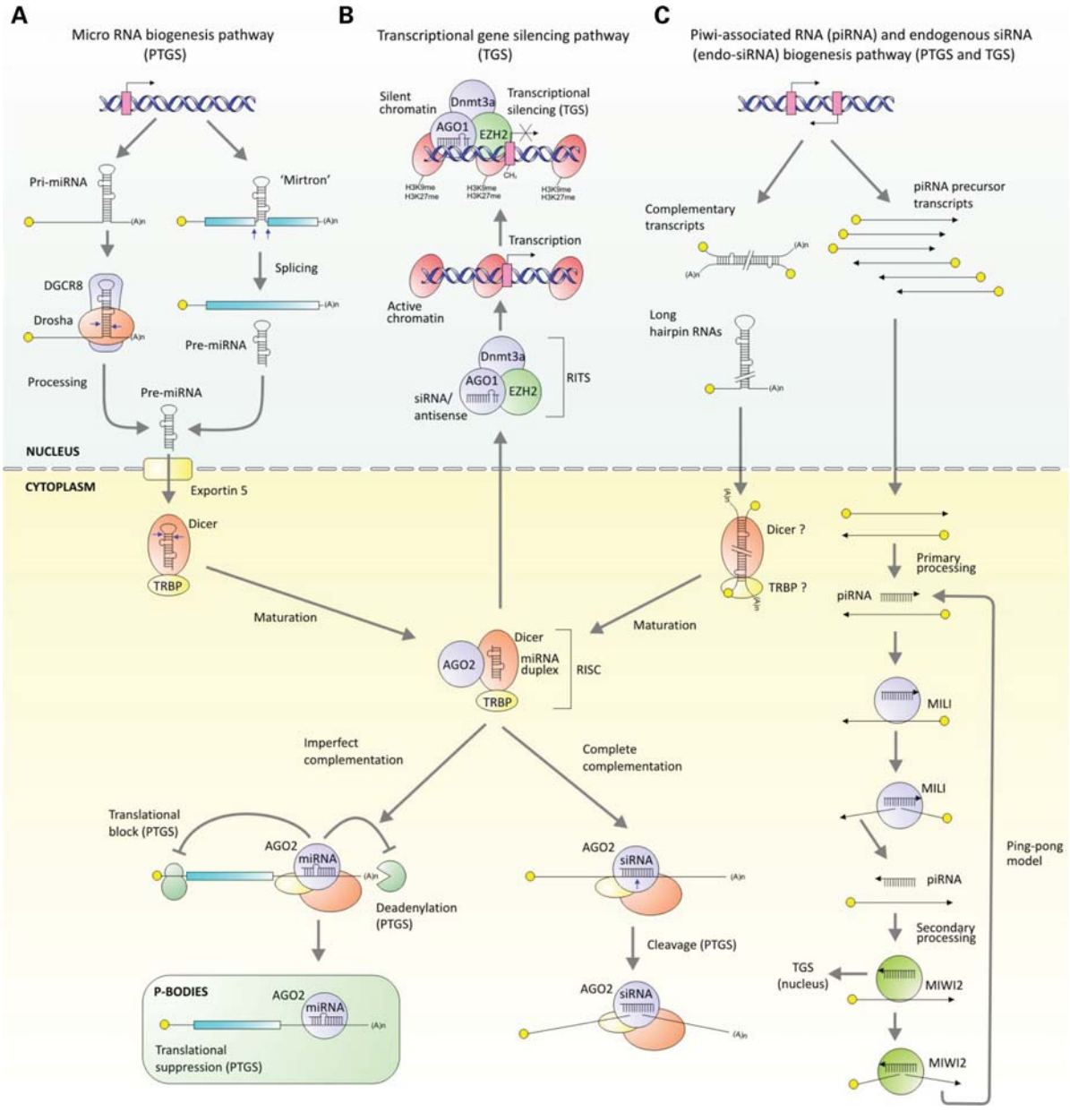

Figure 1. Mammalian RNAi regulatory pathways. (**A**) miRNAs are encoded in pri-miRNAs (124,125). These ~100 nt inverted repeat motifs are usually found embedded once or multiple times within coding or non-coding RNA Pol II-derived transcripts (126). Pri-miRNAs are first processed in the nucleus where their hairpin-like structures are recognized and cleaved by the RNase III enzyme Drosha together with DiGeorge critical region 8 protein (DGCR8), to produce shorter hairpin duplexes known as a 70–80 nt pre-miRNAs (127,128). For a small minority of miRNAs, short intronic sequences, referred to 'mirtrons', can be directly processed by the spliceosome into pre-miRNA-like hairpins without requiring Drosha cleavage (30,129). Spliced lariats are de-branched and likely produce functional pre-miRNAs for export. Pre-miRNAs are exported from the nucleus to the cytoplasm by the exportin-5 (130,131) followed by recognition and cleavage by a second RNase III enzyme, Dicer and its partner, TAR RNA-binding protein (TRBP), to produce a ~22 bp, staggered miRNA/miRNA* duplex with 2 nt 3′ overhangs (132–134). Dicer/TRBP, loads one of the strands, the 'guide strand', into a RISC consisting in its simplest form of Ago2 (135,136) directing cleavage of translational suppression of cognate RNA. (**B**) The mechanism of TGS is poorly understood in mammals but is thought to include a complex consisting (RITS) of Ago1, (and possibly Ago2) a polycomb group component, enhancer of zeste 2 (EZH2) and DNA methyltransferase 3a (Dnmt3a) (21,23,137). Moreover, TGS may require the presence of low-copy promoter-derived transcripts to direct silent heterochromatin marks (H3K9 and/or H3K27 methylation) and DNA methylation at the targeted locus (138). (**C**) Endogenous siRNAs are derived from long hairpin sequences and complementary transcripts which are processed by Dicer into siRNAs. piRNAs are 24–31 nt short RNAs processed from single-stranded precursors derived from transposons or genomic repeat elements in the germline (13). In the 'ping-pong model', primary piRNAs interact with the Piwi protein MILI to cleave a transcript that generates a piRNA for incorporation into MIWI2, which in turn cycles back to produce new MILI-interacting piRNAs (24,25).

of PD patients and in mouse models of dopamine neuron degeneration (41). Interestingly, Kim *et al*. uncover an important negative regulatory relationship in which the essential dopaminergic transcription factor Pitx3 regulates miR-133b transcription which in turn is found to suppress Pitx3 expression, providing an RNA regulatory network that

Table 1. Non-coding miRNAs and neurological disease

Disease	miRNAs implicated	Putative mechanism	Reference
Parkinson's disease	Multiple	Neurodegeneration in dicer knockout mouse	(41)
	miR-133b	Dopamine neurogenesis	(41)
	miR-433	Variation in miR-433 binding site in FGF20 gene leading to increased alpha synuclein levels	(44)
Alzheimer's disease	Multiple	Dysregulation multiple miRNAs in AD patient brains	(48)
	miR-659	Variation in miR-659 binding site in progranulin gene	(46)
	miR-29	Correlate with increased BACE1/β secretase expression	(49)
	miR-298/328	Direct regulation of BACE1 transcript	(50)
	miR-20a	Regulation of APP expression	(51)
	miR-107	Regulation of APP cleaving enzyme 1	(148)
Huntington's disease	Multiple	Interaction of htt protein with Ago2 and localization to P-bodies	(52)
	Multiple	Dysregulated miRNA expression	(55)
	miR-9/miR-9*	Interaction with/regulation of REST and CoREST	(56)
Spinocerebellar ataxias and cerebellar degeneration	Multiple	Purkinje cell ablation of Dicer leads to neurodegeneration	(43)
	miR-19, miR-101 and miR-130	miRNA regulation of ataxin 1 in SCA1	(57)
Other			
Prion-induced neurodegeneration	Multiple	Dysregulated miRNA expression	(58)
Schizophrenia	Multiple	SNPs within or near brain-expressed miRNAs	(59)
Fragile X syndrome	Multiple	Disordered miRNA biogenesis	(60)
Autism	miR-181b	Regulation of genes linked to schizophrenia	(61)
Tourette's syndrome	Multiple	FMRP product of FMR1 gene interacts with miRNAs which assemble into miRNPs containing FMRP which functions as a translational suppressor. Loss of FMRP function results in de-repression of multiple miRNA targets	(149)
	Multiple	Dysregulated miRNA expression, some targeting autism-linked genes Neurexin and SHANK3	(150)
	miR-189	3′-UTR of the SLITRK1 gene contains the binding site of miR-189, which is mutated in some patients	(47)

controls the dopamine neurogenic gene programme. Further evidence linking regulatory miRNAs and PD comes from work identifying variation in the miRNA binding site for miR-433 in the FGF20 gene which is preferentially expressed in the midbrain. Such variation appears to confer increased susceptibility to PD as a result of increased alpha synuclein gene expression, a gene regulated by FGF20 and which is strongly linked to PD through both over-expression and point mutation mechanisms (44,45). Similarly, the recent discovery of variation in the miR-659 binding-site in the 3′-UTR of the progranulin gene as a risk factor for TDP43-positive frontotemporal dementia (46) and an earlier finding that the neurological disorder Tourette's syndrome is associated with a variation in the miR-189 binding site in mRNAs encoding the neuronal proteins Slit and Trk-like 1 (47), together implicates common genetic variation within non-coding regions of disease-related transcripts and regulatory miRNA function as factors in the origin of complex neurodegenerative disease.

Further evidence for altered miRNA expression linked to neurodegenerative disease comes from studies related to AD. Here dysregulated miRNA expression has been documented in AD patient brains relating to the control of cellular pathways involving neurogenesis, amyloid processing, insulin resistance and innate immunity (48). Specific miRNAs have now also been linked mechanistically to AD pathogenesis; for example, loss or reduced expression of the miR-29 cluster in sporadic AD has been found to correlate closely with a subgroup of patients in whom increased BACE1/β-secretase expression was observed (49). BACE1/β secretase is a rate-limiting step for β-amyloid production and its increased expression has been reported among AD patients. Additionally, Boissonneault *et al*. (50) have identified miR-298 and miR-328 which directly interact with the 3′-UTR of the BACE1 transcript. Further, the miR-20a family (i.e. miR-20a, miR-17-5p and miR-106b) has been shown to regulate amyloid precursor protein (APP) expression suggesting a possible role during disease (49,51), a finding corroborated specifically for miR-106b where decreased levels were found by qRT-PCR in the brains of sporadic AD patients.

There is now also accumulating evidence of dysregulated miRNA expression or involvement of regulatory miRNAs in other neurodegenerative diseases, notably hereditary diseases of polyglutamine origin. Interestingly, the huntingtin (htt) protein, which harbours an expanded polyglutamine tract in Huntington's disease (HD), has been implicated directly in the RNAi silencing pathway by virtue of its biochemical interaction with Ago2, an essential PTGS component, and co-localization with Ago2 in P-bodies (52), cytoplasmic sites of RNA metabolism, RNAi and miRNA activity (53,54). In addition, altered expression of a number of neuron-specific miRNAs has been found in both murine and human HD brains, the authors suggesting that this may be due to increased repression by the essential repressor factor REST, also known as neuron-restrictive silencing factor, which itself regulates numerous miRNA transcripts (55). Interestingly, Packer *et al*. (56) have observed downregulation of several miRNAs in HD patient brains and one of these, brain-enriched miR-9/miR-9*, is implicated in the regulation of components of the REST transcription factor complex: REST and CoREST,

providing a possible autoregulatory link between miRNAs and neuron-specific gene transcription networks, which may be defective in HD. Similarly, in the polyglutamine expansion disorder spinocerebellar ataxia type 1 (SCA1) in which a translated CAG repeat expansion is found in the ataxin 1 gene, three miRNAs have now been found to directly co-regulate ataxin 1 expression and hence have potential roles in disease pathogenesis and as possible therapeutic targets (57). Disruption of regulatory miRNA networks has been observed in other neurological disorders including prion-induced neurodegeneration (58) and also in the neurodevelopmental disorder schizophrenia. In the schizophrenia SNPs within or near brain-expressed miRNAs (59), disordered miRNA biogenesis (60) and upregulation of a specific miRNAs shown to regulate genes linked to schizophrenia (61), have all directly implicated regulatory miRNAs mechanistically in the disease.

Thus regulatory non-coding miRNAs are increasingly being associated with the complex aetiologies of neurodegenerative and other major neurological diseases. Moreover, a range of mechanisms are now being uncovered to implicate some of these miRNAs directly in disease pathogenesis including, miRNA dysregulation leading to defects in specific cellular networks. This is seen in neurogenesis and innate immunity pathways that affect the expression of disease-linked transcripts, variations in miRNA binding domains in disease-associated genes and even defects in fundamental regulatory miRNA biogenesis pathways. While no single specific miRNA mutation has yet been associated with neurological disease, these findings suggest that as understanding of involvement of regulatory non-coding RNA networks in neurodegenerative disease accrues, non-coding RNAs will represent at the very least a new category of diagnostic markers for this group of diseases and probably an important regulatory component contributing directly to disease aetiology.

THERAPEUTIC RNAi SILENCING OF DISEASE GENES

Specifically targeted exogenous post-transcriptional degradation of mRNAs in the cytoplasm has been used as a technology to suppress gene expression by introducing dsRNA triggers at different levels of the RNAi pathway (Fig. 2). The most common form of silencing is typically achieved by administering chemically synthesized siRNAs. Most synthetic strategies have focused on producing siRNA mimics by standard phosphoramadite chemistry. While synthetic 19–21 bp siRNAs are loaded directly into RISC, a number of groups are using larger synthetic duplexes (>21 bp) or synthetic precursor-miRNAs (pre-miRNAs), which are first cleaved by Dicer/TRBP, enhancing strand selection upon RISC entry (62–64). For synthetic effector sequences, structures are not confined by canonical symmetrical 19–23 bp natural siRNA parameters. A number of different asymmetric RNA duplexes of between 15–21 bp have been shown to be powerful RNAi inhibitors (65,66). All synthetic siRNAs have the potential to be chemically modified such that they have desirable *in vivo* properties that are not found in their natural counterparts. The most important property derived by chemical modification is the prevention of degradation by serum nucleases. Additional benefits from chemical modifications may result in enhanced guide strand function, decreased immune activation and improved pharmacokinetic properties (reviewed in 67).

Gene-based approaches allow for the expression of hairpin duplexes that structurally mimic the miRNA precursors, pri-miRNAs and pre-miRNAs, to generate exogenous guide strands (Fig. 2). Expressed RNAi effectors have the advantage of being constantly renewed from an expression cassette, thereby providing a more sustained suppression of the target RNA. Moreover, encoded RNAi cassettes can be packaged and delivered by different viral vectors, thereby harnessing the cell-specific targeting and expression characteristics of viruses. The most commonly used expression cassette is a short hairpin RNA (shRNA)-encoding sequence that is inserted downstream of a RNA Pol III promoter. shRNAs are typically expressed from Pol III promoters such as H1 and U6, since these promoters produce defined 5′ and 3′ termini which mimic typical endogenous pre-miRNA characteristics. U1 Pol II promoters and tRNA Pol III promoters have the advantage of producing effective Dicer susbtrates that likely do not use exportin-5 for nuclear export (68–70). Most Pol III and some Pol II promoters cannot be tightly regulated and are therefore constitutively active, which may have undesirable effects (see later). More recent attention has been placed on using pri-miRNA mimics, which follow a more natural maturation pathway (71–73). Pri-miRNAs have the advantage of tissue-controlled expression of RNAi-precursors and can be organized in polycistronic clusters, allowing for the simultaneous inhibition of multiple targets (74,75).

RNAi-BASED THERAPEUTICS FOR NEURODEGENERATIVE DISEASE

While regulatory non-coding RNA networks are increasingly implicated in the complex pathogenesis of specific neurodegenerative diseases, the ability to exploit in parallel the PTGS pathway in a therapeutic context to specifically modify the expression of neurodegenerative disease-associated transcripts will offer novel approaches for developing disease modifying therapies for both familial and sporadic disease (10,11). The major challenges confronting approaches to develop RNA-based therapies are: (i) the relatively poor understanding of complex disease pathways and of appropriate target transcripts in the case of sporadic neurodegenerative disease and (ii) the optimal design and delivery of therapeutic RNAi trigger molecules to the nervous system. However, that such approaches are likely to be of therapeutic benefit for neurodegenerative disease, i.e. that such disease processes might be halted or reversed by targeted gene inhibition, is suggested from studies in conditional transgenic model systems harbouring mutant (expanded) htt transgene (76), a mutant SCA1[82Q] transgene (77) or an over-expressed alpha synuclein allele (78), where in each case switching off the disease-causing transgene resulted in concomitant improvement in disease phenotypes.

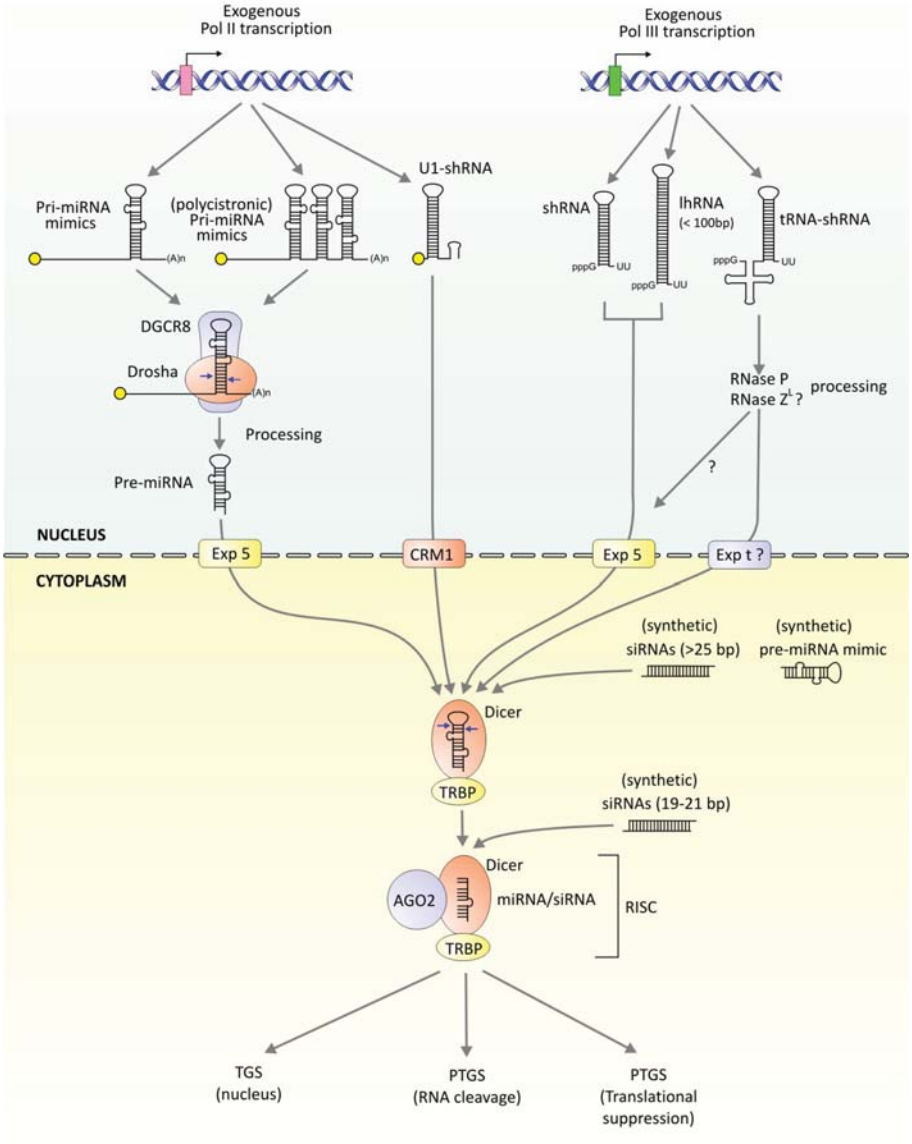

Figure 2. Exogenous RNAi-mediated gene silencing. RNA Pol II-derived transcripts introduce hairpin duplexes which structurally mimic mono or polycistronic pri-miRNAs which are recognized and processed by Drosha/DGCR8 into pre-miRNA-like hairpins. These hairpins are cleaved by Dicer/TRBP following export via exportin-5. The Pol II-generated U1 shRNA transcripts, which contain a 3′ terminal B-box, structurally mimic pre-miRNAs but are likely exported by the CRM1 pathway prior to Dicer/TRBP cleavage (139). RNA Pol III promoters express shRNAs and long hairpin RNAs (lhRNAs) with defined 5′ and 3′ termini. U6 or H1-derived shRNAs and lhRNAs, like pre-miRNAs, consist of 2 nt 3′ overhangs and exit the nucleus via exportin-5. lhRNAs, are processed sequentially by Dicer to produce up to three siRNAs (140,141). $tRNA^{Lys3}$ and $tRNA^{Val}$ Pol III promoters can also be used to produced tRNA–shRNAs for processing in the nucleus by 5′ and 3′ tRNA processing enzymes prior to export (69,142). If unprocessed by RNase Z^L, tRNA–shRNAs may exit the nucleus via exportin-t (143). Synthetic siRNAs or pre-miRNA mimics can be introduced as Dicer substrates (typically >25 bp duplexes) or as 19 bp duplexes for direct loading into Ago2-RISC.

Given the difficulty in defining appropriate therapeutic targets in sporadic neurodegenerative disease, it is unsurprising that most progress in the development of such RNA-based therapies has been made in hereditary neurodegenerative disease, most notably HD. Harper *et al*. (79) provided the first demonstration that targeting mutant human htt in the mouse brain using an adeno-associated virus serotype 1 (AAV1) vector delivered U6 promoter-driven shRNA effector system could yield improvements in HD-associated neuropathology and behaviour. A series of subsequent studies by this and other groups provide further evidence in support of this approach (80–85), however, certain important issues in the development of this therapeutic approach are highlighted. The first is the appropriateness of different animal models, and in particular the use of relatively rapid disease-onset animal models by some groups (81,82) seems simplistic and questionable in the context of complex, chronic neurodegenerative disease. A second important issue concerns the optimal delivery method for therapeutic RNAi effectors. Most investigators have utilized vector expression systems and Machida *et al*.

(84) recently reported improved delivery in an HD model using an AAV5 delivery system. In contrast, DiFiglia *et al*. (81) have shown encouraging data utilizing cholesterol-conjugated synthetic siRNAs. However, questions relating to efficiency of synthetic siRNA delivery to the CNS remain, and in addition whether or not such conjugated siRNAs are likely to prove toxic in human subjects or indeed, how such synthetic compounds might be repeatedly administered to patients over time are major unresolved issues. A further unresolved question relates to whether or not specific silencing of the mutant disease transcript will be required for therapeutic benefit in HD; current work involves indiscriminate targeting of both wild-type and mutant alleles. While several groups have developed successful allele-specific silencing of neurological disease transcripts (86–89) at present there remains no motif for the targeted discrimination of mutant from wild-type HD transcripts, although work to identify possible disease-linked and CAG expansion-linked SNPs is in progress (90). Finally, there is the crucial question of safety and the potential side- and off-target effects of such RNA-based therapies which have been identified in a number of studies (80,85) and are discussed in detail below.

The related spinocerebellar ataxias (SCAs), several of which also have polyglutamine expansions as the causative mutation, have been investigated by a number of groups. Xia *et al*. (91) first demonstrated proof of principle for silencing the human SCA1 disease transcript in cerebellum with concomitant phenotypic benefit. Interestingly, discriminatory SNPs have been identified for SCA3 and SCA7 (92,93), and in the case of the former, allele-specific silencing of mutant ataxin-3 has been demonstrated *in vivo*, albeit via the targeting of a virally expressed mutant transcript (92). Schwarz *et al*. (89) have attempted to understand and define the biochemical parameters for allele-specific siRNA silencing, while in the case of SCA7, Scholefield *et al*. (unpublished data) have similarly defined parameters for shRNA- and miRNA-based allele-specific silencing of a disease-associated SNP in the mutant ataxin-7 transcript, highlighting the importance of structural position 16 mismatches in construct design to achieve optimal allele-specific discrimination and phenotype correction in this particular case. Similar structural constraints at this or other single nucleotide mismatch positions within the effector guide sequence may well apply in other cases. A further strategic option as an alternative to allele-specific silencing is illustrated in the case of SCA6, where Kubodera *et al*. (94) have developed a gene knockdown and replacement strategy whereby both mutant and wild-type transcripts are suppressed and the essential wild-type protein is replaced by co-expression of an siRNA-resistant wild-type mRNA.

Limited progress has been made to date in the RNA-based silencing of targets linked to common sporadic forms and less common familial forms of PD and AD. Sapru *et al*. (95) have successfully targeted the alpha synuclein pathway in the context of PD. While in the case of AD, the amyloid (96,97) and BACE1/β secretase (98) pathways have both been targeted, the latter yielding a striking improvement in disease phenotype.

There are considerable challenges facing the clinical application of exogenous RNAi effector sequences. A number of toxicities are associated with siRNAs and exogenous RNAi precursors. High levels of expressed shRNAs in the liver are known to cause fatalities in mice due in part to the saturation of the endogenous RNAi machinery (99) and McBride and colleagues have observed toxicities arising from shRNA-based vectors in brain (71,85). Expressed shRNAs likely abrogate the function of natural miRNAs (73,99,100). Therefore, careful consideration should be given to the dosage used when applying ectopically introduced RNAi effectors. Pol II promoters, which can be regulated more easily by cell-specific environmental factors, have since become more popular for the expression of pri-miRNA mimics as effective guide strand 'shuttles'. At present there is no evidence to suggest that pri-miRNA shuttles, even if highly expressed, can saturate the endogenous RNAi pathway (71–73). Another area of concern is the possibility of off-target inhibition of unintended mRNAs through interactions between the 6–7 nt seed region of exogenous siRNA guide sequences and target mRNAs. Many off-target effects of this nature have been observed when introducing exogenous siRNAs (101–103). Off-target effects can in some instances be mitigated by introducing specific base modifications within the siRNA duplex (104). However, to determine the full extent of any off-target inhibition, prior screening using candidate RNA and protein expression array analyses may be required (105). Lastly, dsRNA duplexes can potentially activate the innate cellular immune system through the unwanted release of inflammatory cytokines and induction of an interferon response. dsRNAs activate cytoplasmic pattern recognition receptors such as dsRNA-dependent protein kinase receptor and membrane-associated toll-like receptors (reviewed in 106). Careful consideration should be given to the innate immune response, which can mask the effects of RNAi-mediated gene silencing. Recent evaluation of a high-profile study using siRNAs targeted to vascular endothelial growth factor (VEGF) showed that an off-target immune response was responsible for the observed suppression of VEGF (107). Interestingly, ectopic expression of RNAi effectors from vectors, however, is less likely to induce a non-specific immunostimulatory response (108).

FUTURE POSSIBILITIES FOR THE THERAPEUTIC REGULATION OF NEURODEGENERATIVE DISEASE-LINKED MIRNAS

An increased understanding of the role of regulatory non-coding RNAs, and especially miRNAs, in the pathogenesis of sporadic complex neurodegenerative disease will lead to therapeutic possibilities aimed at modulating the activity of these non-coding RNA networks in diseased cells (Fig. 3). miRNA biogenesis and target gene silencing are governed by general and miRNA-specific feedback circuits (reviewed in 13), which are regulated transcriptionally and post-transcriptionally. Ablation of normal miRNA regulation is often associated with disease, as is increasingly being seen for cancer, including nervous system tumours (109,110), and cardiovascular disease (111). While miRNAs are regulated at the transcriptional level, post-transcriptional miRNA regulation during biogenesis suggests a fine-tuning of spatio-temporal miRNA activity and provides the possibility of

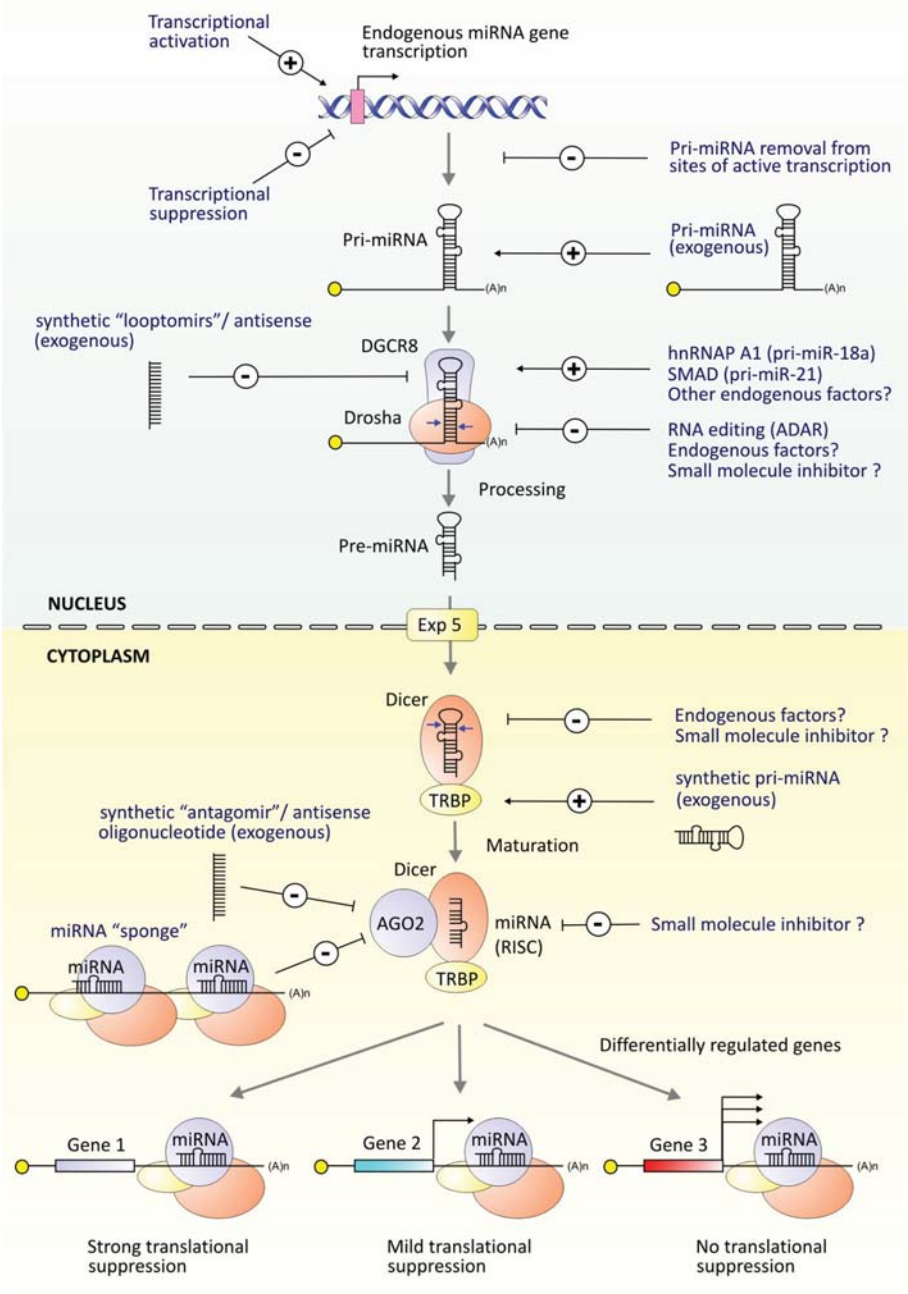

Figure 3. Regulation and modulation of miRNA biogenesis and function. A number of endogenous and exogenous factors can control miRNA biogenesis, and these have the potential to be exploited as novel therapies. Transcription factors act on promoters to activate or suppress endogenous miRNA gene expression. Post-transcriptional control can occur at the level or pri-miRNA processing in the nucleus, or pre-miRNA cleavage in the cytoplasm. In the nucleus, Drosha/DGCR8 preferentially processes pri-miRNAs that are retained at the site of transcription (144), regulating pri-miRNAs co-transcriptionally (145). To stimulate the activity of a specific miRNA, exogenous pri-miRNAs can be introduced as gene-based cassettes or as synthetic pre-miRNAs. Factors which positively regulate specific pri-miRNAs include the SMAD proteins and hnRNAP A1 (112,113), with other positive or negative regulatory factors likely to be discovered in the future. Pri-miRNAs are additionally subject to RNA editing (adenosine to inosine RNA editing), resulting in an unprocessed pri-miRNA or in miRNAs with altered seed regions (146,147). miRNAs can be blocked by 'looptomirs' or short ASOs targeted to the loop of pri-miRNAs (112,113). In the cytoplasm, miRNAs can be blocked by 'antagomirs' or ASOs (120,121). Moreover, expressed sequences that contain multiple miRNA-targets are referred to as 'sponges', blocking translational suppression of endogenous mRNA targets (122). Small molecule inhibitors may be found to block miRNA: mRNA interactions, pre-miRNA maturation or earlier steps in the nucleus. miRNA dysregulation has the potential to affect multiple genes, whose products in turn may positively or negatively feedback to regulate specific miRNA gene transcription or biogenesis.

exogenously controlling specific miRNAs linked to neurodegenerative disease. Currently, factors known to modulate miRNA biogenesis include the RNA-binding protein hnRNAP A1, which facilitates processing of pri-miR-18a by binding to conserved loop sequences (112); and SMAD proteins, which associate with Drosha to process pri-miR-21 in

smooth vascular muscle cells (113). Many more factors are likely to be discovered in future which control specific miRNAs. Interestingly, in human gliomas where miR-21 levels have been reported to be elevated, the suppression of miR-21 in neural precursor cells was shown, together with tumour necrosis factor-related apoptosis inducing ligand (S-TRAIL), to sensitize gliomas for increased apoptotic activity (114). Post-transcriptional control was also shown for precursors of miR-138, which is a miRNA restricted to neuronal cells. The precursor, pre-miR-138-2, is ubiquitously expressed throughout all tissues, indicating cell- or tissue-selective Dicer cleavage of pre-miRNAs in neurons (115). Lee *et al*. (116) showed in a recent study using normal tissues, tumours and cell lines, that a large number of pri-miRNAs are transcribed but are not processed to the mature miRNA, especially in cancer cells, implying that post-transcriptional dysregulation of miRNAs may be at the root of many disease aetiologies.

As miRNA dysfunction in neurodegenerative disease is understood in greater detail, such therapeutic methods to modulate and correct the activity of aberrant nervous system miRNAs will be developed and evaluated. However, validating or blocking direct miRNA–mRNA interactions remains a challenge and tools aimed at inhibiting or augmenting the function of a specific miRNA are useful additions to research and therapeutics (Fig. 3). Mature miRNAs can be inactivated by administering short complementary synthetic antisense oligonucleotides (ASOs) (117). ASOs are usually chemically modified at the 2' sugar moiety or have base changes such as locked nucleic acids, peptide nucleic acid or morpholino bases to improve specificity and serum half-life *in vivo* (114,118,119). Antagomirs are usually cholesterol-conjugated ASOs that are capable of improved degradation of specific miRNAs when administered *in vivo* (120). Recently, ASOs have been shown to block miRNA precursors (112) and miRNAs without degrading the target RNA (121). While a complete mechanism behind ASO-mediated inhibition of miRNA activity remains to be determined, anti-miRNA ASOs are likely to feature as important future therapeutic agents. Expressed sequences consisting of multiple miRNA seed targets, referred to as miRNA sponges, are also effective miRNA inhibitors and likely function by competing with endogenous miRNA targets (122). Perhaps the future lies in finding small-molecule inhibitors of specific miRNAs. A recent chemical screen of 1000 small molecules, identified a potent inhibitor of miR-21, thus paving the way for screening platforms to identify novel inhibitors of other miRNAs (123).

CONCLUSION AND FUTURE DIRECTIONS

Of crucial future importance will be efforts to further understand basic cellular disease mechanisms and the role of non-coding RNA networks in the complex phenotypes of sporadic neurodegenerative disease. Such knowledge will lead increasingly to opportunities to modulate non-coding RNA activity and function in neurodegenerative disease models. It will also to lead to the discovery of novel disease targets for RNA-based silencing methods. Therapies for complex multigenic diseases will likely require modulation of multiple targets or cellular disease pathways. The advent and development of experimental RNA-based therapeutics could lead to opportunities for tackling early causative factors in neurodegenerative disease pathogenesis and the development of a new generation of disease modifying therapies.

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