The Role of MicroRNAs in Spinocerebellar Ataxia Type 3

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Abstract

More than 90% of the human genome are transcribed as non-coding RNAs. While it is still under debate if all these non-coding transcripts are functional, there is emerging evidence that RNA has several important functions in addition to coding for proteins. For example, microRNAs (miRNAs) are important regulatory RNAs that control gene expression in various biological processes and human diseases. In spinocerebellar ataxia type 3 (SCA3), a devastating neurodegenerative disease, miRNAs are involved in the disease process at different levels, including the deregulation of components of the general miRNA biogenesis machinery, as well as in the cell type-specific control of the expression of the SCA3 disease protein and other SCA3 disease-relevant proteins. However, it remains difficult to predict whether these changes are a cause or a consequence of the neurodegenerative process in SCA3. Further studies using standardized procedures for the analysis of miRNA expression and larger sample numbers are required to enhance our understanding of the miRNA-mediated processes involved in SCA3 disease and may enable the development of miRNA-based therapeutics. In this review, we summarize the findings of independent studies highlighting both the disease-related and cytoprotective roles of miRNAs that have been implicated so far in the disease process of SCA3.

Introduction

The central dogma of biology describes the flow of genetic information from the DNA, which is transcribed into RNA, and from the RNA, it is then translated into protein. The number of protein-coding genes in human cells has long been a source of debate. Originally, researchers estimated that the number of human protein-coding genes would fall somewhere between 40,000 and 100,000 [1], and that the majority of RNAs in our cells are protein-coding messenger RNAs (mRNAs). However, sequencing of the human genome revised the number of protein-coding genes to an estimated number below 20,000 [2]. These genes comprise only about 1%–2% of the 3 billion base pairs of DNA, meaning that the remaining 98%–99% of our entire genome must be doing something else than coding for proteins. Remarkably, many more RNAs are being transcribed than proteins being translated. These RNAs are referred to as non-coding RNAs, and they make up a vast majority of cellular RNAs, accounting for more than 90% of all transcribed human RNAs [3]. While it is still uncertain if all these non-coding RNA transcripts are functional, it has been becoming more and more clear over the years that RNA has several additional functions other than only coding for proteins. Examples of functional non-coding RNAs include, but are not limited to transfer RNA ribosomal RNA, long non-coding RNA that are more than 200 nucleotides in length and microRNA (miRNA). Their molecular functions are various, including DNA synthesis, genome stability, and epigenetic modification [4,5]. Most non-coding RNAs assemble RNA–protein complexes or base pair with other nucleic acids to execute their function. Of note, the performance of a functional RNA depends on its three-dimensional structure of RNA, which plays an important role in the recognition of its interacting proteins and nucleic acids. Several RNA

species play important roles not only in healthy tissue but also in disease development. Thus RNA-mediated toxic mechanisms have been suggested to play a role in pathogenesis [6–10] (Neueder, Tabach, Weydt and Schilling et al. from the upcoming Special Issue). In this review, we will specifically focus on the role of miRNAs in spinocerebellar ataxia type 3 (SCA3).

miRNA

miRNAs are endogenously expressed short non-coding RNAs that play an important role in the regulation of gene expression [11–13]. They contain a seed region between positions 2 and 8 from the 5′-end through which they bind to partially complementary sequences in their target mRNAs of protein-coding genes. miRNAs are transcribed from miRNA encoding genes as full-length transcripts that undergo serial cleavage steps to convert into small double stranded RNAs of 18–23 nucleotides. The full-length transcript, also called primary RNA (*pri-miRNA)*, is transcribed by RNA polymerase II and first cleaved by an enzyme complex containing the proteins Drosha and DGCR8 (DiGeorge syndrome critical region in gene 8) into a fragment of approximately 60–70 bp referred to as precursor miRNA (*pre-miRNA*) [14–16]. The pre-miRNA then translocates to the cytoplasm, passing the nuclear pore with the help of exportin 5 [17,18]. In the cytoplasm, a second cleavage step, carried out by the enzyme Dicer in combination with TRBP (trans-activation response RNA-binding protein), produces an 18- to 23-bp RNA duplex of the mature miRNA and its antisense strand (miRNA*), which is released and degraded [19–23]. The mature miRNA then associates with the RNA-induced silencing complex (RISC) to regulate target gene expression. In detail, the miRNA guides the RISC to its complementary target mRNA. Once the miRNA–RISC complex binds its target mRNA, it can either induce degradation of the target mRNA or suppress its translation [24–28]. Around 70% of the miRNAs annotated to date have been detected in the brain, where they play a role in diverse processes including synapse formation, dendritic spine formation, and stress response [29–31]. Interestingly, altered miRNA expression is linked to development of neurological diseases [32–36]. While numerous disease conditions, in which miRNAs are implicated, have been described, here, we will summarize the current knowledge about cellular miRNAs and components of the miRNA biogenesis machinery that have been implicated so far in the disease pathogenesis of SCA3 (Table 1).

SCA3

SCA3 (OMIM No. 109150), also referred to as Machado–Joseph disease, is the most common spinocerebellar ataxia. It is a neurodegenerative disorder that belongs to the family of polyglutamine diseases. The age of onset typically ranges between 20 and 50 years. SCA3 is characterized by clinical variability, with common features including cerebellar ataxia, external ophthalmoplegia, dysarthria, dysphagia, pyramidal signs, dystonia, rigidity, and distal muscle atrophy (reviewed in Ref. [47]). SCA3 is inherited in an autosomal dominant manner with the disease-causing mutation in the coding region of the *Ataxin 3* (*ATXN3*) gene. Specifically, the underlying mutation is an expansion of a CAG trinucleotide motif in exon 10 of the *ATXN3* gene, which is translated into a polyglutamine stretch in the ATXN3 protein [48]. This repeat is expanded to more than 60 repeats in patients, while healthy individuals have 12–44 repeats [49,50]. ATXN3 is a deubiquitinating enzyme [51,52] and has a diversity of interacting proteins [47,53], suggesting that it is involved in several cellular pathways [54,55]. Expansion of the polyglutamine tract renders the mutant ATXN3 protein highly prone to aggregation [56]. Neuronal nuclear and cytosolic inclusions formed by mutant ATXN3 are the pathological hallmark of SCA3. The exact cellular mechanisms leading to neurodegeneration remain unclear, although several hypotheses have been raised to explain the toxic mechanism triggered by mutant ATXN3. These include, for example, transcriptional deregulation, which might be explained by sequestration of transcription factors to the mutant ATXN3 aggregates, or failure of cellular homeostasis, which can be explained for instance by sequestration of cellular chaperones, ubiquitin, or proteasomal compartments (reviewed in Ref. [47]). Several potential therapeutic strategies targeting various known disease-associated processes in SCA3 have been undertaken (reviewed in Ref. [57]). However, currently there is no cure for SCA3 and treatments are of palliative nature only. One possibility for a preventive therapy includes silencing of the *ATXN3* transcript.

RNA interference strategies successfully reduce the pathogenic expression of polyglutamine proteins and neurodegeneration in various polyQ models [58]. In SCA3 cell models, expression and accumulation of mutant ATXN3 are efficiently suppressed using allele-specific, short hairpin RNAs [59]. In a lentiviral rat model of SCA3, non-allele-specific silencing of ATXN3 [60,61] improves behavioral deficits and neuropathology, indicating that silencing of wild-type ATXN3 along with mutant ATXN3 is not deleterious. Furthermore, peptide nucleic acid and locked nucleic acid antisense oligomers targeting CAG repeats were used to suppress expression of mutant ATXN3 in cultured fibroblasts of SCA3 patients [62,63]. Other gene silencing strategies of ATXN3 expression include antisense oligonucleotides targeting

Table 1. Involvement of miRNAs, components of miRNA biogenesis, and 3′UTR of ATXN3 in SCA3 models and SCA3 patients

miRNAs	Species	Cell/tissue	Effects	Ref.
Bantam miRNA	*Drosophila*	Flye eye	Upregulation of bantam represses of mutant ATXN3-mediated toxicity	[37]
miR-34	*Drosophila*	Flye eye	miR-34 mimics extend survival and reduces neurodegeneration	[38]
miR-33, miR-92a	*Drosophila*	Neuron	Trend of overexpression in early disease stage	[39]
miR-181a (a total of 10 deregulated miRNAs)	Mouse	Cerebellum	Upregulated in 2- and 6-month-old transgenic SCA3 mice	[40]
miR-9, miR-181a, miR-494	Mouse	Cerebellum	Downregulated in lentiviral mouse model of SCA3	[41]
miRNA pool	Human	HeLa	Transfection of miRNA pool reverses polyQ-mediated cell loss	[37]
miR-181a	Human	HEK293T	miR-181a mimic targets 3′UTR of ATXN3	[42]
miR-25	Human	HEK293T	miR-25 mimics decrease ATXN3 expression and enhance cell viability	[43]
miR-25	Human	SH-SY5Y	miR-25 mimics decrease endogenous ATXN3 expression	[43]
miR-494	Human	SH-SY5Y	Downregulated in neuroblastoma cells expressing mutant ATXN3	[41]
miR-32, miR-181c	Human	HeLa	miR-32 and miR-181c mimics/precursors reduce ATXN3 expression	[44]
miR-370, miR-543	Human	HeLa	miR-370 and miR-543 mimics/inhibitors target DNAJB1 expression	[45]
miR-181a, miR-494	Human	iPS neuron	Downregulated in iPS neurons derived from SCA3 patients fibroblasts	[41]
miR-370, miR-543 (a total of 90 deregulated miRNAs)	Human	iPS neuron	Upregulated in differentiated iPS neurons derived from SCA3 patients	[45]
miR-32, miR-181c (a total of 30 deregulated miRNAs)	Human	Lymphocyte	Upregulated in lymphocytes from SCA3 patients with low ATXN3 levels	[44]
miR-25, miR-125b (a total of 46 deregulated miRNAs)	Human	Serum	Downregulated in SCA3 patient serums correlating to disease course	[46]
miR-9, miR-181a, miR-494	Human	Cerebellum	Trend of decreased expression in brain samples of SCA3 patients	[41]
Components of miRNA biogenesis and 3′UTR of ATXN3				
Mutants of Dicer and R3D1	*Drosophila*	Flye eye	Reduced processing enhances mutant ATXN3-induced degeneration	[37]
ATXN3 3′UTR added downstream of coding sequence	Mouse	Striatum	Reduces ATXN3 aggregation and neuropathology	[41]
Dicer, DGCR8, FMR1	Mouse	Cerebellum	Transcripts downregulated in lentiviral mouse model of SCA3	[41]
Reduction of Dicer	Human	HeLa	Reduced processing enhances mutant ATXN3-mediated cell loss	[37]
Silencing and inhibition of Dicer and Drosha	Human	HEK293T	Inhibited processing activity increases endogenous ATXN3 expression	[41]
ATXN3 3′UTR added downstream of coding sequence	Human	HEK293T	Reduces expression of ATXN3 and ATXN3 aggregation	[41]

specific splicing signals resulting in exclusion of the CAG repeat containing exon 10 in *ATXN3* mRNA [57]. In addition, artificial miRNAs expressing RNA interfering sequences targeting the 3′UTR of ATXN3 have been developed to reduce mutant ATXN3 suppression of ATXN3 expression, providing a therapeutic strategy even when disease symptoms are already present. In symptomatic SCA3 mice inducibly expressing mutant ATXN3, the progressive neurological phenotype is reverted by turning off the transgene expression [64]. Thus, induction of miRNAs suppressing expression of ATXN3 may represent a promising therapeutic approach. Since ATXN3 is a non-essential protein (knock-out of *ATXN3* does not lead to an obvious phenotype in *Caenorhabditis elegans* and mice [65,66]), reducing the endogenous expression of both mutant and normal allele will most likely have tolerable side effects.

miRNAs in SCA3

The miRNA machinery affects cell viability in SCA3 models

First evidence that miRNA processing and distinct miRNAs play important roles in the pathogenesis of SCA3 came from studies using transgenic SCA3 flies [37]. In *Drosophila*, expression of mutant ATXN3 isoforms leads to partial loss of pigmentation and progressive neurodegeneration [67]. Reducing

the activity of Dicer-1 strongly enhanced neurodegeneration and loss of eye pigmentation in the SCA3 flies [37]. Correspondingly in human cells, the loss of Dicer activity results in a strongly enhanced cell death compared to an only modest reduction in cell viability of HeLa cells expressing polyglutamine-expanded ATXN3. Interestingly, the cell loss in human cells could be partially reversed by transfecting cells with a small RNA fraction containing total miRNAs from HeLa cells, indicating that the enhanced cell death following Dicer reduction can be attributed to the absence of one or more distinct miRNAs [37]. These findings suggest that miRNAs and miRNA processing pathways can modulate the polyglutamine toxicity in both, *Drosophila* and human cells.

Genetic modifier screening for factors modulating polyglutamine toxicity identified the *Drosophila* miRNA bantam as a potent repressor of polyglutamine-induced toxicity in SCA3 flies [37]. Upregulation of bantam suppresses the loss of eye pigmentation and neurodegeneration induced by polyglutamine-expanded ATXN3. Although bantam has been shown to stimulate cell proliferation and suppress apoptosis [68], the study by Bilen *et al*. [37] demonstrated that apoptosis is not involved in the degenerative process induced by polyglutamine-expanded ATXN3. However, the miRNA bantam does not reduce the expression levels or accumulation of polyglutamine-expanded ATXN3, suggesting that bantam functions downstream of polyglutamine-induced toxicity to prevent degeneration. The mechanism of suppression thus remains unclear and so far no analogous miRNA sequences of bantam in human or mouse have been identified. However, the study clearly shows that endogenous miRNAs can improve survival in models of polyglutamine diseases.

In contrast to bantam, upregulation of miR-34 in *Drosophila* expressing polyglutamine-expanded ATXN3 not only extends survival and diminishes neurodegeneration of the flies but also reduces formation of ATXN3-containing inclusions [38]. MiR-34 is a brain-enriched miRNA that regulates age-associated processes and brain integrity in *Drosophila* [69]. Among the age-associated targets of miR-34 is E74A, an essential ETS domain transcription factor involved in steroid hormone pathways [38]. Mutants lacking the conserved binding sites for miR-34 in the 3′UTR of E74A show increased E74A expression, late-onset brain degeneration, and shortened lifespan. However, reducing E74A expression in an SCA3 fly model did not improve neurodegeneration, indicating that other targets apart from E74A are associated with the neuroprotective effects of miR-34 in SCA3 pathogenesis. Members of the miR-34 family regulate numerous targets and influence various cellular processes, such as proliferation, apoptosis, and differentiation [70]. Interestingly, mammalian mir-34 orthologues are highly expressed in the adult brain [71] and have also been shown to be differentially expressed in degenerative disease in humans [72–75]. Interestingly, several preclinical studies using miR-34 mimics have already demonstrated their potential as anticancer therapeutics and are currently being tested in a phase I clinical trial [76].

miRNA expression profiling in SCA3 models

Several *Drosophila* models of neurodegenerative diseases, in which inducible expression of the corresponding disease proteins is targeted to neurons, have been used to profile global miRNA expression by RNA sequencing [39]. Transgenic flies expressing polyglutamine-expanded ATXN3 in adult neurons show a significant reduction of lifespan and a progressive decline of climbing performance starting 23 days after induction of mutant ATXN3 expression. To uncover early dynamic transcriptome changes associated with the initiation of the disease process, flies were induced when 7 days old and RNA was extracted from fly heads 3 days later. Surprisingly, miRNA profiling revealed no significant differences in miRNA expression, suggesting that mutant ATXN3-induced toxicity is not correlated with deregulated expression of miRNAs in early stages of the disease. However, a trend toward overexpression of miR-33 and miR-92a in early stages of the disease was observed in all fly ataxia models (SCA1, SCA3, SCA7) [39]. So far, additional miRNA profiling experiments at different time points during the course of the disease are needed to clarify whether deregulated expression of miR-33 and miR-92a and possibly other specific miRNAs represent a common mechanism involved in pathogenic processes in SCA diseases.

miRNA profiling of transgenic SCA3 mice expressing the entire human SCA3 gene locus including the 3′UTR [77] revealed significantly different expression levels of miRNAs in the cerebellum of SCA3 mice at 2 months of age [40]. Six miRNAs (miR-101a, miR-146b, miR-324-3p, miR-361, miR-365, miR-674) were downregulated and four miRNAs (miR-15b, miR-181a, miR-342-5p, miR-467a) were upregulated in the cerebellum of the SCA3 mice. Interestingly, only the increased expression of miR-181a persisted in the cerebellum of 2- and 6-month-old SCA3 mice suggesting adaptive or reversible changes in the transcriptome that occur in response to the expression of mutant ATXN3 [40].

In the same study, artificial miRNA mimics containing short interfering RNA sequences embedded in a genuine miRNA backbone and targeting the 3′UTR of human ATXN3 were tested for their efficacy to reduce ATXN3 expression. SCA3 mice transduced with recombinant adenoviruses expressing anti-ATXN3 miRNA mimics suppressed expression of mutant ATXN3 throughout the cerebellum of the SCA3 mice. Importantly, SCA3 mice

expressing the anti-ATXN3 miRNA mimic reduced the expression of the most upregulated miR-181a and increased expression of the most downregulated miR-674 in SCA3 mouse cerebella, demonstrating that suppression of ATXN3 expression using an anti-ATXN3 miRNA can partially normalize the deregulated miRNA expression [40]. Since several disease proteins of neurodegenerative diseases have been shown to affect miRNA biogenesis and miRNA expression (reviewed in Ref. [69]), it is also possible that mutant ATXN3 itself contributes to transcriptional deregulation of selected miRNAs.

miRNAs targeting ATXN3

The above-mentioned upregulation of miR-181a in SCA3 mice is particularly interesting since the 3′UTR of ATXN3 contains at least four conserved sites for members of the miR-181 family (miR-181a, miR-181b, miR-181c, miR-181d) (Fig. 1a) all containing the same seed sequence and targeting the same conserved binding sites. Co-transfection of a miR-181a expressing vector together with luciferase reporter constructs containing an intact or mutated seed sequence of one of the predicted miR-181 binding sites in HEK cells repressed the luciferase activity of the construct expressing the intact binding site but not of the mutated construct indicating a specific interaction of miR-181a with the 3′UTR of ATXN3 [42]. Moreover, the 3′UTR of ATXN3 contains one highly conserved binding site for members of the miR-25 family (miR-25/32/92/92ab/363/367) (Fig. 1a). This conserved binding site is targeted by miR-25 as shown by co-transfection of miR-25 mimics and luciferase reporter constructs containing 1531 bp of the 3′UTR of ATXN3 with an intact and mutated miR-25 binding site in different cell types [43]. Furthermore, a recent miRNA profiling study using serums of SCA3 patients revealed a significant differential expression of 46 miRNAs by at least 2-fold in SCA3 patients. Among those, downregulation of miR-25 and

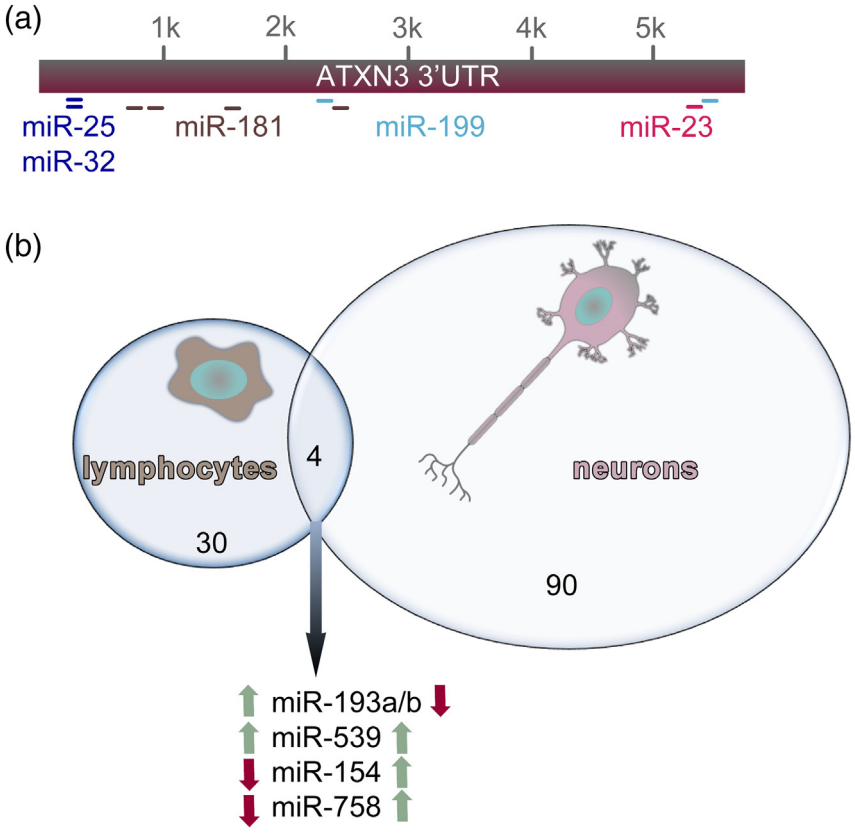

Fig. 1. miRNAs targeting the ATXN3 3′UTR are deregulated in SCA3 cells. (a) Schematic showing the 3′UTR of ATXN3 with binding sites of ATXN3-targeting miRNAs. (b) Expression of several miRNAs is changed in SCA3 cells compared to healthy control cells. Comparison of differences in miRNA expression pattern between lymphocytes and iPSC-derived neurons from SCA3 patients revealed that only four miRNAs are deregulated in both cell types, although only one of these is upregulated in both cell types.

miR-125b in serum of SCA3 patients was found to correlate with the SCA3 disease course and was suggested as potential biomarkers for disease monitoring [46].

These findings are well in agreement with a recent study showing that miR-32 and miR-181c, both members of the miR-25 and miR-181 family, effectively target the 3′UTR of ATXN3 and suppress the expression of ATXN3 in HeLa cells [44]. Luciferase reporter constructs containing 2000 bp of the 3′UTR of ATXN3 and constructs carrying mutated binding sites of miR-25 and miR-181 confirmed that the conserved miRNA binding sites in the 3′UTR of ATXN3 are indeed targeted by the corresponding endogenous miRNAs, as well as by miRNA mimics and precursors of miR-32 and miR-181c [44]. Interestingly, all four members of the miR-181 (miR-181a, miR-181b, miR-181c, and miR-181d) and two members of the miR-25 (miR-25 and miR-32) family were also found to be significantly upregulated by miRNA profiling of lymphoblastoid cell (LC) lines established from lymphocytes of SCA3 patients [44]. This simultaneous upregulation of ATXN3-targeting miRNAs correlates with significantly decreased ATXN3 expression levels in SCA3-LCs, suggesting that members of the miR-25 and miR-181 family as a group collectively contribute to the lowered amounts of ATXN3 mRNA and protein found in SCA3-LCs [44]. In fibroblasts from SCA3 patients, at least a trend toward decreased total expression levels of ATXN3 can also be observed [78,79], suggesting that specific cell types not affected in SCA3 such as lymphocytes and fibroblasts employ compensatory mechanisms including upregulation of miRNAs targeting the 3′UTR of ATXN3 to downregulate the expression of ATXN3. In this regard, even subtle changes lowering ATXN3 expression may be associated with beneficial effects in terms of cell survival and protection.

Compelling evidence that the expression of ATXN3 is controlled by its 3′UTR and endogenous miRNAs has been provided by a recent study of Carmona *et al.* [41]. The insertion of the ATXN3 3′UTR to the coding sequence of mutant ATXN3 leads to a strong reduction of expression and a marked decrease in the number of ATXN3-containing aggregates in transfected HEK cells. Also, HEK cells transfected with a luciferase reporter construct containing 3434 bp of the ATXN3 3′UTR show a significant reduction of the luciferase activity, demonstrating that the expression of ATXN3 is negatively regulated by its 3′UTR. Accordingly, the regulatory effects of ATXN3 3′UTR can be inhibited by genetic silencing and pharmacological inhibition of two key enzymes in miRNA biogenesis, Dicer and Drosha, in HEK cells resulting in a significant increase of the ATXN3 protein levels. Also, in a lentiviral mouse model of SCA3 expressing mutant ATXN3 [60], the addition of the 3′UTR of ATXN3 decreased formation of ATXN3-containing inclusions, neurodegeneration, and neuroinflammation demonstrating that the 3′UTR of ATXN3 and endogenous miRNAs control the expression levels of ATXN3 itself *in vivo* [41]. Together, these findings show that endogenous miRNAs targeting the 3′UTR of ATXN3 regulate the expression of ATXN3. The importance of the 3′UTR in ATXN3 is further highlighted by the finding that early disease onset in SCA3 is associated with two novel SNPs near the conserved miR-25 binding site in the 3′UTR of ATXN3 [80].

By comparing miRNAs predicted to target the 3′UTR of ATXN3 with miRNAs known to be expressed in the human brain, three miRNAs (mir-9, mir-181a, and mir-494) were selected, which efficiently reduced expression of mutant ATXN3 protein and formation of ATXN3-containing aggregates [41]. Surprisingly, the expression levels of miR-9, miR-181a, and miR-494 were found to be generally decreased in both human SCA3 brain samples and models of SCA3. This is in contrast to the increased expression levels of miR-181a found in SCA3-LCs [44] and SCA3 mice [40]. On one hand, this discrepancy may originate from differences in miRNA profiles between cell lineages and tissues, since miRNAs have well-defined developmental and cell type-specific expression patterns [81,82]. On the other hand, mutant ATXN3, depending on the cell type and tissue, may aberrantly contribute to expression changes of specific miRNAs as part of a yet unknown regulatory mechanism controlling the expression or biogenesis of miRNAs [69]. ATXN3-interacting proteins, such as the major transcriptional coactivator CREB binding protein (CBP), are sequestered into ATXN3-containing nuclear inclusions [56,83–85], probably resulting in altered transcription of miRNA genes as shown for Huntington's disease [86]. Interestingly, downregulation of CREB signaling is one of the most affected pathways found in brain tissue of SCA3 mice [87], suggesting that loss of CBP function underlies at least in part the transcriptional dysregulation in SCA3 brain. In line with this, Carmona *et al.* [41] found an altered regulation of genes involved in miRNA biogenesis in the lentiviral mouse model of SCA3, suggesting that crucial steps in miRNA biogenesis may also be affected in SCA3.

miRNAs targeting molecular chaperones in SCA3

A common pathogenic feature in polyglutamine diseases is protein misfolding and aggregation of the polyglutamine proteins. Molecular chaperones such as the co-chaperone DnaJ homology subfamily B member 1 (DNAJB1) are important regulators of protein folding and proteasomal removal of misfolded proteins. In SCA3, DNAJB1 localizes to mutant ATXN3 aggregates [88,89], while over-expression of DNAJB1 reduces polyQ protein toxicity [90]. Recently, a significant upregulation of two miRNAs, miR-370 and miR-543, which efficiently target and reduce DNAJB1 expression, was identified in neurons derived from induced pluripotent

stem (iPS) cells of SCA3 patients by RNA sequencing [45]. The upregulation of miR-370 and miR-543 coincides with decreased DNAJB1 expression, suggesting that both miRNAs act cooperatively in downregulating DNAJB1 expression. In agreement, reduced DNAJB1 expression levels were also found in two different SCA3 mouse models expressing either a cDNA of expanded ATXN3 under the control of the mouse prion promoter or an expanded ATXN3 from the endogenous human ATXN3 gene, respectively [45,91], suggesting that reduced expression of DNAJB1 may represent a common pathomechanistic step in the SCA3 disease process across species. However, whether a similar miRNA-mediated mechanism is involved in the reduction of DNAJB1 in transgenic SCA3 mice remains to be elucidated. Interestingly, downregulated expression of DNAJB1 and upregulation of its targeting miRNAs occurs already at early stages of the disease process, since they were observed under standard culture conditions where differentiated SCA3 neurons do not form ATXN3 aggregates [92]. Thus, the observed mRNA/miRNA changes in iPSC-derived neurons of SCA3 patients are most likely not the result of a compromised misfolded protein clearance due to depletion of essential chaperones but rather point to early disease-related changes in gene expression and gene regulatory networks. The aberrant expression of miRNAs targeting DNAJB1 thus probably represents an important novel aspect of the early disease-associated process in SCA3.

miRNA Expression and Regulation

miRNAs play pivotal regulatory roles in almost all biological processes and their misexpression has been linked to various diseases. Thus, miRNA expression and biosynthesis have to be tightly controlled. However, miRNA expression is regulated at multiple levels and our current understanding of the underlying regulatory mechanisms is still limited. In contrast to intragenic miRNAs encoded and expressed by the host gene, intergenic miRNAs have their own promoters and are expressed independently. In both cases, regulation at the transcriptional as well as at the post-transcriptional level, contributes to the fine-tuned, tissue- and cell type-specific regulation of miRNA expression. Transcriptional regulation includes changes in the promoter methylation, whereas post-transcriptional mechanisms affect miRNA processing and stability. Furthermore, signaling molecules such as hormones or cytokines affect the expression of miRNAs [93]. In SCA17 for instance, aggregation of the polyglutamine-expanded TATA box binding protein (TBP) results in interferon-gamma release, which in turn leads to downregulation of miR-29a/b in a SCA17 cell model [94,95]. Interestingly, dysregulation of miR-29a/b has also been observed in Alzheimer's [96] and Huntington's disease [97], suggesting that neuroinflammatory signaling pathways by cytokines such as interferons could be key players in mediating transcriptional dysregulation of miRNA and neuronal cell death.

Based on these observations of different triggers affecting miRNA expression, it becomes apparent that diverse endogenous and exogenous stimuli used in different experimental settings may induce undesired changes in miRNA expression. For example, treatments used to induce epigenetic changes such as altered methylation of CpG islands or inhibition of histone deacetylases will affect miRNA expression. Similarly, since both intra- and intergenic miRNAs can be regulated by numerous transcription factors, treatments altering transcription factor activity will change miRNA expression [98]. Another important fact is that virally transduced cells can contain viral miRNAs, and thus, viral infections may alter the miRNA profile or change the expression of cellular miRNAs [99]. In addition, transfection of siRNAs can alter miRNA expression levels [100]. Thus, multiple triggers used to manipulate the cell fate can influence miRNA expression levels under different experimental conditions and should be carefully considered during sample preparation and subsequent analysis of the miRNA profiling results.

From a technical viewpoint, profiling of miRNAs is affected by slight deviations of the experimental procedure. This includes the usage of different RNA isolation procedures, densities of the cultured cells, and concentrations of RNA. For example, miRNA profiles differ considerably when the initial amounts of total RNA were different, although the same RNA was used for miRNA extraction [101]. This is because miRNAs are small in size and they lack secondary structures unlike mRNAs, resulting in different degrees of precipitation during RNA extraction [102]. Another issue is degradation by RNases during storage and sampling, which should be inhibited, for example, by the use of RNAse inhibitors. Another important aspect when conducting experiments with miRNAs and their target mRNAs is the RNA turnover kinetics in cells. Interestingly, the stability of miRNAs seems to be generally more robust compared to mRNA [103]. However, although most miRNAs appear to be quite stable, specific miRNAs possess differential stability in human cells. In neurons, miRNAs decay very rapidly and their turnover is dependent on neuronal activity [104]. Given the different half-life times, some RNAs get degraded more rapidly than others. For example, miR-382, an miRNA that contributes to HIV-1 provirus latency, is unstable in human cells [105]. Thus, miRNA and their target mRNA expression studies should be tightly controlled considering, for example, short and similar postmortem intervals or storage of tissue in sample

protection buffers to inhibit degradation by RNAses. In addition, standardized sampling and processing protocols are required, including sample preparation methods, RNA extraction methods, and profiling platforms.

miRNAs as Biomarkers for the SCA3 Disease Course

The high stability and presence of extracellular miRNAs in numerous body fluids such as serum, plasma, saliva, urine, and cerebrospinal fluid make miRNA a good candidate for a biomarker. Circulating miRNAs in serum have been correlated with various diseases [106]. However, one of the most critical problems in utilizing miRNAs as disease markers is the lack of consistency between studies. Different and non-overlapping sets of miRNAs are frequently reported as biomarkers for the same disease [102]. miRNA profiling using sera of 35 SCA3 patients revealed differential expression of 46 miRNAs [46]. Among those, downregulations of miR-25 and miR-125b correlated with the SCA3 disease course and were suggested as potential biomarkers for disease monitoring. However, since some miRNAs are repeatedly observed to be dysregulated in the blood of patients in many distinct diseases [107], it is unlikely that altered expression of two miRNAs is sufficient to yield the required sensitivity and specificity for monitoring disease progression in SCA3. Instead, the combination of expression changes based on multiple miRNAs might be advantageous for disease evaluation. For the classification of most cancer tissues, the combination of 48 miRNAs reached almost perfect accuracy [108]. Compared to other conventional biomarkers studied for polyglutamine diseases (reviewed in Ref. [109]), miRNA expression levels can be measured rapidly and accurately by high throughput sequencing using very low amounts of miRNAs. Also, differentially expressed mRNAs have been suggested as potential blood biomarkers for SCA3. A transcriptome analysis of 42 blood samples from SCA3 patients revealed significant upregulation of 10 mRNAs [110]. One of these transcripts, the tumor necrosis factor superfamily member 14, is also significantly upregulated in blood of SCA3 mice [111]. Compared to the much lower stability of mRNAs in body fluids, extracellular miRNAs are associated with Argonaute2 ribonucleo-protein complexes and protected from nuclease-mediated degradation in the blood and other body fluids [112]. Although the detailed mechanisms regulating cellular release and uptake of selected miRNAs are not completely understood, the stability and diversity of miRNAs point to their being novel and promising biomarkers for the diagnosis of SCA3 disease.

Individual Cell Types from SCA3 Patients Show Different Sets of Deregulated miRNAs

In SCA3 and other neurodegenerative diseases, specific neurons are lost while others remain intact. The reasons for this selective vulnerability are mostly unknown. Thus, an important question in these diseases is the extent to which the identified miRNA changes are related to the disease pathomechanisms in a specific cell type or lineage. For instance, iPS-derived neurons from SCA3 patients show a neuron-specific disease phenotype characterized by the formation of ATXN3-containing aggregates [92], which cannot be observed in other, non-neuronal cell types such as fibroblasts or glia from SCA3 patients. It has been shown that cell type-specific miRNAs participate in the specification of neuronal *versus* glial fates. Moreover, expression of miRNA differs extensively even among neural cell types that are neurons, astrocytes, oligodendrocytes, and microglia [113]. Thus not unexpectedly, miRNA profiling of lymphoblastoid [44] and iPS-derived neuronal cells [45] from SCA3 patients results in different sets and numbers of differentially expressed miRNAs even when the original cell sources used for *ex vivo* expansion were derived from the same SCA3 patients. Comparison of the significantly altered miRNAs between SCA3-LCs (n = 30) and SCA3 neuronal cells derived from iPS cells (n = 90) reveals an overlap of only four miRNAs (miR-193a/b, miR-539, miR-154, and miR-758) (Fig. 1b). Moreover, only one of the overlapping miRNAs, miR-539, is upregulated both in SCA3-LCs and SCA3 neurons, while the other miRNAs show the opposite direction of regulation in LCs and neurons from SCA3 patients. These discrepancies not only reflect the miRNA signature of unique cell types [81,82,114], but may also point at adaptive, cell type-specific miRNA changes contributing to the cellular defense mechanisms against proteotoxic stress. In non-neuronal cells, the combination of various features including the upregulation of ATXN3-targeting miRNAs, rapid cell divisions, and efficient proteolysis machineries may form a defensive wall against the cytotoxic effects of mutant ATXN3. In contrast, post-mitotic neurons may compensate the proteotoxic effects with expression of another set of miRNAs. Depending on the cellular context, these inherent properties most likely define whether a specific cell type is susceptible or not to the toxic burden of mutant ATXN3. Recent transcriptional profiling studies on brain using different mouse models of SCA3 confirm that brain region-specific transcriptional effects correlate with the neuropathologically most affected brain regions in SCA3 patients. For instance, larger fold changes and more differentially expressed genes are found in striatum and brainstem than in the cortex and cerebellum of SCA3 mice indicating diverse effects of mutant ATXN3 in each

brain region [87]. Moreover, transcriptional profiling of the brainstem in a series of mouse models of SCA3 reveals a characteristic pattern of gene expression in the pons that correlates with the degree of ATXN3 aggregation and is primarily linked to oligodendrocytes [115]. Although these studies have not addressed differential expression of miRNAs, these findings clearly show that transcriptional changes in different brain regions occur in the context of the disease and correlate with the capability of a given cell type to compensate for the potential cytotoxic effects of mutant ATXN3.

The increased expression in LCs of several members of the miR-25 and miR-181 family targeting the 3′UTR of ATXN3 possibly contributes to the reduced expression levels of ATXN3 and reduced vulnerability of this cell type toward polyglutamine-induced toxicity [44]. In iPS-derived SCA3 neurons from the same patients, however, none of the ATXN3-targeting miRNAs identified in SCA3-LCs are found among the deregulated miRNAs [45]. Instead two miRNAs, miR-23 and miR-199, predicted to target the 3′UTR of ATXN3, are differentially expressed (Fig. 2). However, since both miRNAs are downregulated in SCA3 neurons, an inverse correlation or reducing effect on the expression levels of ATXN3 is unlikely. However, it remains to be elucidated whether a subset of miRNAs is specifically altered in SCA3 neurons and involved in the protection against the neurotoxic effects mediated by mutant ATXN3. Further validation addressing the identification of key players in the cascade of gene expression and gene networks is required to understand the complex mechanisms taking place in diseased cells.

Conclusions and Current Challenges

Over the past years, evidence emerged that non-coding RNAs including miRNAs play fundamental roles in the fine-tuned, tissue and cell type-specific regulation of gene expression and their deregulation is associated with disease development. With respect to SCA3, miRNAs are involved in the disease process at three different levels: (i) deregulation of the general miRNA biosynthesis machinery possibly induced by recruitment of important transcriptional coactivators to inclusions such as CBP, (ii) cell type-specific miRNAs targeting and controlling the expression of the disease protein in non-affected cells, and (iii) disease-related miRNAs targeting specific molecules of the proteolysis machinery such as DNAJB1 in affected cells (Fig. 3).

SCA3 is a devastating disease, and only symptomatic treatment that does not halt disease progression

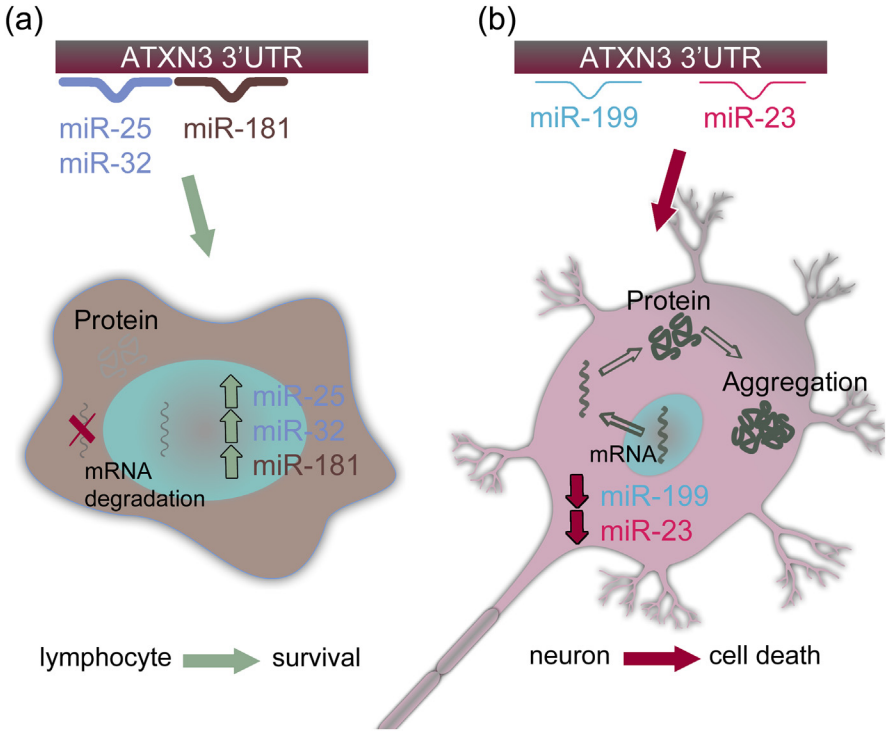

Fig. 2. miRNAs targeting the ATXN3 3′UTR influence ATXN3 expression in SCA3 depending on the cell type. (a) The ATXN3 targeting miRNAs miR-25, miR-32, and miR-181 are upregulated in SCA3 lymphocytes and thus destabilize the ATXN3 transcript. (b) The ATXN3 targeting miRNAs miR-23 and miR-199 are downregulated in SCA3 neurons, allowing expression of mutant protein and subsequent aggregate formation.

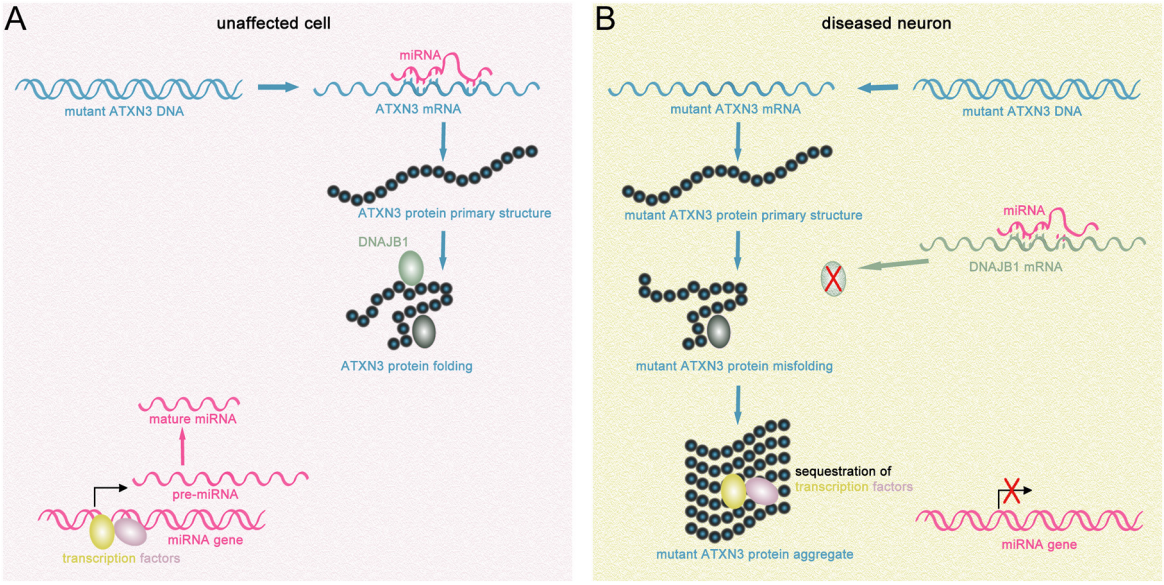

Fig. 3. The influence of miRNAs on SCA3 pathomechanisms. (A) In normal cells, miRNA biogenesis takes place and ATXN3 targeting miRNAs control the level of ATXN3 mRNA and thus protein expression. The normal ATXN3 protein folds into its correct three-dimensional structure with the help of molecular chaperones including DNAJB1. (B) In SCA3 cells, miRNAs contribute to disease development at multiple levels: first, ATXN3 targeting miRNAs are downregulated, allowing an increased expression of mutant ATXN3 protein. Second, miRNAs targeting DNAJB1 are increased, leading to a reduced DNAJB1 protein expression and subsequent misfolding and aggregation of mutant ATXN3 protein. Finally, transcription factors are recruited to the aggregates, disturbing expression and biogenesis of miRNAs.

is available. Thus, the major current challenge is to find a preventive therapeutic strategy. Numerous cellular mechanisms leading to neurodegeneration have been discussed (reviewed in Ref. [47]) and currently are targets of several therapeutic approaches (reviewed in Ref. [57]). However, all these pathomechanisms known to date support the idea that silencing the expression of the ATXN3 transcript represents one possibility for a preventive therapy [60,61,65,66]. Thus, induction of miRNAs that specifically suppress the expression of ATXN3 presents one promising putative therapeutic approach. The limitation of basic research studies investigating the pathomechanisms in SCA3, the role of miRNAs in the disease course, and the development of therapeutic strategies is that they are carried out in experimental cell models or animal models. Thus, a major bottleneck in the development of a novel therapeutic approach is often the translation from findings in cell or animal models to humans. The reasons for this include factors like species-specific regulation of gene expression, cell type-specific properties, or age-related factors that can be modeled in only one cell type using *ex vivo* models. Moreover, several neurodegenerative diseases like SCA3 are characterized by a late onset of symptoms, very often after more than 20 years, a time span that can hardly be covered in cell or animal models of the disease.

Perspectives

The realization that non-coding RNAs play central roles in disease has added another level to our understanding of the pathophysiologic mechanisms in polyglutamine diseases such as SCA3. Among non-coding RNAs, miRNAs are of particular interest for the understanding of complex disorders like polyglutamine diseases because they potentially regulate multiple pathways involved in disease progression and offer a new therapeutic way for disease modification of so far incurable diseases including polyglutamine diseases [106,116]. For instance, the possibility to target miRNA activity by systemic delivery of non-toxic, synthetic miRNA inhibitors and mimics offers a possibility to slow or even halt the disease process. Moreover, high-throughput screening enables to identify small-molecule compounds selectively inducing the expression of regulatory miRNAs altering the expression of specific disease-associated mRNAs such as *ATXN3*. In this regard, it may prove beneficial to simultaneously induce subsets of miRNAs cooperatively targeting the mRNA of a disease protein to increase specificity and avoid adverse events. In future studies, integrated approaches including standardized sampling and processing protocols will be fundamental to understand how cell type-specific miRNA expression is regulated

by various stimuli and how it cooperates with other miRNAs in the disease context.

Declarations of Interest

None.

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Abbreviations used:
mRNAs, messenger RNAs; miRNA, microRNA; SCA3, spinocerebellar ataxia type 3; RISC, RNA-induced silencing complex; LC, lymphoblastoid cell; CBP, CREB binding protein; DNAJB1, DnaJ homology subfamily B member 1; iPS, induced pluripotent stem.

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