REVIEW

Gene Therapy for Polyglutamine Spinocerebellar Ataxias: Advances, Challenges, and Perspectives

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ABSTRACT: Polyglutamine spinocerebellar ataxias (SCAs) comprise a heterogeneous group of six autosomal dominant ataxias caused by cytosine–adenine–guanine repeat expansions in the coding region of single genes. Currently, there is no curative or disease-slowing treatment for these disorders, but their monogenic inheritance has informed rationales for development of gene therapy strategies. In fact, RNA interference strategies have shown promising findings in cellular and/or animal models of SCA1, SCA3, SCA6, and SCA7. In addition, antisense oligonucleotide therapy has provided encouraging proofs of concept in models of SCA1, SCA2, SCA3, and SCA7, but they have not yet progressed to clinical trials. On the contrary, the gene editing strategies, such as the clustered regularly interspaced short palindromic repeat (CRISPR/Cas9), have been introduced to a limited extent in these disorders. In this article, we review the available literature about gene therapy in polyglutamine SCAs and discuss the main technological and ethical challenges toward the prospect of their use in future clinical trials. Although antisense oligonucleotide therapies are further along the path to clinical phases, the recent failure of three clinical trials in Huntington's disease may delay their utilization for polyglutamine SCAs, but they offer lessons that could optimize the likelihood of success in potential future clinical studies.

Key Words: gene therapy; antisense oligonucleotides; RNA interference; gene editing; polyglutamine ataxias

Spinocerebellar ataxias (SCAs) comprise a heterogeneous group of autosomal dominant neurodegenerative diseases, characterized by a progressive cerebellar syndrome usually accompanied by noncerebellar features. The most common SCAs are caused by cytosine-adenine-guanine (CAG) trinucleotide repeat expansions in coding regions of specific genes, which are translated into abnormally large polyglutamine (PolyQ) tracts in the proteins. This is the case for SCA1, SCA2, SCA3, SCA6, SCA7, and SCA17, as well as dentatorubral-pallidoluysian atrophy. Thus, these subtypes are known as PolyQ diseases, a group of neurodegenerative disorders that also include Huntington's disease (HD) and spinobulbar muscular dystrophy.[1-4]

The main physiopathological mechanisms of PolyQ ataxias include toxic gain of function of aberrantly conformed mutant proteins (proteotoxicity), ion channel dysfunction, mitochondrial dysfunction, loss of nuclear integrity, and RNA-based gain of toxic function.[1,2,5] PolyQ proteotoxicity is based in the intrinsically toxic nature of intermediate oligomers and the toxic effects of chronic accumulation of expanded protein aggregates.[6] Abnormal calcium homeostasis disrupts cell signaling, synaptic plasticity, and gene transcription, whereas abnormalities of potassium channels affect neuronal spiking.[4] Mitochondrial dysfunction is reported in experimental models of PolyQ ataxias,[7,8] while oxidative stress markers have been identified in

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patients.[9,10] Also, PolyQ expansions can contribute to abnormal chromatin remodeling, transcriptional dysregulation, and impaired DNA repair.[11]

In RNA toxicity, the expanded repeats of a flawed RNA promote sequestering and subsequent loss of function of distinct RNA binding proteins. Resultant ribonucleoprotein complexes are trapped in the nucleus, where they become toxic.[12] Experimental evidence suggests the contribution of this mechanism to the pathogenesis of SCA3 through recruitment of the nucleolin protein by an expanded *ATXN3* transcript, which in turn arrests ribosomal RNA transcription.[13] Lately, an aberrant interaction between the expanded *ATXN2* transcript and transducin β-like protein 3 (involved in ribosomal RNA processing) has been reported.[14] Interestingly, the expanded antisense transcript of the *ATXN2* gene is assumed to contribute to the pathogenesis of SCA2.[15]

Currently, there is no cure or neuroprotective treatment to slow down the progression of PolyQ ataxias. Only a few symptomatic therapies for improving a patient's quality of life are available.[16] For example, amantadine can improve dystonia and bradykinesia in SCA3.[17] Periodic leg movements are responsive to dopaminergic treatment in SCA2 and SCA3, whereas muscle cramps are improved in both disorders with B-complex vitamins and magnesium, respectively.[17,18] Physical exercises have demonstrated effectiveness in SCA2,[19] SCA3,[20] and SCA7.[21]

Furthermore, several therapeutical approaches targeting the disease proteins are under investigation for the PolyQ ataxias. Among them, the most promising strategies are: stimulation of protein clearance through autophagy; inhibition of toxic protein fragments formation and stimulation of PolyQ aggregates elimination. Moreover, drugs that target other downstream pathogenic mechanisms, such as calcium signaling stabilizers, antioxidant drugs, antiglutamatergic agents, and histone deacetylase inhibitors, are being studied.[5,16]

Undoubtedly, the most promising treatments against PolyQ ataxias are those that target the central pathological mechanisms at early stages of neurodegeneration. In this scenario, gene therapy approaches seem to be hopeful alternatives. In this article, we review the state of the art of development of gene therapies for PolyQ ataxias, with emphasis on nucleotide-based gene silencing of RNA. Furthermore, we discuss some technological issues and main ethical concerns.

Gene Therapy

Gene therapy refers to the correction or deletion of a dysfunctional gene to permanently treat or reverse a disease. This technology uses multiple pathways, such as replacement of a dysfunctional gene with a normal one, homologous recombination and repair of the abnormal gene by selective reversal of the mutation, or selective control of the defective gene expression.[22]

Gene therapy strategies include RNA interference (RNAi)-based tools, antisense oligonucleotides (ASOs),[23] and DNA editing techniques.[5] The therapeutic molecules behind these approaches differ according to the chemical composition, method of release inside cells, and gene targeting mechanisms.[5,24]

RNA Interference

RNAi is a natural, posttranscriptional, sequence-specific gene silencing mechanism elicited in response to double-stranded RNA (dsRNA).[25] The general RNAi mechanism involves cleavage of long dsRNAs by the endonuclease Dicer and the subsequent generation of single RNA fragments. The guide strands of these fragments, considered to be the RNAi molecules, are incorporated into the RNA-induced silencing complex (RISC) to promote endonucleolytic cleavage of the homologous mRNA by the RNase Argonaute 2 (Fig. 1).

The RNAi process is mediated by three functionally different noncoding dsRNA molecules: (1) microRNAs (miRNAs), (2) small interfering RNAs (siRNAs), and (3) short hairpin RNAs (shRNAs).[5,26,27] Although these molecules differ in their cellular biogenesis, they converge into the same RNAi pathway.[28]

miRNAs can be of natural or synthetic origin. Their biogenesis begins with nuclear transcription of a primary miRNA precursor with subsequent cleavage to hairpin-shaped pre-miRNAs by the RNase III Drosha. Later, these pre-miRNAs are exported to the cytoplasm, where they undergo Dicer-mediated final cleavage to miRNAs and incorporation into the RISC.[29] Chemically synthesized miRNAs enhance regulation of natural miRNAs by mimicking the high-level expression of these endogenous molecules.[30]

Distinct to the natural miRNAs, the siRNAs have a synthetic origin and once delivered into the cells by viral or nonviral systems,[23] they enter the RISC directly.[31] In contrast, shRNAs are produced in the nucleus, but they are considered to be exogenous. Their synthesis is guided by synthetic DNA vectors transfected to the cells by delivering plasmids, viral vectors (lentivirus or adenovirus-associated virus [AAV]), or bacterial vectors.[32,33] Because the transgene is integrated into the host genome, the shRNAs are produced for a long period to have a sustained effect.[31,34]

The extent of base complementarity with the target mRNA defines the outcome of the silencing of each RNAi molecule. Because siRNAs and shRNAs are fully complementary to the mRNA sequence, they inhibit the expression of one specific mRNA by endonucleolytic cleavage. In contrast, because the miRNAs have partial

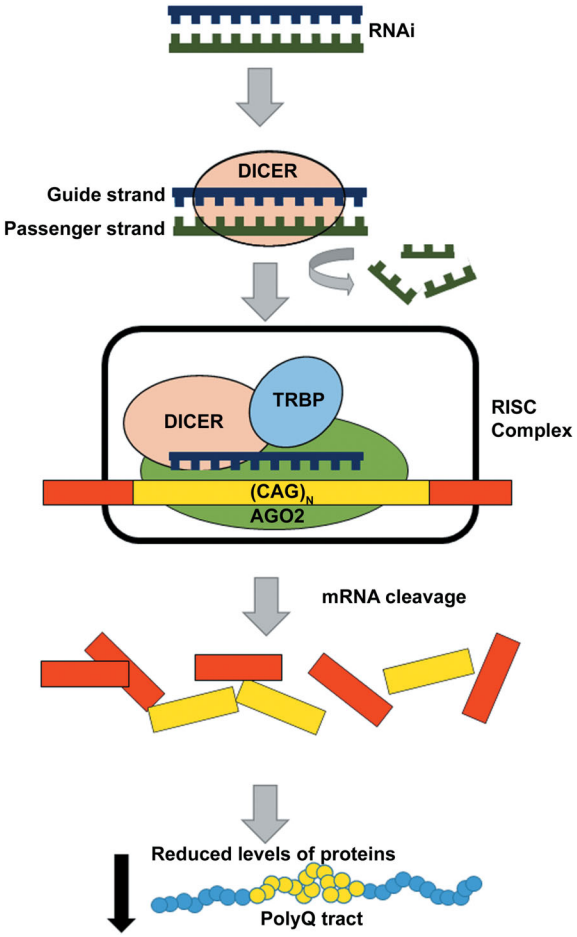

FIG. 1. General mechanism of RNA interference (RNAi). DICER endonuclease cleaves double-stranded RNA (dsRNAs) and generates single RNA strand (siRNA or miRNA). The RNAi strand is loaded into the RNA-induced silencing complex (RISC) and represses translation through cleavage of the target mRNA by the RNase Argonaute 2 (AGO2). Once cleaved, the mRNA cannot be translated, and the levels of the target proteins are reduced (the figure is contextualized to the expanded proteins in polyglutamine [PolyQ] ataxias). TRBP, transactivation response element RNA-binding protein. [Color figure can be viewed at wileyonlinelibrary.com]

complementarity with the 3′-untranslated region (3′-UTR) of the target mRNAs, they primarily cause translational repression to several transcripts at the same time.[30,35-38]

Moreover, the RNAi molecules differ in the extent of genetic silencing they produce. For example, siRNAs are transient and eliminate 30% to 50% of target mRNAs, while miRNAs can silence genes in a greater amount than shRNAs.[39] Therefore, gene suppression with siRNAs may require repeated administration, whereas shRNAs and artificial miRNAs are more long-lasting and can permanently silence a target gene after a single administration.[27]

In addition to the canonical miRNAs, an emerging class of atypical miRNA, called mirtrons, has been described. Their cellular biogenesis involves the splicing of short introns with hairpin-forming potential, which generates mature species that function as typical regulatory miRNAs in a Drosha-independent manner.[40]

RNAi therapy can be applied using non-allele-specific and allele-specific strategies. In the non-allele-specific strategy, the wild-type and mutant alleles are similarly targeted, whereas in the allele-specific approach, only the mutant alleles are silenced. This latter strategy is useful when wild-type protein expression is essential for cellular function, and consequently its suppression can be detrimental for cells.[27] However, allele-specific silencing is more technically challenging, because it requires differences in nucleotide sequence between pathological and nonpathological alleles, such as single-nucleotide polymorphisms (SNPs), and differences in the CAG repeat length for PolyQ disorders.[41-43] Other advantages of RNAi technology are its ease of design, synthesis, and production of the molecular mediators.[24]

However, the clinical application of RNAi therapy faces important challenges, such as poor in vivo stability, the efficacy of the delivery systems, and the existence of off-target effects.[24,36] Also, for neurodegenerative diseases, such as PolyQ disorders, the poor penetration of the blood–brain barrier of RNAi molecules limits their use. Another limitation is potential saturation of endogenous RNAi processing machinery due to excessive delivery of siRNAs to mammalian cells.[44] However, several strategies are currently being developed to minimize these limitations and accelerate the introduction of RNAi-based approaches in clinical contexts.[36]

RNAi Applications in PolyQ SCAs

Studies of RNAi-based therapy in PolyQ ataxias have disclosed promising findings in cellular and animal models,[24] but none of these models have progressed to clinical trials (Table 1). In SCAs, the first in vivo evidence of efficient genetic silencing by RNAi was obtained in SCA1 transgenic mice. This study produced suppression of *ATXN1* transcripts by injecting an AAV vector that expressed shRNA into the medial cerebellar lobe of the mice. As result, the levels of ataxin-1 protein were decreased, and the neuropathological and motor phenotypes were recovered.[45]

The *ATXN1* gene has several miRNA binding sites, which were validated in different transfected human cell lines (HEK293T, HeLa, MCF7) where gene expression decreased in ~60%. Nevertheless, the use of miRNAs increased cytotoxicity because of their detrimental effects on wild-type gene function.[46]

The direct injection of AAV-expressing shRNA into deep cerebellar nuclei of SCA1 knock-in mice promoted ataxin-1 suppression in the cerebellar cortex and the brainstem and preserved motor performance and cellular morphology.[47] In addition, an miRNA (recombinant AAV-miS1) caused partial suppression of the human

TABLE 1 *RNAi therapies for polyglutamine spinocerebellar ataxias*

SCA subtype	RNAi classes	Allele-specific silencing	System	Results	References
SCA1	AAV-shRNA	No	SCA1 transgenic mouse model	Reduction of ataxin-1 inclusions in Purkinje cells. Recovery of motor coordination and cerebellar morphology	Xia et al. (2004)[45]
	miRNAs (miR-19, miR-101, and miR-130 mimic)	No	Transfected HEK293T, HeLa, and MCF7 cells	Reduction of ataxin-1 protein levels (~60%)	Lee et al. (2008)[46]
	AAV siRNAs/shRNAs	No	SCA1 knock-in mouse model ($SCA1^{154Q/+}$)	Reduction of ataxin-1 protein levels. Preserved motor performance and cellular morphology	Keiser et al. (2014)[47]
	AAV-miRNA (miS1)	No	SCA1 transgenic B05 mice	Partial suppression of mutant *ATXN1* gene recovery of SCA1 phenotype	Keiser et al. (2016)[48]
SCA3	Lentivirus-shRNA	Yes	Lentiviral-induced SCA3 rat model	Reduction of ataxin-3 aggregates. Rescue of DARPP-32 neuronal marker	Alves et al. (2008)[49]
	Lentivirus-shRNA	Yes	SCA3 transgenic C57BL/6J mice	Reduction of intranuclear inclusions. Preservation of cerebellar cortex layers. Recovery of motor deficits and anxiety	Nobrega et al. (2013)[50]
	AAV-shRNA shuttled by artificial miRNA (miR-Atx3-148)	No	SCA3/MJD84.2 transgenic mice	Reduction of nuclear accumulation of mutant ataxin-3	Rodríguez-Lébron et al. (2013)[51]
	AAV-shRNA shuttled by artificial miRNA (miRATXN3)	No	YACMJD84.2 transgenic mice	Lifelong suppression of *ATXN3* gene in the cerebellum	Costa et al. (2013)[52]
	Lentivirus-miRNA: mir-9, mir-181a, and mir-494 mimics	Yes	Transfected HEK293T cells Lentivirally induced SCA3 mouse model	Reduction of mutated ATXN3 levels, protein inclusion, and neuronal dysfunction	Carmona et al. (2017)[53]
	SNALPs-siRNAs	Yes	SCA3 transgenic model ataxin-3	Reduction of motor behavior deficit and neuropathology	Conceição et al. (2015)[54]
SCA6	siRNA (siRNA7493)	Yes	Transfected HEK293T cell	Selective silencing of the mutant *CACNA1A* allele	Kubodera et al. (2005)[55]
	AAV9–miRNA: miR-3191-5p	No	AAV-induced SCA6 mouse model	Prevention of Purkinje cell degeneration and motor deficits	Miyazaki et al. (2016)[56]
SCA7	shRNA (shRNA-P16)	Yes	Transfected HEK293 cells	Reduction of mutant ataxin 7 and inclusions	Scholefield et al. (2009)[57]

(Continues)

TABLE 1 *Continued*

SCA subtype	RNAi classes	Allele-specific silencing	System	Results	References
	siRNAs (siR-P16 and siR-atxn7)	Yes (for siR-P16) No (for siR-atxn7)	SCA7 patient-derived fibroblasts	Reduction of expanded ataxin-7 protein (by siR-P16). Reduction of both wild-type and expanded ataxin-7 protein (by siR-atxn7)	Scholefield et al. (2014)[58]
	AAV1–miRNA: artificial miS4 and miC	No	BAC SCA7-92Q transgenic mouse model	Reduction of mutant and wild-type ataxin-7 transcripts in cerebellum (~50%). Significant improvement of ataxia phenotype	Ramachandran et al. (2014)[59]
	AAV1–miRNA: artificial miS4 and miC	No	BAC SCA7-92Q transgenic mouse model	Reduction of mutant and wild-type ataxin-7 alleles in retina (~50%). Normal retinal function	Ramachandran et al. (2014)[60]
	Artificial mirtrons	Yes	Fibroblast cell lines derived from SCA7 patients	Silencing of mutant *ATXN7*	Curtis et al. (2017)[61]

RNAi, RNA interference; SCA, spinocerebellar ataxia; AAV, adeno-associated virus; shRNA, short hairpin RNA; miRNA, microRNA; SNALP, stable nucleic acid lipid particles.

expanded *ATXN1* transcript and reversed the ataxia phenotype in transgenic B05 mice.[48]

The first proof of principle of allele-specific silencing in PolyQ ataxias was provided in an SCA3 rat model.[49] In this study, a lentivirus vector encoding an shRNA directed at an SNP associated with CAG repeat expansions was injected into the striatum of the animals and caused efficient silencing of the human expanded *ATXN3* allele, while preserving the wild-type protein. Consequently, a marked reduction of ataxin-3 aggregates and rescue of DARPP-32 neuronal marker expression was observed.[49] Likewise, the mutant *ATXN3* gene was silenced by the same shRNAi in a transgenic SCA3 mouse with subsequent reduction of intranuclear inclusions, preservation of the cerebellar cortex, and recovery of motor deficits and anxiety in the animals.[50]

Short-term administration of an siRNA shuttled by artificial miRNA in SCA3 transgenic mice promoted a significant decrease in nuclear accumulation of mutant ataxin-3 in the cerebellum.[51] Nevertheless, long-term administration did not reverse the motor deficit despite lifelong suppression of the *ATXN3* gene.[52]

Another study demonstrated that specific endogenous miRNAs targeting sequences in the 3′-UTR are able to downregulate the mutant *ATXN3* gene in HEK293T cells and human neurons differentiated from human induced pluripotent stem cells (hiPSCs) derived from SCA3/MJD patient fibroblasts. Also, injection of lentivirus-encoded miRNAs into the striatum of an SCA3 transgenic mouse model resulted in reduction of nuclear ataxin-3 aggregates and recovery of neuronal dysfunction.[53]

Furthermore, a nonviral vector system for RNAi has been proved preclinically in SCA3. In this case, Conceição et al[54] used stable nucleic acid lipid particles encapsulating siRNAs against the *ATXN3* transcript. Intravenous administration of these liposomal nanoparticles silenced the mutant alleles, reduced neuropathological features, and improved the motor deficits in SCA3 transgenic mice.

In SCA6, allele-specific silencing of the expanded *CACNA1A* gene was reported using an alternative method in which both the nonexpanded and the expanded alleles were suppressed by an siRNA, while the normal protein was restored using an siRNA-resistant wild-type mRNA.[55] In addition, an AAV-associated miRNA prevented Purkinje cell degeneration and reduced motor deficits in a mouse model through modulation of an internal ribosomal entry site located within the *CACNA1A* coding region.[56]

Regarding SCA7, an shRNA binding an SNP linked to expanded alleles caused allele-specific silencing of the mutated gene in human embryonic kidney cells. Consequently, the levels of mutant ataxin-7 were decreased, and the cellular phenotype was restored.[57] Similar findings were obtained in SCA7 patient-derived fibroblasts

using two distinct siRNAs against the 3′-UTR of *ATXN7* transcripts.[58] Moreover, administration of AAV-associated miRNA in an SCA7 mouse model reduced mutated and wild-type ataxin-7 and improved the ataxia phenotype[59] with preservation of the retinal function.[60]

Interesting, another alternative method for allele-specific silencing of mutated genes has been used for SCA7. This strategy combines gene silencing using an artificial mirtron and delivery of a functional copy of the gene. This study found an effective silencing of mutant *ATXN7*, whereas the function of the wild-type protein was preserved.[61]

Recently, a universal shRNA targeting the CAG repeat expansions was generated for *HTT*, *ATN1*, *ATXN3*, and *ATXN7* transcripts, which in turn reduced the levels of mutant huntingtin, atrophin-1, ataxin-3, and ataxin-7 proteins in patient-derived fibroblasts, respectively.[62]

Antisense Oligonucleotides

ASOs are small-size, chemically modified, single-stranded oligonucleotides, generally of 12 to 30 nucleotides in length. ASOs are designed to target both nuclear and cytoplasmic RNAs based on their sequence homology and thus promote gene silencing. This is a hopeful therapeutic platform with potential for treatment of neurodegenerative diseases.[26,63,64] After binding to the target mRNA, the ASOs modulate its expression through different mechanisms[26,65]: (1) transcript cleavage and degradation by RNase H (Fig. 2A), (2) interference with translation by steric blocking (Fig. 2B), (3) modification of RNA splicing (exon skipping or exon inclusion) (Fig. 2C),[66,67] and (4) modulation of mRNA maturation through interference with the polyadenylation site and cap formation (Fig. 2D). An additional mechanism consists of inhibition of endogenous miRNAs.[68]

After binding to the target mRNA, the ASOs form a DNA–RNA duplex that is recognized by the RNase H. Then, this ubiquitous cellular enzyme cleaves the mRNA in the hybrid, but not the ASO.[23] Thus, the resulting fragments of the mRNA are degraded by the exosome complex and by other nucleases.[66] ASOs are then released, enter in another round of hybridization, and target cleavage. In general, the antisense mechanism depends on both the target sequence and the ASO chemical modifications.[69]

Natural unmodified ASOs are susceptible to degradation by nucleases affecting their entrance into tissues.[70] Consequently, chemical modifications of ASOs are optimized to improve drug properties, such as pharmacokinetics, pharmacodynamics, or endocytic entrance.[71] Because each modification confers a unique property to ASOs, the nature of the chemical modification defines its classification. The first generation ASOs contain a substitution of the nonbridging oxygen atoms for a sulfur atom in the phosphodiester bond, which results in a phosphorothioate-modified backbone. This change increases resistance to nucleases and bioavailability, enhancing pharmacodynamics and pharmacokinetic features.[72,73]

Second generation ASOs have structural alterations in the ribose, such as 2'-O-methyl (2'-OMe) and 2'-O-methoxyethyl (2'-MOE) modifications. These changes increase the ASO biological half-life in the central nervous system[26,74] but do not activate RNase H; instead, they inhibit mRNA expression by steric interference with translation.[75]

Another ASO modification, called "chimeric gapmers," involves insertion of 2′-alkyl modifications flanking the central gap where the RNase H is attached. This modification provides more stability, facilitates entry into the cell, and attracts enzymes that degrade the mRNA quickly.[76]

The third generation ASOs are characterized by chemical modifications of the nucleotide in its furanose ring.[77] The most frequently used are peptide nucleic acid (PNA), phosphoramidate morpholino oligomer, and locked nucleic acid, which were developed to improve nuclease resistance, to increase binding affinity, and to enhance pharmacokinetics and biostability.[78]

Similar to RNAi-based silencing, ASO therapy can be achieved using either allele-specific or non-allele-specific strategies. Allele-specific downregulation relies on ASOs targeting SNPs that appear only in the mutant alleles or ASOs that specifically bind to longer CAG repeats in PolyQ diseases.[5]

Although ASOs are efficiently taken up by neurons, the impermeability of the blood–brain barrier hinders the translation of ASO-based therapies to clinical practice. In fact, most anionic ASOs do not cross this barrier or cross it poorly.[73] Thus, ASOs are administered by invasive methods: intraparenchymal injection (into the brain or spinal cord) or injection into the cerebrospinal fluid (intracerebroventricular or intrathecal).[79] Consequently, the development of subcutaneous catheters with intrathecal access has been recommended.[80] Intrathecal administration increases ASO bioavailability in brain and spinal cord, but the invasive nature limits its use.[81] However, other alternatives to increase cellular uptake of ASOs are under investigation. Among them, the combination of ASOs with specific molecules,[81] the use of cell-penetrating peptides, and liposome-mediated delivery are the most promising alternatives.[68,82]

Distinct to therapeutic RNAi molecules, which undergo permanent transgene expression, treatments with ASOs are usually less long-lasting and consequently require several and frequent administrations.

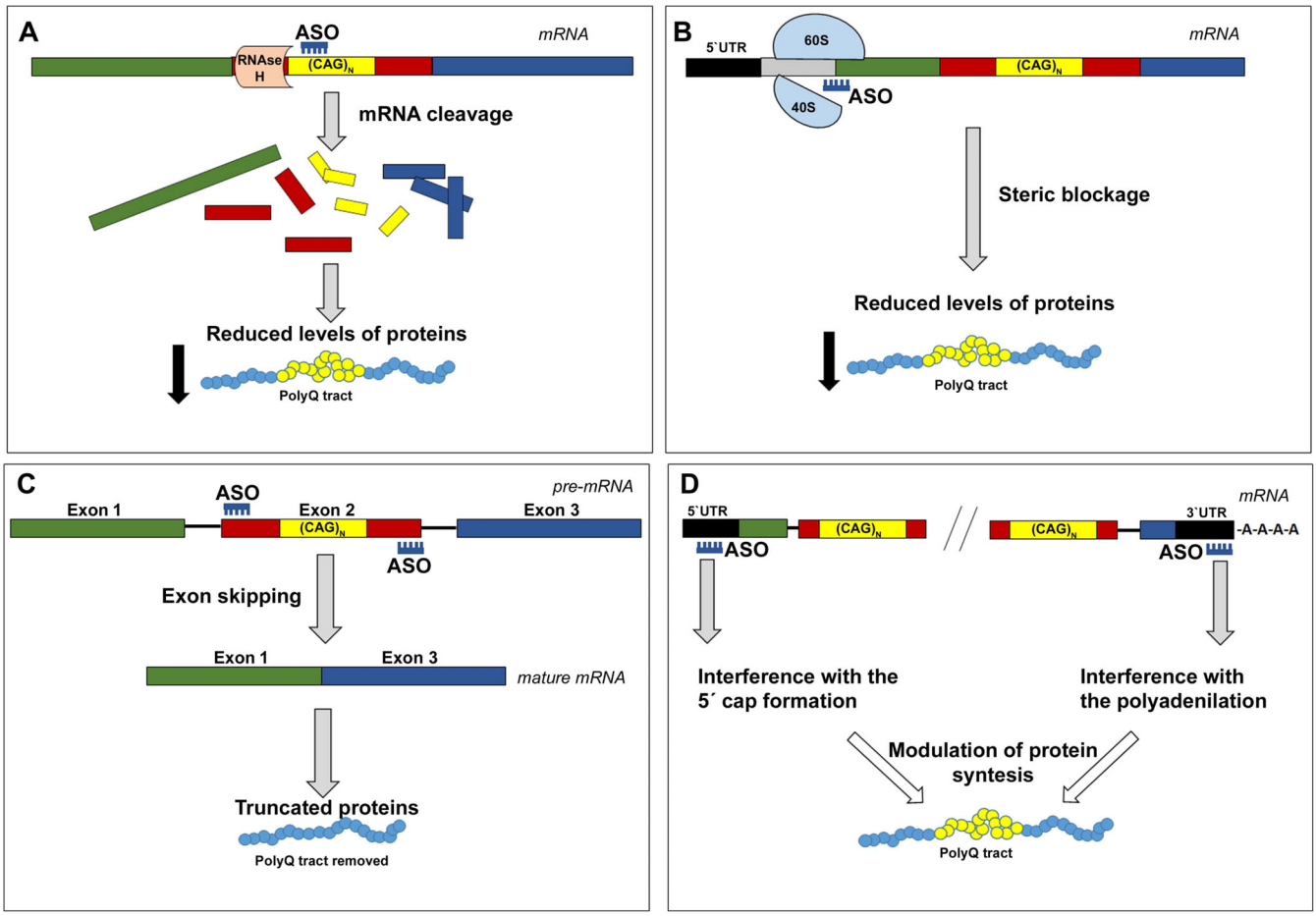

FIG. 2. Mechanisms of action of antisense oligonucleotides (ASOs). (**A**) Transcript cleavage by RNase H. (**B**) Steric blockage. (**C**) Modification of RNA splicing by exon skipping. (**D**) Modulation of protein synthesis by interference with polyadenylation. PolyQ, polyglutamine; 5′-UTR, 5′-untranslated region. [Color figure can be viewed at wileyonlinelibrary.com]

However, ASOs have a wider biodistribution than RNAi in the central nervous system after delivery.

ASO Applications in PolyQ SCAs

The first preclinical study with ASOs for PolyQ disorders was performed in a mouse model of HD. In that study, the animals received intraventricular administration of a phosphorothioate-modified ASO, which in turn caused a significant reduction of the mutant and wild-type huntingtin in the cerebrospinal fluid and recovery from the pathological phenotype.[83] The study set the rationales to evaluate the usefulness of ASO therapy in PolyQ ataxias.[66] Consequently, some studies in cellular and animal models have been developed for these neurodegenerative disorders (Table 2).

For SCA1, allele-specific regulation of the expanded CAG repeat has been applied in a model of patient-derived fibroblasts, leading to significant reduction of the expanded transcript.[84] Likewise, intraventricular administration of an MOE ASO targeting *ATXN1* in an SCA1 knock-in mouse model led to substantial reduction of mRNA levels in the cerebellum, cerebral cortex, and brainstem up to 12 weeks after injection and caused reduction of motor disturbances and lethality in the animals.[85]

In SCA2, intracerebroventricular injection of an MOE gapmer ASO in two transgenic mouse models reduced *ATXN2* mRNA and ataxin-2 levels. This reduction was detected 70 consecutive days after a single injection with significant recovery of motor deficits and restoration of Purkinje cell morphology and firing rate. The SCA2-related proteins expressed in the Purkinje cells were normalized after ASO administration.[86]

Remarkably, the lowering of *ATXN2* mRNA by ASO therapy in a TDP43/ALS (amyotrophic lateral sclerosis) transgenic mouse model decreased TDP-43 aggregation, improved motor function, and increased the survival rate of the animals.[87] Also, ASOs targeting ataxin-2 expression restored nucleocytoplasmic transport in neuronal-differentiated hiPSCs from patients with amyotrophic

TABLE 2 *ASO therapies for polyglutamine spinocerebellar ataxias*

SCA subtype	ASO/mechanism	Allele-specific silencing	Results	System	References
SCA1	2′OMe RNA with phosphorothioate backbone/RNase H-dependent cleavage	Yes	Reduction (~89%) of expanded *Atxn1* mRNA	SCA1 patient-derived fibroblasts	Evers et al. (2011)[84]
	MOE ASO	No	Reduction of *Atxn1* mRNA levels, reduction of motor disturbances, and lethality	SCA1 knock-in mice (*Atxn1*$^{66Q/2Q}$ *and* *Atxn1*$^{154Q/2Q}$)	Friedrich et al. (2018)[85]
SCA2	MOE gapmer ASO/RNase H-dependent cleavage	No	Reduction of *Atxn2* mRNA, improvement of motor function, restoration of Purkinje cell morphology and firing rate	SCA2 patient-derived fibroblasts SCA2 transgenic mice (ATXN2-Q127 BAC-Q72)	Scoles et al. (2017)[86]
SCA3	PNA ASO versus CAG repeats	Yes	Reduction of mutant protein levels	SCA3 patient-derived fibroblasts	Hu et al. (2011)[90]
	MOE ASO/ RNase H-dependent cleavage	No	Reduction of ataxin-3 protein in fibroblasts and in Q84 YAC transgenic mice. Absence of astrogliosis or microgliosis in the animal models	SCA3 patient-derived fibroblasts; Q84 YAC transgenic mice; Q135 cDNA transgenic mice	Moore et al. (2017)[91]
	2′-OMe ASO with phosphorothioate backbone/exon skipping	No	Efficient removal of PolyQ tracts in the protein. Ubiquitin binding function of truncated proteins was preserved. No toxicity in animals	SCA3 patient-derived fibroblasts; C57bl/6j mice	Evers et al. (2013)[92]
	2′- O-methoxyethylribose nucleotides with phosphorothioate backbone/exon skipping	No	Reduction of insoluble ataxin-3 and nuclear accumulation	SCA3 fibroblast SCA3 transgenic MJD84.2 mouse model	Toonen et al. (2017)[93]
	MOE gapmer ASO/RNase H-dependent cleavage	No	Rescue of motor impairment, prevention of ATXN3 nuclear accumulation, recovery of Purkinje cell layer	SCA3 transgenic mice (YACMJD84.2/84.2)	McLoughlin et al. (2018)[94]
	MOE gapmer ASO/RNase H-dependent cleavage	No	Restoring normal excitability of SCA3 Purkinje neurons	SCA3 transgenic mice (YACMJD84.2Q-C57BL/6)	Bushart et al. (2021)[95]
SCA7	Gapmer ASOs (ATXN7-ASO) (CAG-ASO)/RNase H-dependent cleavage	No	Reduction of protein aggregation. Improvement of visual function and recovery of retinal histopathology	SCA7 knock-in mice (SCA7 266Q) SCA7 patient fibroblasts	Niu et al. (2018)[96]
SCA1 and SCA3	(CUG)7 ASO/exon skipping	Yes	Reduction of ataxin-3 protein in fibroblasts and of ataxin-1 and ataxin-3 proteins in the brain of the animals	SCA1 and SCA3 patient-derived fibroblasts SCA3 transgenic mice (MJD84.2) SCA1 knock-in mice (*Atxn1*$^{154Q/2Q}$)	Kourkouta et al. (2019)[97]

ASO, antisense oligonucleotide; SCA, spinocerebellar ataxia; 2′-OMe, 2′-O-methyl modifications; MOE, 2′-O-methoxyethyl modifications; PolyQ, polyglutamine.

lateral sclerosis (ALS).[88] These findings are consistent with the known role of the *ATXN2* intermediate alleles as a risk factor for ALS in humans.[89]

ASO-based therapeutic approaches for SCA3 have been widely studied. The use of a PNA ASO targeting the expanded CAG repeat caused a significant reduction of mutant ataxin-3 levels in patient-derived fibroblasts.[90] Another study with an MOE ASO reported a decrease of mutant ataxin-3 levels in fibroblasts and in two distinct mouse models, in which no evidence of astrogliosis and microgliosis was found.[91]

Regarding the exon skipping mechanism, Evers et al.[92] used a modified ASO to remove the CAG repeat containing region by skipping the 9th and 10th exons of the *ATXN3* pre-mRNA. The truncated protein maintained its ubiquitin binding capacity in SCA3 patient-derived fibroblasts. Intraventricular injection of this ASO in C57bl/6j mice resulted in elimination of the PolyQ-containing exon in the cerebellum without toxicity in the animals. In another study, a truncated ataxin-3 protein without exon 10 led to reduction of insoluble ataxin-3 accumulation in the nucleus of fibroblasts and in transgenic MJD84.2 mice.[93]

A study using an MOE gapmer ASO targeting *ATXN3* transcript in the homozygous MJD84.2/84.2 mice revealed substantial reduction of ataxin-3 and its aggregates for several weeks post-injection, as well as recovery of the Purkinje cell layer in the cerebellum.[94] In another study, the same ASO restored Purkinje cell excitability in YACMJD84.2Q-C57BL/6 transgenic mice through the rescue of transcript levels of two voltage-gated potassium channels.[95]

Regarding SCA7, in 2018, Niu et al.[96] compared the effect of two ASO strategies to treat retinal degeneration. They used an ASO against *ATXN7* mRNA (*ATXN7*-ASO) and another targeting the expanded CAG tract (CAG-ASO). Intravitreal injection of *ATXN7*-ASO in SCA7 knock-in mice reduced *ATXN7* gene expression and protein aggregation in the eyes of the animals. This gene silencing promoted the recovery of retinal histopathology, photoreceptor gene expression, and visual function even when the treatment started after symptom onset. CAG-ASO injection reduced the mutant ataxin-7 aggregates to a lesser extent than the *ATXN7*-ASO, while the levels of the wild-type protein were unchanged.

Following the therapeutic potential of a single CAG repeat-targeting ASO to treat various PolyQ diseases, Kourkouta et al.[97] evaluated a common ASO to downregulate the mutant ataxin-1 and ataxin-3 proteins in vitro and in vivo by exon skipping. This treatment caused reduction of ataxin-3 in fibroblasts and of ataxin-1 and ataxin-3 in the brain of SCA1 knock-in mice and SCA3 transgenic mice, respectively. Hence this study gave a proof of concept about the potential usefulness of universal ASOs for treatment of PolyQ ataxias.

Genome Editing Therapy

Modern gene editing techniques are based on accurate induction of double-stranded breaks (DSBs) at specific target sequences to stimulate the cellular repair machinery.[98] The DSB-based editing mechanism involves engineered endonucleases to introduce site-specific DSBs with high precision, which improves the efficiency of gene targeting through homologous recombination.[99] The main gene editing platforms are the zinc-finger nucleases (ZFNs), the transcription activator-like effector nucleases (TALENs), and the clustered regularly-interspaced short palindromic repeat (CRISPR)/Cas9 systems.[100]

Both ZFNs and TALENs are chimeric enzymes with a DNA binding domain fused with a nonspecific catalytic domain of Fok-I endonuclease. Thus, these dimers can produce DSBs in the DNA target sites. The CRISPR/Cas9 system requires a short guide RNA and Cas9 endonuclease in a ribonucleoprotein complex. In brief, the specific guide RNA recognizes the target DNA sequence and directs the Cas9 nuclease to generate a DBS at this specific site.[101,102]

A common concern about gene editing strategies is the induction of off-target mechanisms as a result of endonucleolytic cleavage of unintended sites with high sequency homology with the target region. However, several strategies have been introduced to minimize these off-target effects, such as precise design of guide RNAs with chemical modifications and Cas9 variants engineered to increase their efficiency toward the target sequence.[103,104]

In addition to off-target effects, the delivery systems of gene editing strategies have an impact on the safety and efficacy of these potential therapeutic tools. Currently, the most used methods are AAV vectors and electroporation or microinjection of target cells. Both methods have their own advantages and challenges, which are influenced by whether the system is ex vivo or in vivo.[104]

Gene Editing Applications in PolyQ SCAs

Because gene editing technology allows a precise and permanent gene correction, it has been considered as a potential approach to remove the CAG repeat mutations in polyQ diseases. Several studies have demonstrated that CAG repeat-targeting nucleases can decrease expanded CAG/CTG sequences in different models as a result of DNA break repair.[27] For example, induction of a TALEN platform into CAG/CTG trinucleotide repeats promoted contraction of the PolyQ tract in a yeast model,[105] whereas the use of ZFNs caused cleavage of CAG repeats in human and

rodent cell lines.[106] Some successful gene editing strategies have been developed in preclinical HD models.[107-109]

For PolyQ ataxias, the proof of concept for gene editing technology was established in SCA3. This study showed successful use of a CRISPR/Cas9 platform targeting the mutant *ATXN3* allele in hiPSCs derived from patients with SCA3. Specifically, the authors used two guide RNAs surrounding the CAG repeat tract in exon 10 of the gene and found a significant deletion of the PolyQ expansion without affecting the pluripotency and cell differentiation of the cellular lines or the ubiquitin binding capacity of the edited ataxin-3.[110] A similar approach was used in fibroblasts from patients with SCA1, where a significant reduction of the mutated ataxin-1 protein was observed with minimal off-target effects[111] (Table 3).

Final Considerations

Although gene therapy strategies have provided promising findings in cellular and animal models of PolyQ ataxias, they have not yet been approved for use in clinical trials, because some technological, ethical, and economic concerns are still unresolved.[112] The technological challenges include the susceptibility of therapeutic RNA molecules to cellular endonucleases, their inability to cross the cell membrane, and triggering of the immune response in the guest organism.[113] Thus, selection of appropriate vectors and delivery methods is a key issue for a successful gene therapy.[79] For example, lentiviruses and AAV are considered suitable vectors for gene therapy in PolyQ ataxias, because they are minimally immunogenic and have been efficiently transduced into the cerebellum.[73] In addition, the use of nonviral vectors is highly recommended, because they are safer than viral vectors and their combination with chemically modified ASOs increases their stability to access the cells.[114] Specifically, the liposome DCL64 is considered a potential vehicle for ASO delivery in PolyQ ataxia treatment, because it allows wide distribution of oligonucleotides in the Purkinje cells after intravenous administration.[82] Other methods for optimizing ASOs and RNAi delivery include development of nanoparticles to envelope the negatively charged molecules to allow them to traverse the target cell membrane.[112]

Another challenge of gene therapy in PolyQ ataxias is the putative deleterious effect of reducing the wild-type proteins by the nonspecific allele strategies because most of these proteins have essential cellular functions and are functionally related to each other in a highly connected ataxia-related protein network crucial for cerebellar function.[115]

For example, knocking out the *TBP* gene,[116] whose CAG expansion produces SCA17, is embryonically lethal in mice, whereas for the *CACNA1A* gene it causes dystonia and late-onset cerebellar degeneration.[117] Furthermore, mice lacking *ATXN1* and *ATXN2* genes showed Alzheimer's disease-related pathogenesis[118] and abnormal fat metabolism,[119] respectively. On the contrary, the loss of wild-type *ATXN3*[120] and *ATXN7*[121] genes seems not to be so problematic. However, the existence of some paralogous genes with high functional homology to PolyQ ataxia genes[115,122-124] could reduce the concern about the non-allele-specific reduction of such genes.

Similar to other therapeutic strategies, the gene therapy approaches must overcome the absence of robust clinical, biological, and neuroimaging biomarkers and the need to conduct trials in large cohorts, especially in prodromal or early clinical stages. In addition, application of protocols combining conventional pharmacological treatments and gene therapy could enhance drug bioavailability.[38]

Some of the technological challenges have been strengthened by the recent interruption of three clinical trials of ASOs in patients with HD.[125] First, the phase III study with the Roche tominersen ASO failed to slow disease progression in spite of a reduction of the mutant huntingtin levels. In fact, a subcohort of patients receiving the drug every 8 weeks showed worse results in motor function and cognition than the placebo group. These negative findings are thought to be caused by lowering of the native huntingtin, inefficient ASO delivery into the target neurons, or inclusion of patients with advanced disease.[126]

TABLE 3 *Gene editing therapies for polyglutamine spinocerebellar ataxias*

Gene editing platform	Gene	System	Results	References
CRISPR/Cas9	*ATXN3*	iPSCs derived from a SCA3 patient	Modified ataxin-3 protein retained ubiquitin-binding capacity	Ouyang et al. (2018)[110]
CRISPR/Cas9	*ATXN1*	SCA1 patient-derived fibroblasts	Downregulation of ataxin-1 protein expression	Salvatori et al. (2020)[111]

iPSC, induced pluripotent stem cell; SCA, spinocerebellar ataxia; CRISPR, clustered regularly-interspaced short palindromic repeat.

The other two unsuccessful clinical trials (phase 1b/2a) used the Wave Therapeutics ASOs WVE-120101 and WVE-120102, respectively.[125] These antisense candidates were designed for allele-specific silencing of expanded *HTT* by targeting two distinct SNPs associated with CAG repeat expansions. However, no significant decrease of the mutant protein or dose-dependent effects was observed in either study, probably because of inefficient target engagement. Also, the open-label extension studies of both trials were discontinued for lack of efficacy. However, a third ASO (WVE-003) targeting a different CAG repeat-related SNP and developed with next generation phosphoramidate (PN) backbone chemistry is currently under clinical study. Researchers are optimistic about this ASO, because it showed increased potency and better pharmacological properties in preclinical studies.

The failure of these clinical trials will probably slow the pipelines of ASO therapy development in PolyQ ataxias, but they offer some lessons learned for upcoming clinical trials. First, non-allele-specific strategies should be avoided in those subtypes in which the loss of wild-type protein produces deleterious phenotypes (SCA17), as well as abnormal motor (SCA6), behavioral (SCA1), or metabolic features (SCA2). However, non-allele-specific strategies could be feasible for SCA3 and SCA7.

A second lesson learned is the need to attain efficient cerebellar engagement of the ASOs. Although preclinical studies have provided evidence of intracerebroventricular targeting of mouse cerebellum (Table 2), the significantly larger size of the human cerebellum and its widespread neurodegeneration pattern, involving both cortical and deep nuclear regions,[2] provides rationales for more efficient ASO delivery systems such as nanocarriers and/or ASOs chemically modified to improve their biodistribution in the cerebellum. Furthermore, better cerebellar engagement could be achieved by increasing the dose of ASOs, but the risks for drug toxicity must be considered. In fact, the use of elevated doses of the tominersen ASO is presumed to have contributed to negative results of the Roche clinical trial.[125] Also, inclusion of patients in early or even prodromal stages of PolyQ ataxias is mandatory for upcoming clinical trials.

Moreover, a phase 1 clinical trial of an ASO (BIIB105) targeting the *ATXN2* gene in patients with ALS with or without intermediate CAG repeat expansions (ClinicalTrials.gov: NCT04494256) is ongoing. In this case, the findings of safety, tolerability, and pharmacokinetic profile of the drug would be valuable for design clinical trials in SCA2 patients. However, the differences in the main neurodegeneration target among both diseases should be considered mainly because of the presumed higher doses required for cerebellar engagement of the ASOs.

The translation of gene therapy research to the clinical context in PolyQ ataxias is not exempt from ethical challenges. First, the putative adverse effects triggered by off-target mechanisms and the lack of drug efficacy mediated by the delivery and biodistribution limitations could negatively unbalance the cost/benefit ratio of the treatments. Another ethical dilemma that may emerge with these treatments is that their effect should depend on disease stage; therefore, they may not be very effective in the most severe cases, who have the highest need for treatment.

Because the PolyQ ataxias are considered rare diseases, they offer low incentives for the pharmaceutical companies, which not only slows down the development of gene therapy treatments but also makes them more expensive. Consequently, the adjustment of the costs to the social and healthcare requirements of the patients is an ongoing challenge.

Altogether, the promising findings of gene therapy in preclinical models of PolyQ ataxias provide rationales for its use in humans, but such introduction into clinical phases is still challenged by some technological, ethical, and economic concerns that need to be appropriately addressed. ■

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Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study. ■

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