

SURVEY AND SUMMARY

Oligonucleotide-based strategies to combat polyglutamine diseases

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ABSTRACT

Considerable advances have been recently made in understanding the molecular aspects of pathogenesis and in developing therapeutic approaches for polyglutamine (polyQ) diseases. Studies on pathogenic mechanisms have extended our knowledge of mutant protein toxicity, confirmed the toxicity of mutant transcript and identified other toxic RNA and protein entities. One very promising therapeutic strategy is targeting the causative gene expression with oligonucleotide (ON) based tools. This straightforward approach aimed at halting the early steps in the cascade of pathogenic events has been widely tested for Huntington's disease and spinocerebellar ataxia type 3. In this review, we gather information on the use of antisense oligonucleotides and RNA interference triggers for the experimental treatment of polyQ diseases in cellular and animal models. We present studies testing non-allele-selective and allele-selective gene silencing strategies. The latter include targeting SNP variants associated with mutations or targeting the pathologically expanded CAG repeat directly. We compare gene silencing effectors of various types in a number of aspects, including their design, efficiency in cell culture experiments and pre-clinical testing. We discuss advantages, current limitations and perspectives of various ON-based strategies used to treat polyQ diseases.

INTRODUCTION

Expansions of short tandem repeat sequences in different genes are responsible for numerous human hereditary neurological diseases. Most of these disorders are caused by the expansion of repeated trinucleotides and are called triplet

repeat expansion diseases (1). Their largest subgroup is polyQ diseases, which are caused by the expansion of CAG repeats present in open reading frames (ORFs) of specific functionally unrelated genes. These disorders include Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), spinal bulbar muscular atrophy (SBMA) and spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7 and 17 (Table 1). Additionally, SCA8 shares some features with polyQ diseases due to the antisense transcription of non-protein-coding gene containing CTG expansion and the translation of antisense transcripts to polyQ proteins (2). The common feature of polyQ diseases is their late onset, as initial symptoms usually appear in affected subjects in their 30s or 40s. The age at onset and the severity of polyQ disorders correlate with the size of the CAG repeat expansion. Typically, normal alleles of polyQ disease genes contain 10–30 CAG repeats, and mutant alleles contain 40–60 repeated units. However, repeats as short as 21 CAG tracts in the *CACNA1A* gene can cause SCA6, and expansions reaching more than 100 repeated units may occur in HD and SCA7 (Table 1). PolyQ diseases also share some pathogenic pathways leading to neurodegeneration. The mutant genes are ubiquitously expressed in the central nervous system (CNS) and peripheral tissues (3), but the pathology develops primarily in distinct brain areas characteristic of each disorder (Table 1). Interestingly, the expression of the mutant gene is usually not much higher in the brain areas mainly affected by the disease than in other brain areas and peripheral tissues. This result suggests that additional factors are required to stimulate pathogenesis. nullnull

Although the polyQ disorders remain incurable and only symptomatic treatment is offered to patients, many drugs are currently being tested to reverse the disease or to slow its progress. The most direct therapeutic strategy is silencing the casual gene expression. For some of these approaches, pre-clinical testing in rodent models is well advanced, and small-scale clinical testing is just beginning. In this review, we briefly refer to the pathogenesis of polyQ diseases and

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Table 1. Brief characteristics of polyQ diseases

Disease	Ref.	Gene	Repeat tract length (normal/mutant)	Expression	Protein/size/function	Main site of pathogenesis	mutCAG-binding factors
DRPLA: dentatorubral-pallidoluysian atrophy	(288,289)	<i>ATN1</i>	6–36/49–88	Ubiquitous	Atrophin-1/~125 kDa/transcription regulation	Globus pallidus, subthalamic nucleus, dentate nucleus, white matter	?
HD: Huntington's disease	(290)	<i>HTT</i>	6–35/36–121	Ubiquitous, high in neurons	Huntingtin/~350 kDa/embryonic development, neurogenesis	Striatum, globus pallidus, substantia nigra	MBNL-1, SRSF6, U2AF65
SBMA: spinal and bulbar muscular atrophy	(291)	<i>AR</i>	6–37/38–62	Ubiquitous	Androgen receptor/~110 kDa/steroid-hormone activated transcription	Spinal anterior horn, facial nucleus, skeletal muscle	?
SCA1: spinocerebellar ataxia type 1	(292)	<i>ATXN1</i>	8–44/45–82	Ubiquitous	Ataxin-1/~85 kDa/transcription regulation, alternative splicing	Purkinje cells, dentate nucleus, brainstem, spinal cord	?
SCA2: spinocerebellar ataxia type 2	(293–295)	<i>ATXN2</i>	13–31/36–63	Ubiquitous, high in Purkinje cells	Ataxin-2/~140 kDa/mRNA maturation and translation, stress-granule formation, endocytosis, Ca-mediated signaling	Cerebellum (Purkinje cells), pons, inferior olives, thalamus, substantia nigra	?
SCA3: spinocerebellar ataxia type 3	(296)	<i>ATXN3</i>	12–44/60–84	Ubiquitous	Ataxin-3/~30 kDa/ubiquitin-mediated proteolysis, chromatin remodeling	Substantia nigra, cranial nerve motor nuclei, striatum	MBNL-1, U2AF65, NCL
SCA6: spinocerebellar ataxia type 6	(297)	<i>CACNA1A</i>	4–18/21–33	Ubiquitous, but predominant in brain	Calcium channel alpha 1A subunit/~30 kDa/calcium-dependent processes	Purkinje cells, dentate nucleus, inferior olive	?
SCA7: spinocerebellar ataxia type 7	(298)	<i>ATXN7</i>	4–34/37–306	Ubiquitous, high in brain and testis	Ataxin-7/~90 kDa/transcription regulation	Purkinje cells, pons, dentate nucleus, inferior olive, retina	?
SCA17: spinocerebellar ataxia type 17	(299,300)	<i>TBP</i>	29–42/45–63	Ubiquitous	TATA-binding protein/~40 kDa/transcription regulation	Purkinje cells, striatum, cerebral cortex	?

present the current status of therapeutic strategies, especially the oligonucleotide (ON) based techniques. We also discuss issues important for the design and further testing of various treatment approaches and conclude with our thoughts on their clinical perspectives.

PATHOGENESIS OF POLYQ DISEASES

In the traditional view of gene expression, the expanded CAG*CTG repeats present in mutant polyQ disease genes are transcribed into CAG repeats in RNA and translated into polyQ tracts in the encoded proteins (Figure 1). The abnormal protein–protein interactions triggered by mutant proteins were long thought to be the only factor responsible

for the pathogenesis of polyQ diseases. In recent years, however, several examples of mutant transcript toxicity were demonstrated, and additional toxic RNA and protein entities were identified.

Protein toxicity

Proteins implicated in polyQ diseases differ in size and cellular function and are mainly involved in the regulation of transcription (Table 1) (4). The toxicity of mutant proteins in polyQ diseases is thought to result primarily from a gain-of-function mechanism triggered by protein misfolding and aggregation (5) (Figure 1). The nuclear ubiquitin-positive aggregates of mutant proteins are the hallmark of

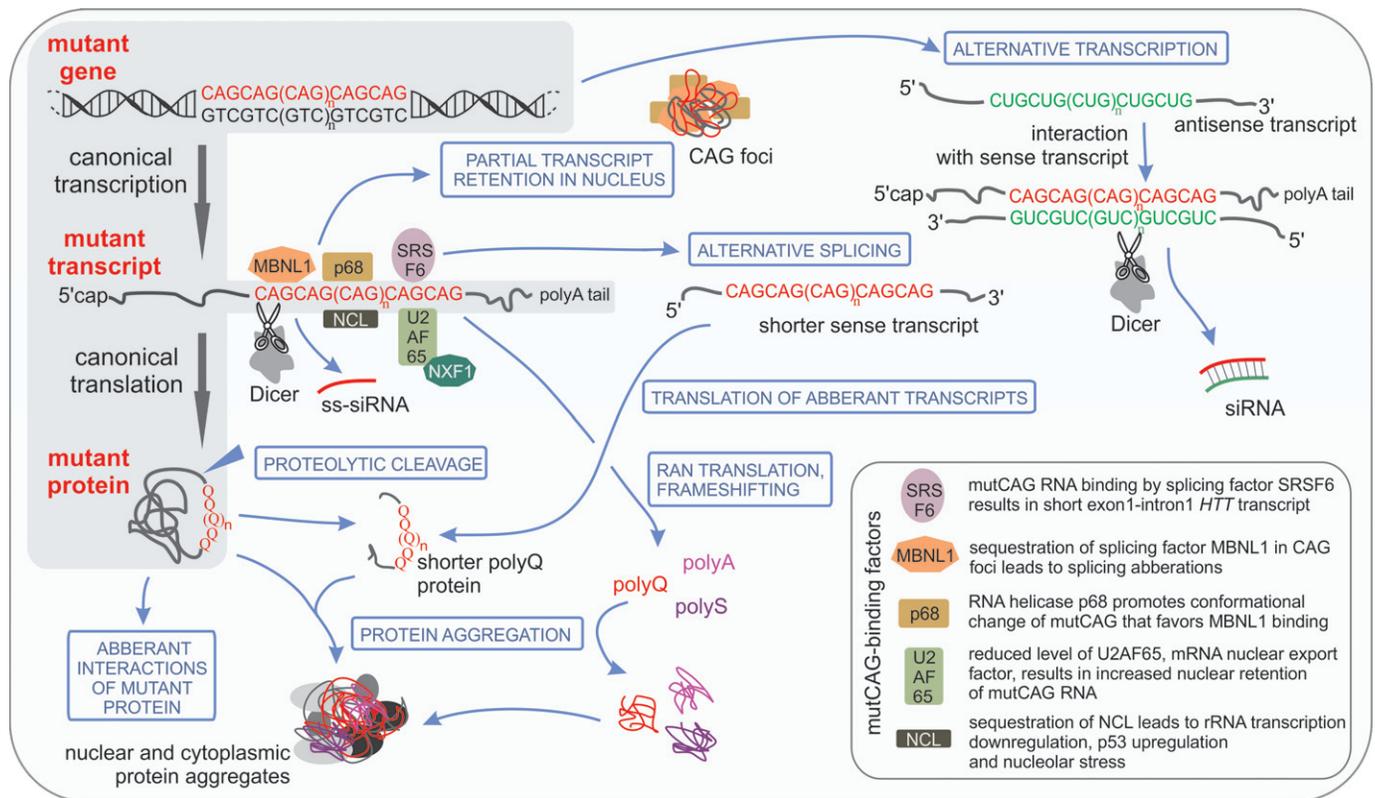


Figure 1. Toxic entities in the pathogenesis of polyQ diseases. The main products of the mutant gene are the mutant transcript containing the expanded CAG repeats and the mutant protein containing the expanded polyQ tract. The hallmarks of primary toxic events are nuclear aggregates containing mutant RNAs (CAG foci) or mutant proteins (full length or fragments). The interactions and events leading to the production of additional toxic entities from the mutant transcript and mutant protein are indicated. The antisense transcription of the mutant polyQ gene may result in transcripts containing expanded CUG repeats. siRNAs generated by the RNase Dicer from the expanded tracts may interact with complementary sequences in the transcriptome and cause the downregulation of the expression of numerous genes. Aberrant translation may lead to peptides containing polyA, polyS or polyQ tracts. See the text for more details.

polyQ disorders (6–9). There is no clear answer yet as to whether neuronal intranuclear inclusions (NIIs) play an essential role in the pathogenesis or partially protect cells from cytotoxicity. Several studies on cellular and animal models showed no clear correlation between inclusion formation and cell death (10–12). Importantly, mutant protein fragments containing polyQ tracts are more toxic than full-length proteins and might be crucial for pathogenesis (13). Such fragments, which are mainly the products of cleavages by caspases, were identified for huntingtin, atrophin-1, ataxin-2, ataxin-3 and ataxin-7 (14–16). In agreement with these observations, more severe phenotypes are observed in animal models of polyQ diseases that express fragments of mutant genes than in models expressing full-length proteins (17).

Post-translational modifications are also significant contributors to polyQ protein toxicity (18). The phosphorylation of the polyQ protein may affect its proteolytic cleavage, as well as influence SUMOylation and ubiquitinylation. Interestingly, specific post-translational modifications can affect the toxicity of polyQ proteins in opposite ways, e.g. huntingtin phosphorylation at Ser421 results in a decrease in NII formation and reduces toxicity (19), whereas phosphorylation of ataxin-1 at Ser776 is required for SCA1 pathology (20). In the case of ataxin-1, the importance of

Ser776 phosphorylation comes from the fact that it triggers a structural change in the region in which several functional signals are localized (21,22).

Not only new protein–protein interactions are triggered by mutant proteins, interactions characteristic of wild-type proteins can also be either enhanced or attenuated in case of mutant polyQ proteins (23). The beta-sheet conformation and coiled-coil structure are thought to mediate aggregation, as well as interactions with other proteins (24–28). Such altered protein–protein interactions were characterized very extensively for huntingtin (29–32), and they are thought to be crucial for the vulnerability of specific types of neurons to degeneration. The other examples include the following: ataxin-1 association with transcription factor RORalpha (33) and translational repressor Capicua (34) and ataxin-7 interactions with transcription coactivator complex STAGA (35,36). The formation of additional toxic protein products may be triggered by the expanded CAG repeat tracts in transcripts. These extra proteins include frame-shifted polypeptides (37–39) and products of repeat-associated non-AUG (RAN) translation (40,41) (Figure 1).

RNA toxicity

Growing evidence supports the importance of mutant transcript toxicity in the pathogenesis of polyQ diseases. Expanded CAG repeats in transcripts form hairpin structures containing periodic A-A mismatches (42–47). A similar hairpin structure, formed by CUG repeats (48), triggers the major pathogenic mechanism in myotonic dystrophy type 1 (DM1) (49), the disease caused by a CTG expansion in the 3' UTR of the *DMPK* gene (50). The hallmark of CUG repeat toxicity is the formation of nuclear foci by mutant transcripts and sequestered MBNL1 protein (51). Ribonucleoprotein foci formation and MBNL1-dependent deregulation of alternative splicing were also observed in HD and SCA3 cells (52). The toxicity caused by expanded CAG repeat RNA was demonstrated using genetic constructs containing mutant CAG repeat tracts expressed in different model organisms (53). Experiments performed in *Drosophila* compared the effects of transcripts that were translated with those that were not and contained pure or CAA-interrupted CAG repeats encoding polyQ tracts (54–56). Significant toxicity was reported for translated and untranslated CAG repeat tracts, but it was not observed for untranslated CAA-interrupted tracts, which do not form stable hairpin structures (57). Pathogenic features were also observed in a transgenic mouse model in which the expression of an expanded untranslated CAG repeat tract was directed to muscle (58). A comparison of two HD mouse models, which contained different patterns of CAA-interrupted CAG repeat tracts, supported the contribution of CAG RNA toxicity to the pathogenesis of polyQ disorders (59). Using a *Drosophila* SCA3 model and a HD mouse model, the involvement of the NXF1/U2AF65 RNA export pathway in RNA-mediated toxicity was demonstrated (60). The interaction of mutant CAG repeats with nucleolin was shown to induce nucleolar stress, leading to apoptosis in *Drosophila* and human cellular models of SCA3 as well as in HD mouse model (61). Helicase p68 was also shown to colocalize with expanded CAG repeats and increase MBNL1 binding to mutant transcripts (62). Moreover, splicing factor SRSF6 was reported to interact with expanded tracts in HTT transcripts, which results in short HTT sense transcripts being translated into toxic peptides (63). Furthermore, other toxic RNA entities were identified: antisense transcripts (64) and short CAG repeat RNAs (65,66) (Figure 1).

THERAPEUTIC TARGETING OF MUTANT GENES AND THEIR EXPRESSION PRODUCTS—AVENUES FOR POLYQ DISEASES

Taking advantage of the fact that each polyQ disease is monogenic, a rational therapeutic strategy could be designed to lower the causative gene expression. As proof of the concept, the inducible expression of a mutant transgene was turned off in rodent models of HD and SCA1, and recovery from the disease could be seen (67–70). This result included the reversal of aggregate formation and improvement in the motor phenotype.

The possible ways to block the pathogenic pathway in polyQ diseases include the following: genome editing,

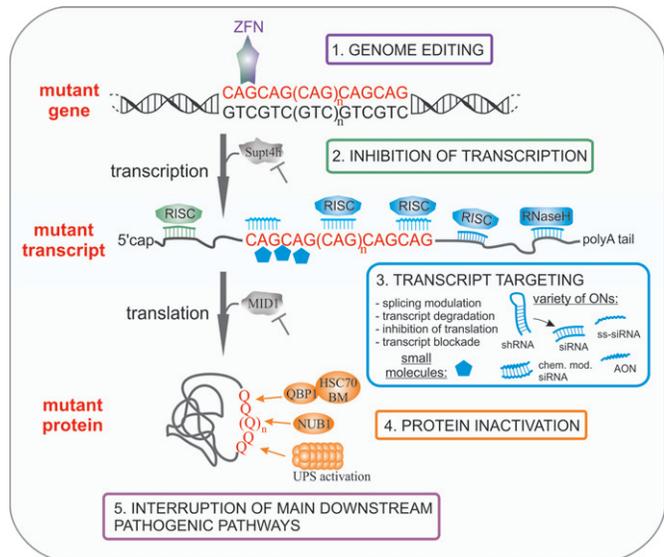


Figure 2. Strategies directed at the elimination of toxic entities in polyQ diseases. The main steps of mutant gene expression at which therapeutic intervention may be applied are indicated. The possible interventions include: 1. editing the CAG expansion in the mutant gene, 2. inhibiting mutant gene transcription, 3. interacting of potential drugs with mutant transcript leading to its degradation or inhibition of translation, 4. inactivating the mutant protein by its degradation or blockage and 5. targeting the main downstream pathogenic pathways. See the text for more details.

transcription inhibition, transcript degradation or modification, translation arrest, and inhibition or degradation of toxic protein (Figure 2). To prevent the activation of pathogenic pathways, the mutation should be eliminated or blocked at the earliest step of mutant gene expression, i.e. in the DNA sequence. The technologies designed for DNA editing are developing rapidly (71–73) and were already tested for therapeutic use (74–79). In the case of polyQ diseases, gene editing tools can be directly targeted to expanded CAG sequences in genomic DNA. Zinc finger nucleases that recognize and cleave CAG repeat sequences were tested in mammalian cells (80). More recently, the CAG repeat-targeting zinc finger proteins (ZFPs) were used in a R6/2 HD mouse model to achieve efficient and specific repression of mutant *HTT* (81). The ZFP-mediated mutant transgene repression likely involved steric blockade formation for RNA polymerase during transcription and resulted in a substantial correction of the molecular and behavioral pathogenic hallmarks. In attempts to therapeutically regulate gene transcription, the promoter sequence may be targeted with short RNA duplexes (82–85). The feasibility of this approach is supported by growing evidence that core RNA interference (RNAi) proteins are present and operate in the nucleus (86–89). It is not clear whether the observed changes in the histone code are caused by short RNAs targeting DNA or RNA (sense or antisense) covering the promoter sequence, but clearly Argonaute proteins are involved in these processes (90,91). In the case of polyQ diseases, this strategy would likely result in silencing both alleles of the gene.

The prevailing strategy for the therapeutic regulation of gene expression is transcript targeting with ON-based

tools (Figure 2). This regulation takes advantage of various mechanisms, which are determined by the ON type, its features such as structure, chemical modification and target localization and its dependence on specific cellular proteins (92–94).

Antisense oligonucleotides (AONs) may be designed to target pre-mRNA for its correction. Specific AONs can modulate splicing, e.g. exclusion of exon containing mutation or skipping transcript region encoding protein portion implicated in pathogenesis (95–97). This strategy was tested for SCA3 and HD in cell culture and initially assessed *in vivo* with AONs modified with 2'-*O*-methyl (2'OMe) and containing a phosphorothioate (PS) backbone. The AONs directed the exclusion of two *ATXN3* exons that contained the CAG repeat tract while the reading frame and functional domains of ataxin-3 were preserved (98). In the HD model, the AONs induced the skipping of the exon 12 fragment of the HTT transcript that encodes a crucial cleavage site (99). This resulted in a protein resistant to caspase-6 cleavage, which is responsible for generating more toxic truncated huntingtin.

ON-based approaches that have been developed to promote specific mRNA cleavage include mainly RNase H-activating AONs and RNAi-based tools. AONs directing transcript degradation by RNase H now constitute the majority of ON-based drugs in clinical trials (100). RNase H is expressed at relatively low levels, localizes to the nucleus and cytoplasm and mediates the cleavage of the RNA in an RNA–DNA heteroduplex (101). In mammalian cells, the 15–20 nt long chemically modified AONs are used for RNase H1 activation, and the cleavage site is located between the 8th and 12th nucleotides from the 3' DNA terminus of the heteroduplex substrate (102–104).

RNAi has been widely explored for post-transcriptional gene silencing in functional assays as well as for therapeutic purposes. The RNAi effectors are short interfering RNAs (siRNAs), which are ~21 nt duplexes (105,106). siRNAs share many features with microRNAs (miRNAs), which regulate the expression of the majority of genes in human cells (107). Both siRNAs and miRNAs are activated through the RNA-induced silencing complex (RISC), which contains the core Argonaute (AGO) protein (108,109). siRNAs are usually fully complementary duplexes containing 2-nt 3' overhangs. Also siRNAs typically show perfect complementarity to the target sequence and activate AGO2-directed transcript cleavage. This cleavage site is located opposite of the internucleotide bond between the 10th and 11th nucleotides from the 5' terminus of the siRNA strand (110). miRNAs are usually not fully complementary duplexes; they form mismatches in the interaction with target, and trigger mainly mRNA deadenylation and degradation or translation inhibition (111–113).

Another type of regulatory ONs are those that do not induce mRNA degradation but are transcript blockers and may affect translation, the local structure or interactions with proteins. One possibility is that translation arrest by AONs targets translation initiation, which results in the formation of a steric blockade to mRNA scanning (114,115). Translational inhibition may also be induced by miRNA-like siRNAs, in a RISC-dependent process, which was tested for polyQ diseases and will be described in detail

in the next section ('CAG repeat-targeting'). Moreover, the ON-based blockers may be designed to inhibit toxic interactions of specific proteins with mutant transcripts. Various tools targeting the expanded CUG repeat tract in DMPK transcripts were tested. Morpholino oligomers and AONs containing 2'OMe and a PS backbone or locked nucleic acid (LNA) residues caused the reversion of splicing defects and myotonia in DM1 mouse models (116–118). This reversion was achieved by blocking the expanded CUG repeat interactions with the splicing factor MBNL1.

In another strategy, structure-specific small-molecule drugs are designed or selected for targeting expanded repeats in transcripts (119). An extensive high-throughput search was performed for CUG repeat-binding drugs as potential therapeutics for DM1, taking advantage of the stable hairpin structure formed by the expanded CUG repeats (120–126). The crystal structures of the CUG and CAG repeat duplexes (127–130) were very useful in the design of this type of drugs. The bis-benzimidazole-derivatives were shown to efficiently bind to expanded CAG repeats in the transcript (131). More recently, the benzylguanidine-containing small molecule was described as an efficient inhibitor of the expanded CAG repeat RNA interaction with MBNL1, which resulted in the reversal of the alternative splicing defects in cell culture (132). Nevertheless, it seems that transcript targeting by small-molecule drugs could only inhibit RNA toxicity in polyQ disorders.

Another way of interfering with the expression of a mutant gene is targeting specific protein factors involved in the transcription and translation of the mutant entities. In the case of polyQ diseases, the transcription elongation factor Supt4h was shown to be required for the transcription of long CAG repeat tracts (133). The downregulation of Supt4h by siRNAs in cultured neuronal cells resulted in a decrease in the mutant huntingtin level. In another recent study, the translation regulatory protein complex containing the MID1 protein was demonstrated to stimulate the translation of CAG repeat expansions and was suggested to be a potential therapeutic target (134).

Some other therapeutic strategies, which will be only briefly mentioned here, are focused on the elimination of protein toxicity by targeting mutant proteins. These approaches include (i) inducing protein degradation and (ii) inhibiting processes of aggregation, proteolytic cleavage and post-translational modifications of mutant protein (135,136). In spite of the inconsistent data regarding the toxicity of polyQ aggregates, the therapeutic potential of decreasing polyQ aggregation was shown upon increasing chaperone activity (137) or using specific inhibitors (138–140). Small molecule drugs were screened for altering polyQ aggregation and enhancing the clearance pathway (141). Direct polyQ protein degradation may be enhanced by stimulating the ubiquitin–proteasome system (142) or autophagy (143). For the degradation of mutant huntingtin, the polyglutamine binding peptide 1 (QBP1) was fused with the binding motif of heat shock cognate protein 70 (HSC70) (144). The fusion protein was intrastrially delivered in an rAAV vector and ameliorated the disease phenotype in the HD mouse model. Recently, the NUB1 factor was shown to lower the mutant huntingtin level by enhancing its proteasomal degradation (145). The stimulation of NUB1 was

achieved by the treatment of cell culture with interferon. Structural motifs such as beta-sheet conformation of the polyQ tract or coiled-coil structure, which are common in polyQ proteins, could also be targeted to diminish polyQ pathology. In the case of particular polyQ proteins, specific domains or structural motifs could also be blocked to prevent abnormal interactions, e.g. blocking the AXH domain of ataxin-1 (146,147). Apart from targeting mutant transcript and protein in polyQ diseases, it can be advantageous to overexpress the normal allele of a mutant gene as was shown for ataxin-1-like (Atxn-1l) overexpression in SCA1 mouse models (148,149). Elevated Atxn1l levels suppressed SCA1 neuropathology likely by displacing mutant ataxin-1 from its native complex with Capicua (34).

Therapeutic approaches that focus on the inhibition of the main downstream pathogenic pathways in polyQ diseases are described elsewhere (150–154). These neuroprotective strategies include the correction of mitochondrial dysfunction, calcium dysregulation or neuroinflammation.

ON-BASED STRATEGIES FOR SILENCING MUTANT GENES CAUSING POLYQ DISEASES

Allele-selective versus non-allele-selective strategies

Therapeutic strategies for suppressing causative gene expression in polyQ diseases may be designed to target both alleles of the gene or only the mutant allele. In considering the requirement of allele selectivity in therapeutic gene downregulation, the cellular function of the wild-type protein is an important factor (Table 1). The key question is whether partial loss of the wild-type protein could be tolerated in adult life? Some light on this issue was shed by studies employing conditional knock-out mouse model of *HTT*. Using this model, an essential role of huntingtin, mainly in the CNS, was shown (155,156). Thus, decreasing the level of wild-type protein may pose a risk to patients. Moreover, the wild-type protein function may decrease the toxicity of the mutant protein, as demonstrated for SCA3, in a mechanism involving ubiquitin-mediated proteolysis (157), and for HD (158). In the study of HD mouse model, loss of wild-type *Htt* was shown to negatively affect neuropathology, motor dysfunction and lifespan of mice (158). These results could explain the disease phenotype in rare homozygous SCA3 and HD patients with two mutant alleles, whose phenotype is more severe than that of patients with a single mutant allele (159,160). However, the overexpression of wild-type ataxin-3 in SCA3 rat model was found not to mitigate the pathology (161).

In the next subsections, we first present the results of targeting a sequence that is present in both alleles of polyQ-related transcripts, which may be referred to as non-allele-selective targeting. Then, we continue with the description of single nucleotide polymorphism- (SNP-) and CAG repeat-targeting approaches, which are designed to preferentially silence the mutant allele.

Non-allele-selective targeting

The pre-clinical testing of various ON-based therapeutic tools, performed in rodent models of polyQ diseases, was the most extensive testing so far for the non-allele-selective

strategy (Table 2, 'Non-allele-selective targeting'). In the majority of cases, the mutant human transgene was targeted and silenced, taking advantage of sequence differences between mouse and human homologues. In these studies, much attention was paid to demonstrate the reversal of the molecular and behavioral pathogenic hallmarks. nullnull

A study by the Davidson group in the SCA1 mouse model provided the first *in vivo* evidence for efficient gene silencing in a polyQ disorder (162). A few studies were performed using transgenic HD models and *HTT* transgene-specific RNAi triggers: shRNAs delivered in AAV vectors (163–166) or siRNAs (167,168). Locally delivered RNAi triggers caused a substantial reduction in transgene expression, which resulted in reduced neuropathy and positively affected the behavioral phenotypes of the mice. In a very extensive study, the mutant *HTT* transgene was silenced with AONs in three mouse models of HD (169). Treatment with a 2'-O-methoxyethyl- (2'MOE-) modified gapmer (Figure 3A) caused a reduction in the huntingtin level throughout the CNS up to ~25% and resulted in the improvement of motor coordination. Recently, artificial miRNA mimics targeting the 3' UTR of the mutant *ATXN3* transgene and delivered in AAV were tested in a transgenic SCA3 model (170,171). Constructs based on miRNA precursors, which are considered safer, were effective in transgene silencing in the cerebellum, but no correction of the phenotype was reported, likely due to the insufficient distribution of the inhibitor across the brain (170).

In several experiments, the silencing of the normal endogenous *HTT* and *ATXN3* was performed in order to test the non-allele-selective strategy straightforwardly. In rodent models, normal *Htt* or *Atxn3* was inhibited usually together with the mutant human transgene, while only endogenous *HTT* was silenced in non-human primates. Studies performed in HD mice treated with RNAi triggers demonstrated that lowering the wild-type protein to 30–40% of control level was well tolerated (166,172,173). Similarly, in a study performed in SCA3 rat model, silencing of endogenous *Atxn3* to ~30%, together with mutant *ATXN3*, was shown to reduce neurodegeneration and suggested feasibility of inhibition of both alleles (161). Moreover, it was shown that silencing of endogenous *Atxn3* in wild-type and SCA3 rats did not cause additional neuropathology (161). The RNAi-mediated silencing of endogenous *HTT* in non-human primates was described in three independent studies (174–176). A partial reduction in huntingtin after the injection of shRNA in the AAV vector to putamen was well tolerated, as shown by the analyses performed up to 6 weeks after treatment (174). siRNA delivery for a period of 4 weeks to the *Rhesus* brain by infusion pump, resulting in a 50% decrease in the huntingtin level, was shown not to cause any significant histopathological changes (175). shRNA delivered in the AAV vector caused a 45% reduction in *HTT* expression, while no histopathological changes occurred in the brain and no motor abnormalities were detected even 6 months after the treatment (176). *HTT* silencing was also performed in non-human primates by AON administration. Huntingtin downregulation up to 30% of the control level was achieved, but the long-term safety of this suppression was not analyzed (169). In spite of the lack of adverse effects

Table 2. Testing ONs in therapeutic strategies against polyQ diseases

Disease/Gene	Silencing agent	Model	Main results	Ref. Year
Non-allele-selective targeting				
SCA1/ <i>ATXN1</i>	shRNA in AAV1 vector	Transgenic mouse model (human ataxin-1 with 82Q)	ICB injection resulted in a reduction in the accumulation of mutant ataxin-1 and an improvement of motor coordination	(162) 2004
HD/ <i>HTT</i>	shRNA in AAV1 vector	Transgenic mouse model (HD-N171–82Q)	Reduced inclusions in stratum, correction of disease phenotype after IST injection	(163) 2005
HD/ <i>HTT</i>	siRNA	Transgenic mouse model R6/2	IVT infusion of siRNAs in the liposome complex reduced huntingtin inclusions, improved motor coordination and prolonged lifespan	(167) 2005
HD/ <i>HTT</i>	shRNA in AAV5 vector	Transgenic mouse model R6/1	Reduced NIIs and disease phenotype ameliorated after IST injection	(164) 2005
HD/ <i>HTT</i>	shRNA in AV vector	Transgenic mouse model R6/2 and model induced by the AAV vector containing the human <i>HTT</i> fragment	IST injection resulted in a reduction in huntingtin inclusions	(165) 2007
HD/ <i>HTT</i>	Cholesterol-conjugated siRNA	Model induced by the AAV vector containing the human <i>HTT</i> fragment	Inhibition of neurodegeneration and improvement in motor coordination by IST injection	(168) 2007
HD/ <i>HTT</i>	miRNA-based shRNA in AAV2/1 vector	Knock-in mouse model CAG140 and a transgenic mouse model (N171–82Q)	miRNA-based constructs were found to be safer than typical shRNAs after IST injection and caused decreased striatal toxicity, improvement in motor coordination and a prolonged life span	(166,247) 2008 2009
HD/ <i>HTT</i>	shRNA in LV vector	Mouse and rat models induced by the injection of the LV vector containing a human <i>HTT</i> fragment (with 82Q)	The silencing of <i>mHTT</i> only or together with normal <i>Htt</i> resulted in a decrease of huntingtin inclusions after IST injections	(172) 2009
SCA3/ <i>ATXN3</i>	shRNA in LV vector	Rat model induced by the injection of the LV vector containing human <i>ATXN3</i> (with 72Q)	Silencing of both, normal endogenous <i>Atxn3</i> and mutant transgene, by IST injections of vectors resulted in neuropathology reduction and did not cause any significant side effects	(161) 2010
HD/ <i>HTT</i>	siRNA	Non-human primates	Convection-enhanced delivery of siHtt by IST (putamen) injection resulted in the widespread distribution throughout the striatum and efficient huntingtin silencing	(175) 2011
HD/ <i>HTT</i>	miRNA-based shRNA in AAV2/1 vector	Non-human primates	<i>HTT</i> suppression after IST (putamen) injection did not cause any neuronal degeneration or behavioral abnormalities, and no immune response to AAV vector was detected	(174) 2011
HD/ <i>HTT</i>	shRNA in AAV vector	Non-human primates	The efficient downregulation of normal <i>htt</i> in the striatum after IST (putamen) injection, without adverse behavioral effects and histopathological changes in brain tissue	(176) 2012
HD/ <i>HTT</i>	AON	Transgenic mouse models: YAC128, BACHD, R6/2 and non-human primates	RNase H-activating AONs, delivered by IVT infusion, mediated <i>HTT</i> transgene silencing throughout the CNS and improvement in motor coordination	(169) 2012
SCA1/ <i>ATXN1</i>	Artificial miRNA in AAV vector	B05 mouse model	The efficient knockdown of a transgene in the cerebellum, after ICB injections of vectors, improved neuropathology and rescued the behavioral phenotype	(149) 2013
SCA3/ <i>ATXN3</i>	Artificial miRNA mimics in AAV2/1 vector	Transgenic mouse model (SCA3/MJD84.2)	Effective downregulation of ataxin-3 in the cerebellum after ICB injections of vectors, but no corrected phenotype was reported	(170,171) 2013

Table 2. Continued

Disease/Gene	Silencing agent	Model	Main results	Ref. Year
HD/ <i>HTT</i>	shRNA in AAV9 vector	Transgenic mouse models: BACHD and N171-82Q	Systemic delivery of viral vectors by intra-jugular vein injection reduced <i>mHTT</i> expression in brain and peripheral tissues, prevented inclusion formation in key brain regions and prevented severe weight loss	(274) 2014
HD/ <i>HTT</i>	miRNA-based shRNA in AAV2/1 vector	Transgenic mouse model YAC128	Reduction of striatal huntingtin aggregates and improvements in behavioral deficits after IST injections	(173) 2014
Polymorphism site-targeting				
SCA3/ <i>ATXN3</i>	siRNA, shRNA in AV vector	Cos-7 and HeLa cells transfected with plasmids containing a SNP site	Selective suppression of mutant ataxin-3 by targeting a SNP site located just after a CAG repeat tract	(183) 2003
SCA3/ <i>ATXN3</i>	siRNA	HEK293T cells transfected with plasmids containing a SNP site	Inhibition of mutant ataxin-3 by targeting a SNP site located just after a CAG repeat tract	(184) 2004
HD/ <i>HTT</i>	siRNA	HeLa cells transfected with plasmids containing a SNP site	Five SNPs targeted, mismatch position 16 described as providing high discrimination	(190) 2006
HD/ <i>HTT</i>	siRNA	Patient-derived fibroblast cell lines	One SNP targeted and allele-selective siRNA selected	(191) 2008
SCA3/ <i>ATXN3</i>	shRNA in LV vector	Rat model induced by the injection of the LV vector with human <i>ATXN3</i>	Selective silencing of mutant transgene, after IST injections of vectors, resulted in a decrease of neuropathological abnormalities	(186) 2008
HD/ <i>HTT</i>	siRNA	Patient-derived fibroblast cell lines	Selective suppression of mutant huntingtin by targeting a polymorphic site of a 3-nt deletion	(301) 2009
HD/ <i>HTT</i>	siRNA	NIH3T cells transfected with plasmids containing a SNP site, patient-derived fibroblast cell lines	Three SNPs targeted and allele-selective siRNAs selected	(192) 2009
HD/ <i>HTT</i>	siRNA	Patient-derived fibroblast cell lines	Three SNPs targeted, five allele-selective siRNAs were estimated to be useful for three-quarters of HD patients	(193) 2009
SCA7/ <i>ATXN7</i>	shRNA, pri-miRNA-based hairpins	HEK293T cells transfected with plasmids containing a SNP site	pri-miRNA mimics targeted mutant <i>ATXN7</i> transcript efficiently and decreased mutant protein aggregation	(188) 2009
HD/ <i>HTT</i>	siRNA	HeLa cells transfected with plasmids containing a SNP site and patient-derived lymphoblast cell lines	Four SNPs targeted and allele-selective siRNAs selected	(194) 2010
HD/ <i>HTT</i>	AON	Patient-derived fibroblast cell lines, cultured primary neurons and BACHD and YAC128 mouse models	Effective and selective (~5-fold) silencing of <i>HTT</i> transgene in a model containing a targeted SNP variant using AON modified with PS, MOE, S-cEt and delivered by IST injections	(195) 2011
SCA3/ <i>ATXN3</i> , HD/ <i>HTT</i> , SCA1/ <i>ATXN1</i>	siRNA	Patient-derived fibroblast cell lines	For each SNP targeted allele-selective siRNAs were identified that preferentially inhibited the expression of the mutant allele	(179) 2012
SCA3/ <i>ATXN3</i>	shRNA in LV vector	Transgenic mouse model (expressing truncated ataxin-3 with 69Q in Purkinje cells)	Reduction in Purkinje cell pathology and improvement in motor coordination after allele-selective transgene silencing using vectors delivered by ICB injections	(187) 2013
HD/ <i>HTT</i>	AON	Transgenic mouse model Hu97/18	AON modified with PS, S-cEt and 2'MOE, showed >100-fold selectivity in the silencing of mutant <i>HTT</i> and efficient and selective huntingtin suppression in CNS after delivery by IVT infusion	(196) 2013
CAG repeat-targeting				
SBMA/ <i>AR</i>	dsRNA (81 bp)	<i>Drosophila</i> S2 cells and HEK293T cells	Non-allele-selective silencing of normal and mutant <i>AR</i> transgenes	(302) 2002

Table 2. Continued

Disease/Gene	Silencing agent	Model	Main results	Ref. Year
SCA1/ <i>ATXN1</i>	shRNA in AAV vector	HEK293T or PC6-3 neuronal cells transfected with plasmids encoding normal and mutant ataxin-1	shCAG, targeting CAG repeat region was used as a positive control of silencing, both alleles were silenced efficiently	(162) 2004
HD/ <i>HTT</i> , SCA3/ <i>ATXN3</i>	AON	Patient-derived fibroblast cell lines	Selective inhibition of mutant huntingtin and ataxin-3 with PNA- and LNA-modified AONs (selectivity fold > 6)	(201,303) 2009
HD/ <i>HTT</i>	AON	Patient-derived fibroblast cell lines	Selectivity of mutant <i>HTT</i> silencing even >6-fold for AONs with modifications LNA, cEt, carba-LNA, cEt-PS, MOE-2'F-PS	(202) 2010
HD/ <i>HTT</i>	siRNA	Patient-derived fibroblast cell lines	Selectivity of mutant <i>HTT</i> silencing > 40-fold for siRNA containing mismatches with the target sequence	(199) 2010
HD/ <i>HTT</i>	siRNA	Patient-derived fibroblast cell lines	Selective inhibition of mutant huntingtin using CAG/CUG duplexes with specific mutations	(200) 2011
SCA3/ <i>ATXN3</i>	AON, siRNA	Patient-derived fibroblast cell lines	Selectivity of mutant ataxin-3 silencing 5-fold for PNA modified AON and 16-fold for siRNAs	(204) 2011
HD/ <i>HTT</i>	AON	Patient-derived fibroblast cell lines	AONs modified with LNA and containing oligospermine conjugate for efficient delivery	(304) 2011
HD/ <i>HTT</i>	AON	Patient-derived fibroblast cell lines	High allele selectivity was demonstrated for morpholino-modified AON	(179) 2012
HD/ <i>HTT</i>	ss-siRNA chemically modified	Patient-derived fibroblast cell lines and knock-in mouse model HdhQ150/Q7	Selectivity of mutant <i>HTT</i> silencing > 30-fold in cell culture, specific <i>HTT</i> silencing in mouse brain by ss-siRNAs delivered by IVT infusion	(206) 2012
HD/ <i>HTT</i> , SCA3/ <i>ATXN3</i>	siRNA with abasic substitutions	Patient-derived fibroblast cell lines	RNA duplexes containing centrally located abasic residues (and additional 2'OMe and PS modifications) showed allele selectivity in <i>HTT</i> and <i>ATXN3</i> silencing	(305) 2013
HD/ <i>HTT</i> , SCA3/ <i>ATXN3</i>	siRNA with UNA substitutions	Patient-derived fibroblast cell lines	siRNAs containing single UNA-modified nucleotides in antisense strand showed >40 and >10-fold selectivity for mutant <i>HTT</i> and mutant <i>ATXN3</i> , respectively	(306) 2013
SCA3/ <i>ATXN3</i>	ss-siRNA chemically modified	Patient-derived fibroblast cell lines	Selectivity of mutant <i>ATXN3</i> silencing >35-fold for selected ON	(207) 2013
HD/ <i>HTT</i>	sd-siRNA	Patient-derived fibroblast cell lines	Guide strand-only siRNAs showed high selectivity, >60-fold, in <i>HTT</i> silencing	(208) 2013

The table is divided into the three parts, depending on the strategy type, which include the targeting of the following: the sequence harboring the polymorphism site, CAG expansion or another region of the transcript sequence. In each part of the table, the studies are placed in the order from the oldest studies to the most recent. The specific ON-based tools and models used in the studies are listed, together with brief descriptions of the main results. The selectivity of the silencing is based on the ratio of IC₅₀ for inhibiting the normal allele versus IC₅₀ for the mutant allele. For description of ON administration *in vivo*, the abbreviations are used: IST, intrastriatal; IVT, intraventricular; ITH, intrathecal; ICB, intracerebellar.

on the phenotype observed after silencing the endogenous *HTT* in animal models, a serious concern remains regarding human cases in which longer-lasting gene inhibition will be required.

In another strategy, the silencing of both alleles of the causative gene is combined with the exogenous expression of a version of the normal allele that has been engineered to be resistant to the silencing agent (177). This gene-knockdown and replacement strategy was tested for SCA6 and SCA3 in cell culture models (178,179). In these studies, the expression of both alleles of the *CACNA1A* and *ATXN3* endogenes was silenced in human cultured cells using siRNAs. For other disorders, such as *retinitis pigmentosa*, there

are reports of testing the replacement strategy *in vivo* (180). In principle, with the use of this approach, a similar effect could be achieved as with the allele-selective approaches, i.e. the elimination of the mutant allele only. However, the replacement strategy is more demanding as both the silencing agent and the expression vector for the normal allele must be efficiently delivered, and the exogenous protein has to be expressed at an appropriate level.

SNP-targeting

Allele-selective silencing may be achieved by targeting ONs to transcript regions harboring SNP variants that dis-

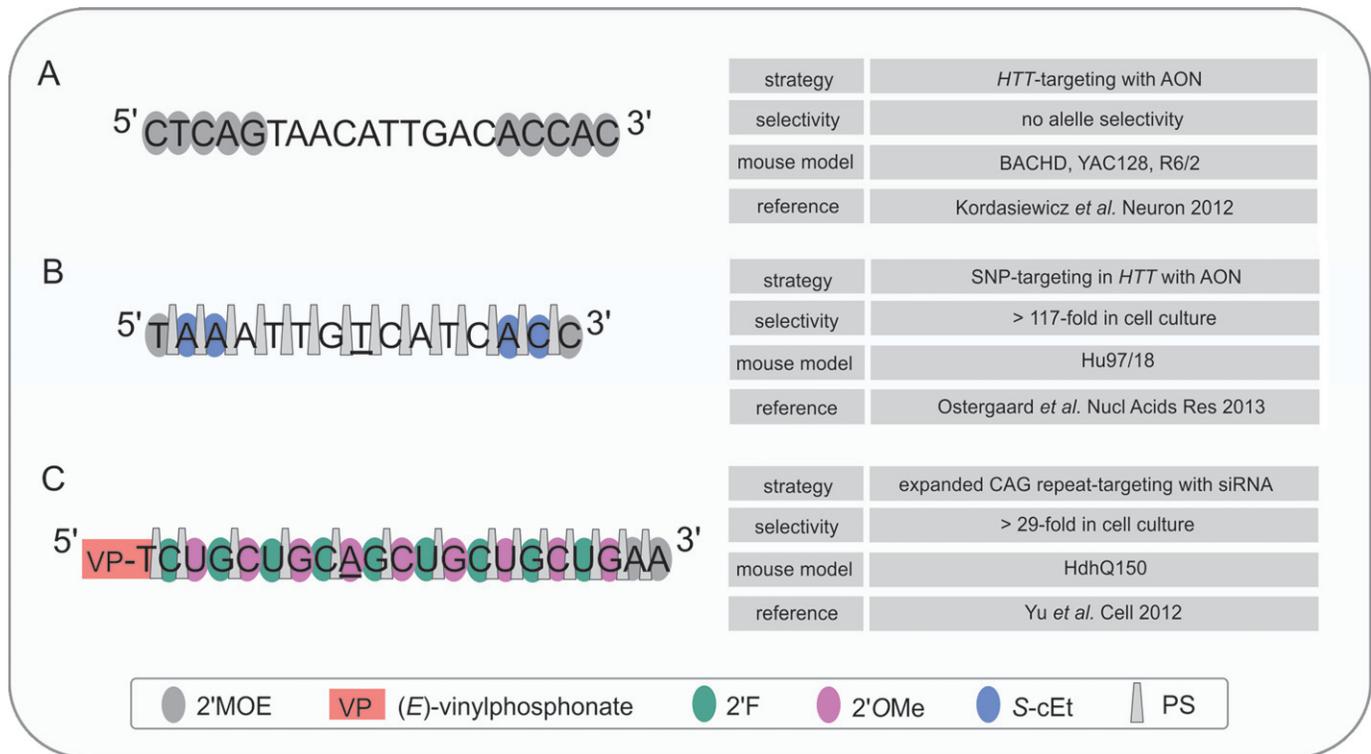


Figure 3. The most advanced ON-based tools for silencing the *HTT* gene tested in HD mouse models. The sequences with a schematic representation of chemical modification patterns are given for three selected ON-based silencing tools (A, B, C) tested as potential therapeutics for HD. (A) represents the non-allele-selective approach for *HTT* silencing, while (B) and (C) are designed to preferentially target the mutant allele by the SNP-targeting strategy (B) or by CAG-targeting (C) (nucleotides essential for the selective activity of these ONs are underlined). (A) and (B) are AONs that activate RNase H for transcript degradation, while (C) is ss-siRNA that activates an RNAi-based mechanism. In the experiments in HD mouse models, all ONs were delivered by intraventricular infusion. See the text for more details.

tinguish between the normal and mutant alleles of the causative gene. It is advantageous if the SNP is highly heterozygous in the population and/or is associated with the expansion mutation. This configuration allows the design of a single ON inhibitor for a group of patients carrying such a variant. The therapeutic ON is designed to form a canonical base pair with the SNP variant present in the mutant allele, which triggers transcript cleavage and degradation, whereas the mismatch present in the interaction with the normal allele should render it resistant to cleavage.

Design rules for the SNP-targeting ONs that were described for siRNAs refer mainly to mismatch type and localization (181,182). The central positioning of a purine-purine mismatch is recommended in the interaction of the antisense strand of the siRNA with the normal transcript. However, the sequence flanking the SNP variant may strongly influence the silencing efficiency and selectivity. Therefore, the design rules are rather rough guidelines, and often a set of siRNAs needs to be tested to find efficient and allele-selective inhibitors.

In the majority of studies that used SNP-targeting to silence the polyQ disease genes, the ONs were designed to function using the RNAi pathway (Table 2, 'Polymorphism site-targeting'). The first studies of this type were performed using cellular models of SCA3 (183,184). The targeted SNP is located at the 3' end of the CAG repeat tract, and its G and C variants are strongly associated with the normal and

mutant *ATXN3* alleles, respectively (43,185). The designed siRNAs included strong G-C pairing in the interaction with the mutant allele and a strong G-G mismatch in the interaction with the normal allele, which allowed for highly preferential mutant allele silencing (179,183,184). The SNP-targeting strategy was also tested using shRNA in rat and mouse models of SCA3 (186,187). shRNAs were delivered in LV vectors by injection to the striatum. Two months after the injections, a significant decrease in the formation of disease-associated inclusions and the correction of neuronal dysfunction was observed in the rat model (186). The substantial reversal of the neuropathological phenotype was also observed in a severely affected mouse model (187). The SNP site was also targeted in a cellular model of SCA7 (188). The exogenous *ATXN7* transcripts containing different SNP variants were selectively targeted by siRNAs expressed from pri-miRNA-based hairpin constructs.

SNP-targeting was most extensively investigated for HD, for which approximately 10 different SNPs were targeted to silence mutant *HTT* expression. The relatively long sequence of the *HTT* gene gives the opportunity to identify many SNP variants in patients' cells (189). High allele-discriminatory properties of siRNA were observed for two localizations of mismatch with the target: the central and 16th nt positions (counting from the 5'-end of the antisense strand) (179,190–194). In a comprehensive study, five allele-selective siRNAs were designed for common variants

of three SNPs to cover approximately three-quarters of the HD population (193). Furthermore, RNase H-activating SNP-targeting AONs were described by the Hayden group (195) and the Seth group (196) as selective inhibitors of mutant *HTT* expression. In the first study, the most efficient AONs were PS substituted 19-mers with nine DNA residues in the gap and five *S*-constrained-ethyl (*S*-cEt) motifs on each wing (195). The AONs were injected into the striatum of animals from two HD mouse models, in which different SNP variants were identified in the transgenes. The expression of mutant human *HTT* was efficiently silenced only in the BACHD model, in which the targeted SNP variant was present and the selectivity of silencing was assessed to be ~5-fold (195). More recently, the researchers from Isis Pharmaceuticals in collaboration with the Hayden group demonstrated the power of chemistry in designing an AON that targeted the same SNP variant with >100-fold selectivity in suppressing mutant huntingtin (196). Such a dramatic improvement in allele selectivity was achieved by iterative redesigning of the specific chemical modification pattern in AON, which included the base, backbone and sugar modifications. The best selectivity was achieved with a 15-mer containing 2'MOE, *S*-cEt and PS modifications (Figure 3B). This ON was tested in a new humanized mouse model of HD (197), and efficient *HTT* silencing across brain tissue was reported, together with high tolerability for AON administration.

CAG repeat-targeting

Allele-selective CAG repeat-targeting takes advantage of differences in the length of the repeat tracts in normal and mutant alleles. This approach is challenging because typical ON-based silencing tools are relatively short, and their binding sites are present not only in the mutant transcript but also in the normal allele and numerous other transcripts containing CAG repeat tracts. Furthermore, transcripts containing CUG repeats may be targeted by the sense strand of siRNA duplexes composed of CAG and CUG repeat strands. There are ~200 and ~100 mRNAs in human cells that contain repeat tracts composed of at least six units of CAG and CUG repeats, respectively (198). Due to this fact, it was thought that CAG repeat-targeting strategy cannot be selective enough to be used for therapeutic purposes. This pessimistic scenario was realized when RNAi triggers composed of fully complementary CAG/CUG repeats, shRNA (162) and siRNA (199,200) were used, which resulted in the non-selective downregulation of normal and mutant transcripts. These ONs activated the typical RNAi pathway, i.e. the mechanism of specific transcript cleavage, which turned out to be indiscriminatory for longer and shorter repeat tracts. However, recent studies have shown that high selectivity for mutant allele silencing can be achieved, and this approach is now regarded as feasible (Table 2, 'CAG repeat-targeting').

The allele-selective silencing of polyQ genes by repeat-targeting was achieved by different types of ONs, which reduced the mutant protein level without transcript degradation. The first inhibitors of this type were chemically modified translation blockers described by the Corey group (201). The most efficient inhibitors were CTG PNA

oligomers conjugated with lysine residues, which showed >6-fold selectivity for mutant huntingtin and ataxin-3 inhibition. In further studies, similar effects were obtained with ONs modified with LNA, cEt, carba-LNA, MOE-2'F-PS, 2'OMe-PS (202) and morpholino oligomers (179). These extensively chemically modified oligomers are thought to act as efficient blockers for the translation of the mutant polyQ protein (Figure 4).

Furthermore, various siRNAs targeting CAG repeat expansion were described, and some of them showed significant preference in the silencing of mutant alleles (Table 2, 'CAG repeat-targeting'). The idea of Corey's group was to weaken the siRNA-target interaction by introducing base substitutions to the siRNA, resulting in one or more base mismatches with the target sequence (199). The substitutions were of different types, and in the most selective siRNAs, they were located in the central region. The same effect was obtained by our group by placing base substitutions in the 3' portions of the CUG repeat strands (200). In our design, the wobble base pairs were formed in the interaction of the siRNA antisense strand with its CAG repeat target. In both siRNA designs, the formation of mismatches with the target resulted in miRNA-like activity of these siRNAs (199,200,203). The CAG/CUG miRNA-like siRNAs were first described as an allele-selective tool for HD (199,200) and soon after for SCA3 (204).

Researchers from Isis Pharmaceuticals developed a pattern of chemical modifications that was introduced to single-stranded siRNAs (ss-siRNAs) to ensure their high stability and activity *in vivo* (205). The same pattern was used for the efficient inhibition of huntingtin expression by Corey *et al.* in collaboration with Isis (206). The ss-siRNAs composed of CUG repeats contained both base substitutions in the central region of ON, generating mismatches with targeted CAG repeats, as well as a specific pattern of 2'F, 2'OMe, PS and 2'MOE modifications (Figure 3C). Some ss-siRNAs showed high allele selectivity in silencing mutant huntingtin expression in HD fibroblasts and were successfully tested in a HD mouse model (206). More recently, similar ONs were tested for *ATXN3* silencing in SCA3 fibroblasts, and allele selectivity was also achieved (207). Interestingly, the CAG repeat-targeting ss-siRNAs tested as RNAi-activating inhibitors of ataxin-3 translation were also shown to be RNAi-independent activators of the alternative splicing of the *ATXN3* transcript, resulting in CAG repeat skipping.

Most recently, we have developed self-duplexing siRNAs (sd-siRNAs) as another type of allele-selective CAG repeat-targeting ONs for HD (208). The CUG repeat single strands to which one, two or three U to A or U to G substitutions were introduced in either the central or 3' region formed guide-strand-only duplexes functioning as miRNAs. In that study, the first repeat-targeting shRNA showing allele selectivity was also described (208).

CAG repeat-targeting siRNAs are not typical RNAi triggers, and their silencing mechanism, as well as basis for allele selectivity, needs further investigation. The involvement of the AGO2 and GW182 proteins in the silencing mechanism has already been established (203). The hairpin structure likely formed by the expanded CAG repeats in cells is not stable enough to resist the attack by RISC

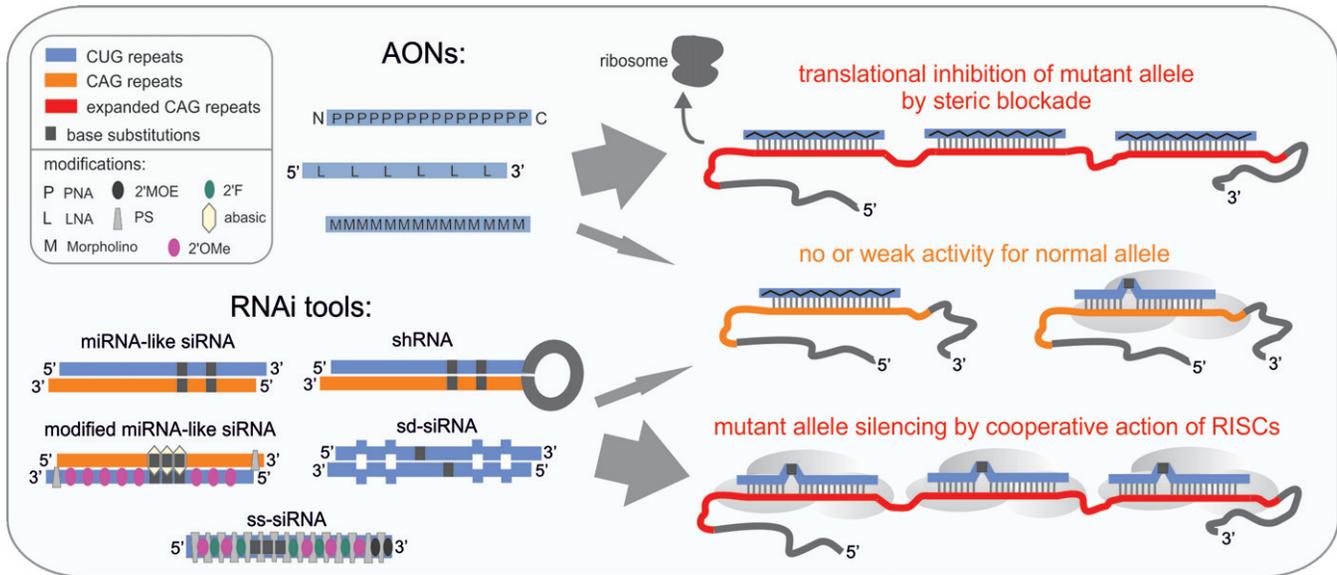


Figure 4. The variety of CAG repeat-targeting ON-based tools showing preferential activity for the mutant allele. On the left side, various CAG repeat-targeting ONs and oligomers are presented. These molecules include a group of AONs, PNA and morpholino oligomers, as well as LNAs and RNAi-based tools including siRNAs, which are delivered as synthetic RNAs or expressed as shRNAs in cells. Specific chemical modifications, as well as the positions of base substitutions, are marked (see figure legend in the left upper corner). In some ONs, the CAG strand must have been included, while other ONs are composed of the CUG repeats only. On the right side, the interactions of ONs with CAG tracts in normal (marked in orange) and mutant alleles (marked in red) are presented. Only the binding of several ONs to the expanded CAG repeat tract in mRNA results in efficient gene silencing. The major mechanism is translational inhibition, which may occur by a steric blockade formed by AONs or by the RISC machinery recruited by siRNAs. See the text for more details.

(47,199,200,209). The molecular basis of allele selectivity is thought to result from the cooperative action of multiple RISCs residing on expanded CAG repeats (203) (Figure 4). The single RISC bound to shorter repeats is easily stripped by translating ribosomes. This result also explains the minimal sequence-specific off-target effects in repeat-targeting, which was previously considered a serious issue.

CELLULAR AND RODENT MODELS USED TO EVALUATE THE ON-BASED THERAPEUTIC TOOLS

Cellular models of polyQ diseases are valuable in the initial screening of ONs. Fibroblast cells derived from polyQ disease patients are a very convenient model that is extensively used in evaluating the allele-selective approaches. These cells contain endogenous mutations, express both normal and mutant alleles and are relatively easy to culture and transfect. Even in testing the therapeutic reversal of the polyQ pathogenic hallmarks, fibroblasts may be a useful model, as some pathogenic events occur in these cells (52,210,211). Nevertheless, in recent trend, cultured neuronal cells are being used. These cells are more adequate, as they better represent brain cells, which should be mainly targeted in therapeutic treatment. The relevant neuronal cells may be derived from brain tissue of polyQ rodent models. Human neuronal cells may be obtained from patient fibroblasts by their reprogramming to induced pluripotent stem cells (iPSCs), followed by differentiation to neurons. The first example of the use of iPSC-derived neurons for modeling polyQ disorders was described in the study of SCA3 pathogenesis (212). In the most extensive study published to date, a set of iPSCs obtained from HD patient-derived

fibroblast cells was generated and analyzed in the context of pathogenesis (213). Several studies on polyQ disorders were performed to characterize the iPSC models and the neuronal cells derived from them (214–219). With regard to testing therapeutic approaches, the cultured mouse neurons, as well as human neuronal cell models derived from embryonic stem cells and iPSCs, were used for the validation of specific polyQ protein-targeting strategy for HD (145). These cells were also used in cell-replacement strategies after their genetic correction and differentiation into neuronal cells (220–222). The use of neuronal cell culture models is anticipated to increase rapidly in the coming years, both for the studies of pathogenesis, as well as for testing therapeutic drugs.

The *in vivo* testing of ONs targeting polyQ diseases was performed mainly in transgenic rodent models. The experience gathered in testing ONs in rodent models of HD, SCA3 and SCA1 can be useful in the design of strategies for other polyQ diseases. There is a variety of models available that differ in their genetic and phenotypic characteristics and are used based on their validity for different approaches (17,223,224). PolyQ rodent models differ in the severity of their phenotypes, which depend on the specific features of the mutant exogene, mainly the length of the whole exogene and its repeated sequence, as well as its expression level. There are important issues to consider in testing the allele-selective silencing of causative genes *in vivo*. Due to sequence differences between rodent and human orthologs, the designed ONs are often specific only for human transgenes (186,187). A model best suited for testing the allele selectivity of targeting should contain two alleles of full-length human sequences, the normal version and the mu-

tant with the pathogenic repeat tracts, as well as the relevant SNP variants. However, such a model might present a mild phenotype; therefore, the simultaneous use of a model with a faster and stronger phenotype may be required. Most of the full-length HD models contain only mutant transgenes and include knock-in types with chimeric sequences under mouse promoters or transgenic types with full-length human *HTT* (17). Recently, a transgenic mouse model of HD with two human *HTT* alleles (containing 18/97 CAG repeats and a human promoter) was created (197) and used in the successful testing of AON in an allele-selective strategy (196) (see the ‘SNP-targeting’ section). The development of new cellular and mouse models is underway for better evaluation of ON-based therapeutic strategies, and models of larger mammals are also under investigation (225–227).

COMPARISON OF DIFFERENT ON-BASED STRATEGIES

In the harnessing of ON-based tools for therapy, a number of important issues still need to be addressed. The high efficiency of ONs is required, which depends on their delivery, uptake and stability. Low doses and appropriate delivery are needed to prevent toxicity. Two strategies are being tested to ensure long-lasting activity of ONs: (i) incorporation of chemical modifications to increase ON stability and (ii) delivery of silencing reagents in genetic vectors (compared in Table 3). These strategies differ in the design of the relevant therapeutic tools and in the obstacles that need to be overcome in pre-clinical testing.

Design

Various features determined by the nucleotide sequence of the ON are important for its activity. The extensive relevant information was gathered for siRNAs and used to establish rules for effective siRNA design (228). The guidelines for siRNAs involve their nucleotide composition, their duplex thermodynamics and the structure of the duplex ends (229–231). The stable structure formed by the siRNA guide strand or AON (232), as well as the stable structure of the target sequence (233–235), reduces the efficiency of transcript inhibition. However, some AONs were shown to be more sensitive to target structure than siRNAs (236), and finding active AONs required more effort, i.e. a larger set had to be tested. The siRNA hybridization with the target is facilitated by RISC, which contains helicase activity, but it is likely that the ability of AON to find the target sequence is also stimulated by yet unknown proteins (237).

Another aspect of ON design is the selection of the chemical modification pattern. The AONs require suitable chemical modifications for activity. Chemical modification not only increases the biological stability of the AON and the efficiency of its hybridization with the target sequence, but may also be critical for its effective delivery and low toxicity. These issues will be discussed in the following subsections. The variety of chemical modifications introduced into ON include internucleotide bonds (e.g. PS, boranophosphate), sugar units (e.g. 2'OMe, 2'OMOE, 2'F and LNA) and nucleobases (e.g. 5-bromouracil and diaminopurine). PS linkages were applied to the first generation of RNase

H-activating AONs, and they improved their cellular stability and increased their binding to serum proteins *in vivo*. However, the efficiency of PS-modified AONs was not satisfactory due to decreased affinity for the target sequence and the need to use high doses, which often resulted in toxic effects. In the second generation of AONs, the modifications of the ribose moiety increased AON stability and its binding to the target sequence, which resulted in higher efficiency of those inhibitors (238,239). LNA modification, the representative of bicyclic sugar modifications, improves nuclease resistance, decreases immune stimulation and strongly increases binding affinity. The development of 2'MOE-modified AONs by Isis was crucial for the improvement in potency in the second-generation AONs. Furthermore, the efficiency of siRNAs was increased by their appropriate chemical modifications (240–242). The central regions of RNaseH1-activating AONs and AGO2-activating siRNAs are generally less extensively modified, as cleavages triggered by these enzymes are very sensitive to the modification status of the central nucleotides. PS, 2'F and 2'OMe modifications are well tolerated by RNase H and AGO (Figure 3). The unmodified siRNAs outperform first-generation AONs in their efficiency in cell culture experiments, but the second-generation AONs are as active as siRNAs (237).

Some AON modifications are still more drastic and have the sugar-phosphate backbone replaced by a peptide (PNA) or morpholino moiety. These oligomers are very resistant to cellular nucleases, and their activity results from strong binding to a complementary sequence in the transcript. They do not trigger transcript cleavage but form a blockade for translation or redirect splicing. Thus far, testing this alternative splicing modulation by morpholino oligomers is well advanced for disorders such as Duchenne muscular dystrophy (243).

The gene silencing reagents are very often expressed in cells from a genetic vector. However, this approach is applicable to RNAi tools only, as they are efficient as non-chemically modified RNAs. These gene inhibitors include shRNAs and shmiRs (pri-miRNA-based constructs) (244,245), which are processed in cells to short RNAi triggers by the miRNA biogenesis machinery. Typical shRNA were found more efficient than shmiRs (246), but they also exerted more toxic effects than shmiRs, what was directly compared *in vivo* in HD mouse model (247). Several types of viral vectors were used to deliver shRNA and shmiR expression cassettes to target polyQ-related gene expression: AAV, AV and LV (Table 2).

The increased understanding of the potential roles of mutant transcripts in the pathogenesis of polyQ diseases prompts researchers to develop therapeutic strategies leading to the elimination of toxic RNAs. AGO- and RNase-H-activating ONs (compared in Table 4) are typically designed to induce transcript degradation. In these cases, the level of toxic RNA and protein components are reduced in the cell. However, some of the CAG repeat-targeting reagents were described to decrease the mutant protein only, i.e. PNA and morpholino oligomers, as well as RNA duplexes acting as miRNAs. These reagents, however, bind to repeat tracts in the mRNA to induce translational inhibition, and this mechanism may diminish RNA toxicity, e.g. by competing

Table 3. Comparison of chemical and genetic approaches for ON delivery

	Chemically modified ONs (AONs and siRNA)	Genetic vectors (viral)
Design	<ul style="list-style-type: none"> • Variety of chemical modifications available • The chemical modification pattern must be carefully optimized for high stability, efficiency and lack of toxicity • Can be obtained by specific ligands or adaptors 	<ul style="list-style-type: none"> • Expression level can be determined by the choice of the promoter • Heterogeneous cleavage of pri- or pre-miRNA-based constructs generates isomiRs, which is considered a drawback • Can be obtained by vector tropism
Neuronal tissue specificity		
Distribution in brain	<ul style="list-style-type: none"> • Free uptake by neuronal cells when delivered intra-CNS 	<ul style="list-style-type: none"> • Brain transduction observed for some vectors in rodents and non-human primates but still less dispersed compared to synthetic ONs • Possibly long-term
Lifespan after delivery	<ul style="list-style-type: none"> • Much higher than for unmodified ONs but still transient • Require repetitive administration 	<ul style="list-style-type: none"> • Appropriate for permanent treatment • Dosage control is more problematic
Safety	<ul style="list-style-type: none"> • Transient activity may be regarded as an advantage concerning the safety • Direct control of dosage 	<ul style="list-style-type: none"> • Possible mutagenesis and immunogenicity
Other	<ul style="list-style-type: none"> • Requires large-scale chemical synthesis 	<ul style="list-style-type: none"> • Difficulties in large-scale manufacturing

with specific cellular proteins for the interaction with the CAG repeat expanded tract.

Off-target effects

The required efficiency of gene inhibition is usually achieved by an excess of ON over the target. This approach raises specificity problems because in human transcriptomes, there are multiple sequences that are partially complementary to the ON sequence. Some of these unintended interactions are productive, causing sequence-specific off-target effects. The AONs are considered more selective than RNAi tools. The RNase H cleavage mechanism is very sensitive to base mismatches, and only one, two or at most three mismatches are tolerated. The AGO-mediated silencing mechanism is more tolerant for siRNA-target mismatches, as these are typically present in miRNA-mRNA interactions. For most miRNAs, base-pairing in the seed region (nucleotides 2–8 from the 5' end of short RNA) is crucial for activity (248,249), and therefore, complementarity in this region is mainly used for predictions of potential off-targeting by siRNA (250,251). The siRNA designs aimed at reducing off-target effects (252,253) included shortening the passenger strand (254,255) and introducing chemical modifications into the sense strand of the siRNA to render it inactive.

Another issue is off-targeting, which is independent of the ON hybridization to transcripts. The competition of exogenous RNAi triggers with endogenous miRNAs for RISC charging may cause alterations in natural miRNA regulatory networks (256,257). Moreover, ONs of various types may interact with other cellular and extracellular proteins and induce toxic effects. Many proteins are involved in the activation of the immunological response to exogenous nucleic acids (258,259). Furthermore, specific immunostimulatory sequence motifs were identified, which should be avoided in the ON design (260–263). This issue is the subject of several reviews (264–266), and relevant information is gathered in the RNAimmuno database (263).

Delivery

The efficient delivery of ONs can be facilitated by their specific chemical modification, conjugation or formulation and

by using an appropriate administration route. Most of the described pre-clinical testing of the ON-based approaches for polyQ diseases focused on the local delivery of inhibitors to the brain. The tests were performed in rodent models of polyQ diseases and in non-human primates. The viral vectors containing shRNA-encoding cassettes, and likewise, synthetic siRNAs or AONs, were either locally injected or infused into CNS (Table 2). The specific injection site was typically the brain region primarily affected by the disease, i.e. the cerebellum in the case of SCA1 and the striatum in HD models. The brain distribution of the injected viral vectors showed relatively high transduction efficiency, although the vector was not dispersed widely and equally throughout the tissue (163,164). In several recent studies, chemically modified AON and ss-siRNA inhibitors without any formulation were infused into cerebrospinal fluid (CSF), which resulted in their more widespread and uniform distribution across the brain (169,196,206). In spite of this success, further research was conducted to enhance the delivery of various types of inhibitors directly to the brain (267,268,269).

Regarding the less invasive systemic or intrathecal delivery, the improved pharmacokinetic properties of ONs may prevent their rapid excretion and increase their uptake. The general barriers after systemic administration are: (i) clearance from blood via the kidney, (ii) degradation by nucleases in plasma, (iii) crossing the capillary endothelium (extravasation to other organs than liver, kidney and spleen), (iv) cellular uptake from the extracellular matrix, (v) escape from endosomes, (vi) reaching a specific cell compartment (270,271). Crossing the blood-brain barrier is the major hurdle for the ONs to be delivered systemically in the case of CNS diseases. In several studies, the methods were tested for the efficient delivery of the ONs to the brain by systemic administration, with the promising example of delivery in exosomes (272,273). Recently, the intra-jugular vein delivery of AAV9 vectors for *HTT* silencing was described by the McBride group (274). In this report, the effective transduction of multiple brain regions and peripheral tissues was demonstrated. This treatment resulted in the reduction of neuropathology in HD mouse models, although it did not prevent motor deficits.

Table 4. Comparison of the mechanisms of gene expression downregulation in terms of their therapeutic utility

	AONs (RNase H-activating)	RNAi
Activity Design	<ul style="list-style-type: none"> • Nuclear and cytoplasmic • Chemical modifications required 	<ul style="list-style-type: none"> • Mainly cytoplasmic • Active <i>in vivo</i> as chemically modified or in genetic vectors • More limited chemical modifications can be introduced • Mainly double-stranded (larger molecules) • Design rules are well established
Efficiency	<ul style="list-style-type: none"> • Can be more extensively chemically modified than siRNA • Single-stranded (smaller) • Relatively large set of ONs must be tested to find active ones • Lower or equal to that of siRNAs in cell culture, depending on the chemical modification pattern used • Better efficiency <i>in vivo</i> than siRNAs • Might be more sensitive for target structure than siRNAs • It is unknown whether it finds and hybridizes to targets unassisted 	<ul style="list-style-type: none"> • Very high efficiency in cell culture, due to typical catalytic mode of action • Moderate sensitivity for target structure • RISC complex facilitates finding targets and hybridizing
Off-target (sequence specific)	<ul style="list-style-type: none"> • Moderate risk 	<ul style="list-style-type: none"> • High risk as activity is based on ‘seed’ pairing • Improvement in siRNA design overcame some off-targeting • Heterogeneous cleavages in shRNAs and shmiRs generate unwanted siRNAs • Possible disturbance of the endogenous miRNA pathway
Off-target (non-sequence specific)	<ul style="list-style-type: none"> • High risk of immunostimulatory responses through interactions with toll-like receptors (TLRs) 	
Strategy development	<ul style="list-style-type: none"> • Generally more advanced in clinical trials, as developed since the late 1970s • For polyQ disorders tested in last few years 	<ul style="list-style-type: none"> • Activation of pathways by dsRNAs • Developed since 2000s • Extensively tested in cell culture and rodent models of polyQ diseases

Table 5. Comparison of allele-selective approaches for polyQ diseases

	SNP-targeting	CAG-targeting
Available tools	<ul style="list-style-type: none"> • siRNAs/shRNAs 	<ul style="list-style-type: none"> • siRNAs/shRNAs (miRNA-like, sd-siRNA, ss-siRNA)
Design	<ul style="list-style-type: none"> • AONs (RNase H – activating) • Requires the identification of SNP variants 	<ul style="list-style-type: none"> • AONs (blockers of translation, splicing modifiers) • The RNAi-based approach requires the positioning of base mismatches with target
Efficiency of silencing	<ul style="list-style-type: none"> • May be influenced by the sequence in the region of the SNP variant 	<ul style="list-style-type: none"> • Comparable with specific sequence-targeting
Selectivity of silencing	<ul style="list-style-type: none"> • May depend on the types of SNP variants present in the mutant and normal alleles 	<ul style="list-style-type: none"> • Based on multiple binding sites in the expanded repeat tract • Depends on the difference between the repeat tract length in the normal and mutant alleles
Off-targeting	<ul style="list-style-type: none"> • Standard risk 	<ul style="list-style-type: none"> • Risk of targeting numerous transcripts containing normal CAG repeat tracts

CLINICAL ASPECTS

Still, some limitations must be overcome for therapeutic use of ONs. They are mainly due to shortcomings in the efficiency and safety of ONs in clinical trials. This comprises problems regarding efficient ON delivery to targeted tissues without causing any adverse effects.

There are additional issues to be considered while taking ONs from pre-clinical testing to human trials for the development of treatments available to patients. The basic evaluation of ON potency as a therapeutic agent is the analysis of targeted gene downregulation. For a more complete assessment of ON properties *in vivo*, specific analyses need to be performed to observe the potential reversal of selected markers of the molecular pathogenesis of polyQ diseases. From a clinical point of view, it is challenging to develop methods for the validation of therapeutic benefits

that would not be harmful and could be used in human trials (275,276).

The question remains whether it is sufficient to suppress mutant gene expression locally in the regions where the disease primarily develops or whether it is necessary to target either the whole CNS or also the peripheral tissues. As significant dysfunctions in the periphery were reported in patients and in mouse models of HD and other polyQ disorders (277–282), systemic delivery seems to be the best option, provided it also successfully delivers ONs to the CNS.

Another important question is at what stage of disease is it necessary to intervene and silence the gene expression so the pathology will completely, or at least partially, reverse? Treatment at the late stage of disease poses the risk that some toxic events will become irreversible. Results from an inducible mouse model of SCA1 indicate that the pathogenic effects of the mutation in ataxin-1 accumu-

late starting from postnatal development (33). The recovery from disease was observed in several inducible rodent models when the mutant transgene was turned off at an early or middle stage of the polyQ disease (67,68,70,283,284). Nevertheless, full recovery was not achieved when the transgene expression was halted at a late stage of the disease (68,284). Similar results were obtained with ON-based silencing of the mutant transgenes in rodents (Table 2), but partial recovery even from a severe phenotype was reported (187). Considering the human lifespan, it is preferable that a single administration of a drug provides long-term mutant gene silencing. In this context, the result obtained by Cleveland *et al.* is very promising. Using HD mouse models, the authors demonstrated that AONs intraventricularly infused into the CSF caused phenotypic recovery lasting much longer than mutant gene downregulation (169). This finding, named ‘huntingtin holiday’ (285), is one of the major breakthroughs in HD research and a very good message for HD patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this review, we gathered the existing information and discussed the progress of research aimed at developing therapeutic approaches for polyQ diseases. We presented and compared the two most advanced gene inhibition strategies, antisense and RNAi, which show many similarities, including their therapeutic potential.

The AONs directing transcript degradation by RNase H have been explored as potential drugs since the late 1970s, whereas RNAi has been used for post-transcriptional gene silencing since the early 2000s. Therefore, AON tools are more advanced in pre-clinical and clinical testing, but the experience gathered in testing AONs is in many respects useful for the development of RNAi-based strategies (286). This advantage refers to the general administration, distribution, metabolism and excretion characteristics of ONs, which can be relevant in many ways for both the AONs and siRNAs (259,287). As a result, rapid progress in advancing siRNAs to clinical trials is expected in coming years.

At this point, it is difficult to say which ON-based therapeutic strategy is best suited for polyQ diseases. The existing strategies offer great opportunities for therapy but also have limitations. The allele-selective SNP- and CAG repeat-targeting strategies (compared in Table 5) are regarded as more safe. CAG repeat-targeting strategy, which was considered some years ago a ‘mission impossible’, turned out to be not only feasible but also very promising according to the first *in vivo* studies in mouse models of HD (206). However, a full assessment of the CAG repeat-targeting strategy needs to be accomplished. The potential advantage of this strategy is its universality, as one drug could possibly be used to treat most of the polyQ disorders.

The ON-based strategies are more advanced than other strategies capable of interfering with mutant gene expression. ONs may be designed for any transcript and may function by many mechanisms. Other strategies, such as genome editing, that are aimed at repairing a mutant gene or preventing its transcription are very attractive but still far from clinical applications. The clinical application seems to be still more distant for the cell therapy of polyQ diseases.

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