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Therapy for dominant inherited diseases by Allele-Specific RNA Interference: Successes and Pitfalls

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Running Title: Therapy by allele-specific RNAi

Abstract: RNA interference (RNAi) is a conserved mechanism for post-transcriptional gene silencing mediated by messenger RNA (mRNA) degradation. RNAi is commonly induced by synthetic siRNA or shRNA which recognizes the targeted mRNA by base pairing and leads to target-mRNA degradation. RNAi may discriminate between two sequences only differing by one nucleotide conferring a high specificity of RNAi for its target mRNA. This property was used to develop a particular therapeutic strategy called “allele-specific-RNA interference” devoted to silence the mutated allele of genes causing dominant inherited diseases without affecting the normal allele. Therapeutic benefit was now demonstrated in cells from patients and animal models, and promising results of the first phase Ib clinical trial using siRNA-based allele-specific therapy were reported in Pachyonychia Congenita, an inherited skin disorder due to dominant mutations in the *Keratin 6* gene. Our purpose is to review the successes of this strategy aiming to treat dominant inherited diseases and to highlight the pitfalls to avoid.

Keywords: Allele-specific silencing, Dominant inherited diseases, Pitfalls, RNA interference, Single nucleotide substitution, Gene-based therapy

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing first characterized in plants as an anti-virus or anti-transgene defense mechanism [1]. Thereafter, RNAi was shown to induce gene-selective inactivation in a wide variety of eukaryote cells, including mammalian cells [2, 3]. RNAi is classically induced by chemically synthesized small interfering RNA (siRNA) of 19-21 nucleotides with two 3' overhanging nucleotides or by short hairpin RNA (shRNA) synthesized from RNA polymerase III promoters and converted into siRNA by the endogenous cell machinery. The siRNA recognizes the targeted messenger RNA (mRNA) by base pairing of the seed region located from the second to the seventh nucleotide of the siRNA antisense strand referred to as "guide strand". The annealing directs the cleavage of the mRNA after the tenth nucleotide of the siRNA guide strand [4] in the RNA-induced silencing complex (RISC). A fascinating aspect of RNAi stems from its high specificity for the targeted mRNA sequence. This property led to the development of the allele-specific RNAi (AS-RNAi) as a therapeutic strategy for dominant inherited diseases by targeting the mutated allele without affecting the wild type (WT) allele. Proof of concept for a therapeutic use of AS-RNAi was first established in 2002 against dominant mutations of oncogenes or tumor suppressors [5, 6] (Table **S1**) and, thereafter, rapidly achieved for monogenic dominant inherited diseases [7-10]. During the last decade, therapeutic benefit was also demonstrated in patient-derived cells and animal models, and promising results of the first clinical trial were reported [11]. In this review, we aim at reviewing the state of the art of this strategy applied to treating dominant inherited diseases. The review of the literature also highlights potential limitations and pitfalls to avoid.

AS-RNAi, a versatile strategy for different types of dominant mutations

The specificity of RNAi-inducing molecules (siRNA and shRNA) led to the development of AS-RNAi for different types of mutations responsible for dominant inherited diseases (Fig. **1**). The majority of the AS-RNAi was developed for silencing of mutated alleles differing from the WT alleles by a single nucleotide substitution (Tables **1**, **2** and **S1**), by targeting directly pathogenic missense mutations or disease-associated single nucleotide polymorphisms (da-SNP) in particular cases of triplet repeat diseases. However, AS-RNAi technology was used in several other molecular contexts (Fig. **1** and Table **S2**). Efficient AS-RNAi was achieved for targeting three-nucleotide deletions [7, 12, 13], multiple nucleotide deletions [14] or substitutions [15-21] and disease-associated splicing isoforms [22-25]. If the nucleotide repeats responsible for triplet repeat diseases are not considered as suitable targets for AS-siRNA [8] because identical target sequences are present in normal and

mutated alleles, Li *et al.* [26] reported effective AS-RNAi against the CAG expansion of the disease-related allele of *Ataxin 3* (ATXN3) using siRNA covering the extremity of the expanded region and its flanking sequence. In that case, WT and mutated sequences differ only by the number of repeated motifs while the target sequence of the siRNA is the same in both transcripts. The different repeat sequence lengths may probably induce structural changes allowing allele-specificity in this particular case. More recently, such AS-silencing was achieved using RNA duplexes targeting exclusively the repeated sequence [27-29] but probably by a “miRNA mimicking mechanism” which does not involve mRNA cleavage. Regardless, these results suggest that allele-specific silencing may be envisaged in diseases with nucleotide expansion even in absence of da-SNP. Nevertheless, we will largely focus this review on the AS-RNAi targeting single nucleotide substitutions.

How to develop AS-RNAi targeting single nucleotide substitutions causing dominant inherited diseases

For targeting single nucleotide substitutions, a perfect AS-siRNA has to achieve effective knock-down of the mutated allele without affecting the normal allele. With this objective, AS-siRNA is designed to fully match the mutated sequence but harbors one mismatch against the WT. The position of the mutated nucleotide in the siRNA, the nature of the mismatch against the WT sequence, and the flanking sequences may influence efficiency and specificity of silencing. Because all these parameters are largely dependent on sequence and structure of a given mRNA, no definitive rules have been established to date for the design of AS-siRNA and development of efficient molecules has relied on empirical testing. Using 19-base-pair siRNA, 19 possible single-mismatched siRNA exist relative to the position of the mutated nucleotide (Fig. 1). Several studies have reported the results from systematic screening for the 19 possible siRNA (Fig. S1) [30-38]. From these studies, central mismatches (from positions 8 to 14) were associated with AS-RNAi with highest specificity at position 10. These data highlight the crucial role of the central region of the siRNA and the fact that AS-RNAi appears more efficient when the siRNA is designed to block the cleavage of the WT allele rather than its annealing. The conclusions from systematic screening may be refined by overall analysis of the 87 single-mismatched siRNA or shRNA reported in the Table S1. The central region proves to be a master region to develop AS-siRNA with the highest specificity reached at position 10 (19 out of 87; *i.e.* 22% of the reported AS-siRNA), followed by positions 9 and 11 (16% each). By adding siRNA mismatched at the position 16, these four siRNA (9, 10, 11, and 16) represent 63% of the already reported efficient molecules. Not only the position but also the nature of the mismatch between siRNA and WT sequences may influence AS-RNAi efficacy with theoretically

purine:purine (pu:pu) mismatches introducing the largest possible destabilization compared to pyrimidine:pyrimidine (py:py) or purine:pyrimidine (pu:py) mismatches. By definition, in this particular case, the nature of the mismatch is fixed by the mutated nucleotide. It is noteworthy that siRNA with single mismatches at positions 9, 10, 11, and 16 have been shown to discriminate mutated and WT alleles whatever the nature of the mismatch (Table **S1**).

With the objective to increase allele-specificity, a second mismatch was introduced in the siRNA or shRNA. In that case, the RNAi-inducing molecule harbors one mismatch relative to the mutated sequence but two against the WT. Twelve successful examples of double-mismatched siRNA are indicated in the Table **S1** using 19-base pair siRNA including 10 cases in which the first mismatch is located at position 9 or 10. In these cases, the most efficient positions for the second mismatch are 12, 13 and 14 and the majority (6 out of 10) introduces a purine:purine mismatch. Nevertheless, introducing a second mismatch does not always increase AS-RNAi efficiency compared to single-mismatched siRNA [39-43].

Huang *et al.* analyzed silencing properties of siRNAs on 230 reporter constructs and confirmed that some positions are more sensitive than others to target a given mismatch [44]. This study and the successful examples now available for 31 distinct mRNA (Table **S1**) suggest a two-step strategy in order to develop AS-RNAi. Single mismatched siRNA at positions 9, 10, 11, and 16 should be privileged as a first screening strategy whatever the nature of the mismatch. In case of low allele-specificity, a second purine:purine mismatch may be introduced at positions 12, 13, or 14 in addition to the first mismatch at positions 9, and 10. These 10 different siRNA sequences (out of the 667 possible single and double-mismatched siRNA) may facilitate the development of AS-RNAi as they represent 65% of the efficient AS-siRNA already reported. This strategy may be associated with the recently developed formulas for calculating allele-discrimination [45] in order to optimize determination of allele-specific siRNA.

Proof of concept of AS-RNAi in patient-derived cells and animal models

Phenotype reversion in patient-derived cells and/or animal models is a crucial step for preclinical development. This was achieved *in vitro* for numerous disease-related genes mainly in patient-derived fibroblasts (Table **1**). Interestingly, induced pluripotent stem cells (iPSCs) derived towards neuronal cells as an Amyotrophic lateral sclerosis model [42] or toward cardiomyocytes as a Long QT syndrome model [46] were recently used to study AS-RNAi in functionally relevant disease models. Validation of AS-RNAi in pertinent

disease-related cells appears particularly important in absence of primary cultures from the affected tissue and iPSC represent probably a model of choice.

In vivo AS-RNAi was reported for the first time in 2003 by Ding and collaborators after co-injection of WT and mutated SOD1 constructs with shRNA in mice [10]. Beyond the first *in vivo* proof of concept, this pioneer study demonstrated the rapid silencing of the mutated allele evidenced 24 hours post-transfection. Afterwards, different types of animal models (transgenic mice or vector-mediated overexpression of mutated transcripts) were developed in order to study the feasibility of AS-RNAi *in vivo* (Table 2). These models confirmed the rapid down-regulation of the mutated alleles [31, 34] and highlighted several important features of *in vivo* AS-silencing. First, early treatment in pre-symptomatic animals is able to prevent the appearance and/or the progression of the disease [21, 47-50]. Second, rescue of phenotype is also possible when treatment is started in symptomatic mice [51]. Third, the therapeutic effect is rapid as evidenced after 2-4 weeks of treatment [50, 51] without evident long-term toxicity [21]. Fourth, a low therapeutic threshold was demonstrated as incomplete reduction of the mutated allele was sufficient for therapeutic benefit [48, 49]. The last point may be of importance for particular mutations for which highly efficient AS-siRNA could not be easily designed.

In addition to these helpful experimental *in vivo* models, therapeutic potential of AS-RNAi was also investigated in Knock-in mouse models expressing disease-causing mutations in the endogenous mouse genes to recapitulate more closely the human heterozygous conditions. This was achieved in a mouse model of the Apert syndrome expressing a *Fgfr2* (Fibroblast growth factor receptor type 2) mutation [20], in two models of neuromuscular disorders due to *Ryr1* (Ryanodine receptor type 1) mutations [19], and in one model of Hypertrophic cardiomyopathy due to a *Myh6* (Myosin heavy chain 6) mutation [52]. Among these, the Knock-in-*Myh6* mice represent the only case of mutation due to a single nucleotide substitution (in the three other models, the mutations come from double or triple substitutions). AS-RNAi in these knock-in models confirmed the safety, the rapidity, and efficacy of this therapeutic strategy to rescue or prevent the phenotype. A low therapeutic threshold was evidenced in the *Myh6* model as a reduction of 28.5% of the mutated allele benefited the mice whereas a reduction of 50% led to a partial rescue in one *Ryr1* model. In addition, AS-RNAi only delayed the cardiac phenotype but was unable to rescue an established cardiomyopathy in the *Myh6* mice and a dissipation of protective effect over time was noticed [52]. These findings that may be gene- and/or disease-specific reveal the importance of functional validation in pertinent Knock-in mouse models for each targeted dominant disease.

Clinical trials in humans

Pachyonychia congenita (PC) is a highly disabling autosomal dominant skin disorder due to mutations in the *Keratin 6* gene (*KRT6A*). The clinical manifestations of PC include painful plantar calluses which have been targeted successfully by AS-RNAi in a clinical trial reported in 2010 [11]. Treatment consisted of 17 weeks of intra-callus injections performed twice-weekly with dose escalation from 0.1 mg to 17 mg of siRNA against the mutated Krt6a mRNA. Clinical improvement with callus regression was noticed from 10 weeks after the first injection and was maintained during a relatively long period of 14 weeks, *i.e.* 7 weeks after the last injection. Despite the spatially and temporally limited effects, which highlight the importance of the delivery method for future trials, this first report of AS-RNAi in human are promising for PC and other dominant inherited diseases.

Pitfall and limitation

Several specific aspects of the AS-RNAi technology, which may limit development of future effective treatments for dominant inherited diseases, require consideration.

- 1) Probably the most important point is that the expected result of this approach is to retain only 50% expression from the spared WT allele of the gene of interest . Consequently, absence of haploinsufficiency needs to be clearly established. In this context, *in vitro* knock-down studies, investigation of heterozygous knock-out animal models when available and analysis of the spectrum of gene mutations and genotype-phenotype correlation in patients are indispensable.
- 2) Another serious limitation is the restriction in the choice for the targeted sequence in the region of the mRNA harboring the mutation. This may limit efficiency of RNAi as all the regions of a given mRNA are not similarly sensitive to RNAi and may restrict efficacy and allele-specific silencing. In other words, AS-RNAi will not be possible in all cases. However, this problem may be overcome if targetable SNP is present on the mutated allele.
- 3) It is important to take into account the possibility that the AS-RNAi molecule can inhibit the translation of the WT allele whereas the quantified WT mRNA level remains unchanged [15]. This may be due to a “microRNA effect” affecting the translation as already described for mismatched siRNA [53]. Consequently, allele-specific properties should not be established exclusively by measuring the mRNA expression level but need to be combined to protein expression and protein activity when possible.

4) A loss of allele-specificity with increased dose of RNAi-inducing molecule [54] or an arrest of protective effect over time may appear [52]. This highlights the importance of the choice of the vector, mode of delivery and tight control of the delivered amount of future therapeutic molecules *in vivo*. In this context, miRNA-based hairpins have been used in order to express AS-RNAi-inducing molecules [25, 39]. This approach may offer an attractive alternative for *in vivo* tissue-specific expression compared to siRNA or shRNA and to avoid potential toxicity of shRNA expression *in vivo* [55, 56].

5) In order to facilitate the screening for AS-siRNA *in vitro*, artificial assay systems with reporter genes have been developed. Using this screening procedure, siRNA maintaining their allele specificity against the *bona fide* mRNA target were identified [15, 30, 33, 36, 57, 58]. However, significant discrepancies have been shown comparing reporter systems and full-length mRNA or even between two different reporter systems [34-37, 39, 43, 59]. Consequently, screening for AS-siRNA on the full-length natural target (endogenous or overexpressed) should be privileged.

6) The best AS-siRNA sequences identified *in vitro* are often incorporated into plasmids or viral vectors to be expressed *in vivo* as shRNA. The shRNAs are known to retain the efficacy and allele specificity of the original siRNA [8, 10] but some divergences may exist between siRNA and the corresponding shRNA [9, 12, 18]. Indeed, shRNA are processed by the endogenous Dicer RNase to generate siRNA. Given that Dicer cleavage site in the shRNA loop is difficult to predict that may introduce one or two-nucleotide differences between the expected and the produced siRNA. Consequently, even if several examples showed that AS-siRNA and AS-shRNA are similarly efficient, it would be important to validate shRNA constructs *in vitro* before starting *in vivo* studies in animals.

7) By definition, the AS-RNAi strategy is a mutation-specific approach. This may be interesting for mutation hot spots concentrated in a reduced number of nucleotides allowing development of common molecular tools for a large cohort of patients. In contrast, this may require personalized therapy by developing specific tools for mutations affecting few patients.

8) Another limitation comes from the study of Jiang and collaborators [52] which stress the necessity to start treatment at a pre-symptomatic stage. In such cases, AS-RNAi could be limited to familial forms of diseases receiving an early genetic diagnosis. Development of preclinical studies in knock-in animal models appears crucial to determine the optimal timing of treatment.

9) Interferon response classically attributed to long double-stranded RNA has not been reported in AS-RNAi to date. Nevertheless, some siRNA [60] and shRNA [61] have been shown to induce this response. Consequently,

monitoring a possible interferon response should be included, especially for *in vivo* studies, to avoid misinterpretation of treatment efficacy.

Concluding remarks

Despite of the possible limitations indicated above, the AS-RNAi technology emerged during the last decade as a powerful strategy for dominant inherited diseases. However, some aspects still need to be resolved especially those concerning *in vivo* delivery. Similar to all the siRNA-based therapeutics, using the best vector for delivering the optimal dose will probably be the key bottleneck when systemic delivery is required for widespread dissemination or in contrast for limited expression in one affected tissue. Also, the long-term consequences of chronic stimulation of the RNAi pathway (potential toxicity or immunological side effects as reviewed in [62]) as well as off-target effects need to be carefully investigated. Continuous improvements to overcome these limitations and increase efficacy of RNAi-based therapeutics, including chemical modifications, are occurring and will benefit the AS-RNAi strategy. Furthermore, development of preclinical testing in animal models of dominant inherited diseases are now crucial in order to deepen the specificities of the AS-RNAi approach *in vivo* such as long-term maintenance of allele-specificity. Nevertheless, the proof of concept for AS-RNAi therapy now available for numerous dominant inherited diseases strongly suggests that the promising result of the first clinical trial for Pachyonychia Congenita paves the way for future successful clinical trials.

Conflict of interest

There is no conflict of interest to disclose.

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Figure 1: AS-RNAi targetable mutations. For targeting a single nucleotide substitution, the 19 possible siRNAs are indicated relative to the position of the mutated nucleotide. This specific nucleotide determines the position of the mismatch between the siRNA and the WT mRNA. P10 indicates the siRNA mismatched at position 10. nt: nucleotide. Specific nucleotides of the mutated mRNA are indicated in blue.

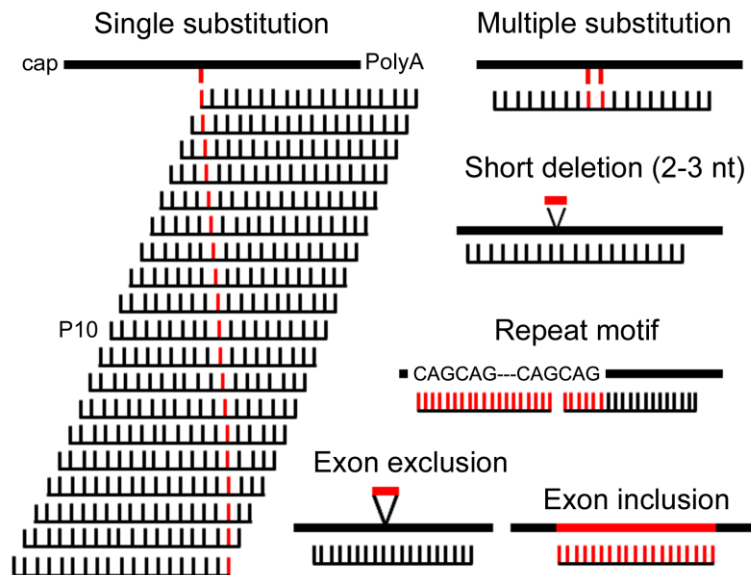


Table 1: Single nucleotide substitutions targeted by Allele-specific RNAi in patient-derived cells

Gene	Disease	Targeted change	Model	Phenotype reversion	References
ACVR1	FOP	p.R206H	Primary MSC	yes	[63]
ACVR1	FOP	p.R206H p.G356D	Lymphoblastoid cells	yes	[64]
ATXN1	SCA1	da-SNP	Fibroblast	-	[65]
ATXN3	SCA3	da-SNP	Fibroblast	-	[65]
ATXN7	SCA7	da-SNP	Fibroblast	yes	[54]
COL1A2	OI	da-SNP	Bone-derived cells	-	[66]
COL3A1	vEDS	p.G252V	Fibroblast	yes	[35]
COL6A1	UCMD	p.G284R	Fibroblast	yes	[43]
GNE	Sialuria	p.R266Q	Fibroblast	yes	[67]
HTT	HD	da-SNP	Fibroblast	-	[68]
HTT	HD	da-SNP	Fibroblast	-	[58]
HTT	HD	da-SNP	Lymphoblastoid cells	-	[69]
HTT	HD	da-SNP	Fibroblast	-	[65]
HTT	HD	da-SNP	Neural stem cells	yes	[48]
KCNH2	LQT2	p.A561T	iPSC-derived cardiomyocyte	yes	[46]
KRT12	MECD	p.L132P	Corneal epithelial cells	-	[70]
PSEN1	AD	p.L392V	Fibroblast	yes	[32]
TARDBP	ALS	p.M337V	iPSC-derived neural stem cell	-	[42]
TGFBI	LCDI	p.R124C	Corneal epithelial cells	-	[38]

GNE: UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, HTT: Huntingtin, HD: Huntington's disease, ATXN1: Ataxin 1, ATXN3: Ataxin 3, ATXN7: Ataxin 7, SCA1: Spinocerebellar ataxia 1, SCA3: Spinocerebellar ataxia 3, SCA7: Spinocerebellar ataxia 7, TARDBP: TAR-DNA Binding protein (TDB-43), ALS: Amyotrophic lateral sclerosis, PSEN1: Presenilin 1, AD: Alzheimer's disease, ACVR1: Activin A receptor type 1, FOP: Fibrodysplasia ossificans progressive, COL1A2: Collagen type I alpha 2 chain, OI: Osteogenesis imperfecta, COL3A1: Collagen type 3 alpha 1, vEDS: Vascular type of the Ehlers-Danlos syndrome, COL6A1: Collagen type VI alpha 1, UCMD: Ullrich congenital muscular dystrophy, KCNH2: Potassium channel voltage-gated subfamily H member 2, LQT2: Long QT syndrome type 2, KRT12: Keratin 12, MECD: Meesmann epithelial corneal dystrophy, TGFBI: TGFβ-induced gene, LCDI: Lattice corneal dystrophy type I, da-SNP: disease associated-Single Nucleotide Polymorphism, iPSC: induced pluripotent stem cell, MSC: mesenchymal stem cells.

Table 2: Single nucleotide substitutions targeted by Allele-specific RNAi in animal models

Gene	Disease	Targeted change	Model	Comment	Phenotype reversion	Ref.
ATXN3	MJD/ SCA3	da-SNP	Lentivirus-mediated expression of mutant and shRNA in rat brain	Single injection in striatum	Reduce the formation of histological lesions 8 weeks post-injection	[47]
ATXN3	MJD/ SCA3	da-SNP	Lentivirus-mediated expression of human full length mutant ATXN3 and AS-shRNA	Single intracerebellar injection in pre symptomatic mice	Prevent pathological lesions, neuronal loss, and motor impairments 10 weeks post-injection	[50]
ATXN3	MJD/ SCA3	da-SNP	Lentivirus-mediated expression of shRNA in transgenic mice expressing the human mutant	Single intracerebellar injection in pre symptomatic mice.	Decrease in histological lesions, prevent neuronal loss and alleviate motor impairments 10 weeks post-injection	[51]
HTT	HD	da-SNP	Lentivirus-mediated expression of shRNA in transgenic mice expressing full-length human mutant or in rat expressing human reporter construct	Single injection in striatum. Mice injected with shRNA against the da-SNP or the WT nucleotide	Decrease in formation of histological signs in rats 8 weeks post-injection	[48]
KRT6A	PC	p.N171K	Bioluminescence mouse model	Single intradermal injection of siRNA and reporter constructs in footpad. Live imaging at 24, 48 and 72h. Rapid down expression (24h post injection)	-	[31]
KRT9	EPPK	p.R163Q	Bioluminescence mouse model	Single intradermal injection of siRNA and reporter constructs in footpad. Live imaging at 24 hours post-injection	-	[34]
Myh6	HCM	p.R403Q	MHC ^{403/+} knock-in mice	Single injection in thoracic cavity of siRNA-expressing AAV9	No reversion 2 months post-injection in symptomatic mice. Prevent cardiomyopathy in presymptomatic mice (8 weeks post-injection in 1-day-old mice). Arrest of	[52]

					protective effect over time (at 11 months of age).	
SOD1	ALS	p.G93A	Transgenic mice expressing the mutant	Crossing with shRNA transgenic mice	Delayed onset and extended survival	[49]
SOD1	ALS	p.G93A	Co-injection of WT-, mutant- and shRNA-expressing plasmids in mice	Single systemic injection in tail vein. Evaluation in liver 48 hours post-injection	-	[10]
SOD1	ALS	p.G93A	Transgenic mice expressing mutant	Single systemic injection in tail vein of shRNA-expressing AAV6 in 6-week-old presymptomatic mice.	No benefit for onset and progression of motor deficits probably due to insufficient neuronal transduction	[71]

Myh6: Myosin heavy chain 6, HCM: Hypertrophic cardiomyopathy, ATXN3: Ataxin 3, MJD/SCA3: Machado-Joseph disease/Spinocerebellar ataxia 3, SOD1: Superoxide dismutase 1, ALS: Amyotrophic lateral sclerosis, HTT: Huntingtin, HD: Huntington's disease, KRT9: Keratin 9, EPPK: Epidermolytic palmoplantar keratoderma, KRT6A: Keratin 6a, PC: Pachyonychia congenita, da-SNP: disease associated-Single Nucleotide Polymorphism.

Supplementary Table 1: Overview of AS-RNAi targeting single nucleotide substitutions.

Gene	Disease	Targeted change	Silencing molecule	Mismatch position	Model	Type of mismatch	Comment	Phenotype reversion	References
ACVR1	FOP	p.R206H	siRNA	10, 11	j	py:py		yes	[63]
ACVR1	FOP	p.R206H p.G356D	siRNA siRNA	9/14 10/13	j	9 pu:py/14 pu:pu, 10 pu:py/13 py:py		yes	[64]
APP	AD	p.V717I	shRNA	9	f	pu:py			[16]
ATXN1	SCA1	da-SNP	siRNA	10	j	pu:py		-	[65]
ATXN3	MJD/ SCA3	da-SNP	siRNA/shRNA	10	b	pu:pu		-	[8]
ATXN3	MJD/ SCA3	da-SNP	siRNA	7/8 11	a	7 pu:pu 8 pu:pu 11 pu:pu		yes	[8] [26]
ATXN3	MJD/ SCA3	da-SNP	shRNA	11	a,i	pu:pu		yes	[47]
ATXN3	MJD/ SCA3	da-SNP	shRNA	11	e	pu:pu		yes	[51]
ATXN3	MJD/ SCA3	da-SNP	shRNA	11	i	pu:pu		Yes,	[50]
ATXN3	MJD/ SCA3	da-SNP	siRNA	9, 10	j	pu:pu		-	[65]
ATXN7	SCA7	da-SNP	siRNA	16	j	pu:pu		yes	[54]
ATXN7	SCA7	da-SNP	shRNA/miRNA	16	b	pu:py		yes	[39]
CHRNA1	SCCMS	p.S226F	siRNA	10	a	pu:py		-	[9]
CHRNA1	SCCMS	p.S226F	shRNA	9	a	pu:py		-	[9]
COL1A2	OI	da-SNP	siRNA	7	j	pu:py		-	[66]
COL3A1	vEDS	p.G252V	siRNA	10	j	pu:pu	Syst. Screen.	yes	[35]
COL6A1	UCMD	p.G284R	siRNA	8, 8/13	j	8 pu:py, 13 py:py		yes	[43]
GNE	Sialuria	p.R266Q	siRNA	10	j	pu:py		yes	[67]
HBB	SCA	p.E6V	siRNA	10	a, f	pu:pu		-	[57]
HBV-P	HBV	p.M204I	siRNA	16	f	pu:pu		-	[72]
HTT	HD	da-SNP	siRNA	4	j	pu:pu		-	[68]
HTT	HD	da-SNP	siRNA	10	j	pu:py		-	[58]
HTT	HD	da-SNP	siRNA	10	j	pu:py		-	[69]

HTT	HD	da-SNP	siRNA	16	j	pu:py		-	[65]
HTT	HD	da-SNP	shRNA	10,11	e, j	pu:py		yes	[48]
HTT	HD	da-SNP	siRNA	10/5	c	10 pu:py, 5 py:py			[73]
KCNH2	LQT2	p.A561T	siRNA	13	j	py:py		yes	[46]
K-RAS	Cancer	p.V12L	shRNA	9	h	pu:pu		-	[6]
KRT12	MECD	p.L132P	siRNA	9	j	pu:py		-	[70]
KRT12	MECD	p.L132P	siRNA	5, 9, 10, 11, 12, 14, 15	a, b, c	pu:py	Syst. Screen. only si9 studied in the 3 models	yes	[33]
KRT12	MECD	p.R135T	siRNA	5, 6, 8, 9, 13, 14	a, b, c	pu:pu	Syst. Screen. only si5 studied in the 3 models	-	[37]
KRT5	EBS	p.S181P p.N193K	siRNA siRNA	11 8	a	pu:py py:py		yes yes	[36]
KRT6A	PC	p.N171K	siRNA	4, 10, 12	b, g	py:py	Syst. Screen.	yes	[31]
KRT9	EPPK	p.R163W p.R163Q p.M157V p.M157T	siRNA siRNA siRNA siRNA	3 13 11 16	b, c, g	pu:py pu:py pu:py pu:py	Syst. Screen.	-	[34]
LRRK2	PaD	p.R1441G p.R1441C	shRNA shRNA	11 11	c	py:py pu:py		-	[59]
MAPT	FTDP17	p.V337M	siRNA	9/12	b	9 pu:py 12 pu:pu		-	[8]
MAPT	FTDP17	p.V337M	siRNA/shRNA	10	a	pu:py		-	[17]
Myh6	HCM	p.R403Q	shRNA	16/20	k	16 pu:py 20 py:py	21 base-pair shRNA	yes	[52]
p53	Cancer	p.R248W	siRNA	9	b	pu:py		-	[5]
PIK3CA	Cancer	p.E545K p.H1047L	siRNA siRNA	10, 16 11	a	pu:py pu:py		-	[44]
PRNP	PD	p.P102L	siRNA/shRNA	9/12, 9/13, 9/14, 9/17	a, c	9 pu:pu, 12 pu:pu, 13 py:py, 14 pu:pu, 17 py:py		-	[74]
PRNP	PD	p.P105L	siRNA	9, 10/14, 10/15	a, c	9 and 10 pu:py, 14 pu:pu, 15 pu:pu		-	[74]

PSEN1	AD	p.L392V	siRNA	10	j	py:py		yes	[32]
PSEN1	AD	p.L392V	siRNA	8, 9, 10, 11	c	py:py	Syst. Screen.	-	[32]
SNCA	PaD	p.A30P	shRNA	10, 13, 14	b, c	pu:pu		-	[40]
SNCA	PaD	p.A53T	siRNA	9	a	pu:py	21-base-pair siRNA	-	[41]
SOD1	ALS	p.G85R	siRNA	10	a	pu:pu		-	[10]
SOD1	ALS	p.G85R	siRNA	5, 9, 10, 12, 13, 14, 16	c	pu:pu	Syst. Screen.	-	[30]
SOD1	ALS	p.G93A	siRNA/shRNA	10, 16	a, d	pu:pu		-	[10, 75]
SOD1	ALS	p.G93A	shRNA	11	e	pu:pu	Crossing with shRNA transgenic mice	yes	[49]
SOD1	ALS	p.G93A	shRNA	9	e	pu:pu		No benefit	[71]
TARDBP	ALS	p.M337V		9	j	pu:py		-	[42]
TGFBI	LCDI	p.R124C	siRNA	14	j	pu:py		-	[38]
TGFBI	LCDI	p.R124C	siRNA	8, 14, 16	a, c	pu:py	Syst. Screen.	yes	[38]
TTR	FAP	p.V30M	siRNA	10	a, b	pu:py			[76]

Model: a: cells transfected with WT or mutated transcripts, b: co-transfection of WT and mutants *in vitro*, c: cell-free assay or reporter assay *in vitro*, d: co-transfection of WT and mutants *in vivo*, e: Transgenic mice expressing WT or mutated transcripts, f: stably transfected cells with mutated transcripts, g: reporter assay *in vivo* in mice, h: cell lines endogenously expressing WT or mutant, i: overexpression of mutated transcript *in vivo*, j: patient-derived cells, k: knock-in mouse model. Mismatch positions are indicated relative to the most 5' nucleotide of the antisense strand. In case of double mismatch, the position of the mutated nucleotide is indicated in bold. Syst. Screen: Systematic screening; *i.e.* the 19 possible siRNA with the mutated nucleotide at each position were tested. pu: purine base, py: pyrimidine base, da-SNP: disease associated-Single Nucleotide Polymorphism, SOD1: Superoxide dismutase 1, ALS: Amyotrophic lateral sclerosis, APP: Amyloid precursor protein, AD: Alzheimer's disease, ATXN3: Ataxin 3, MJD/SCA3: Machado-Joseph disease/Spinocerebellar ataxia 3, GNE: UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase, HTT: Huntingtin, HD: Huntington's disease, ATXN1: Ataxin 1, SCA1: Spinocerebellar ataxia 1, ATXN7: Ataxin 7, SCA7: Spinocerebellar ataxia 7, TARDBP: TAR-DNA Binding protein (TDB-43), PSEN1: Presenilin 1, ACVR1: Activin A receptor type 1, FOP: Fibrodysplasia ossificans progressive, COL1A2: Collagen type I alpha 2 chain, OI: Osteogenesis imperfecta, COL3A1: Collagen type 3 alpha 1, vEDS: Vascular type of the Ehlers-Danlos syndrome, COL6A1: Collagen type VI alpha 1, UCMD: Ullrich congenital muscular dystrophy, KCNH2: Potassium channel voltage-gated subfamily H member 2, LQT2: Long QT syndrome type 2, KRT12: Keratin 12, MECD: Meesmann epithelial corneal dystrophy, TGFBI: TGFβ-induced gene, LCDI: Lattice corneal dystrophy type I, Myh6: Myosin heavy chain 6, HCM: Hypertrophic cardiomyopathy, MAPT: Microtubule-associated protein Tau, FTDP17: Frontotemporal dementia with parkinsonism linked to chromosome 17, CHRNA1: Acetylcholine receptor alpha subunit, SCCMS: Slow channel congenital myasthenic syndrome, TTR: Transthyretin, FAP: Familial amyloidotic polyneuropathy, HBB: Hemoglobin beta locus, SCA: Sickle cell anemia, KRT6A: Keratin 6a, PC: Pachyonychia congenita, KRT5: Keratin 5, EBS: Epidermolysis bullosa simplex, PRNP: Prion protein, PD: Prion diseases, PIK3CA: Phosphoinositide-3-kinase catalytic alpha polypeptide, HBV-P: HBV polymerase gene, HBV: Hepatitis B Virus infection, PSEN1: Presenilin 1, AD: Alzheimer's disease, LRRK2: Leucine-rich repeat kinase 2, SNCA: Alpha-synuclein, PaD: Parkinson's disease, KRT12: Keratin 12, KRT9: Keratin 9, EPPK: Epidermolytic palmoplantar keratoderma.

Supplementary Table 2: AS-RNAi targeting multiple nucleotide changes responsible for dominant diseases.

Gene	Disease	Targeted change	Silencing molecule	Model	Phenotype reversion	Reference
APP	AD	2-nucleotide substitution	siRNA	c	-	[15]
APP	AD	2-nucleotide substitution	shRNA	a	-	[16]
APP	AD	2-nucleotide substitution	siRNA/shRNA	a	-	[17]
APP	AD	2-nucleotide substitution	shRNA	e	yes	[21]
ATXN3	MJD	CAG repeat	siRNA	a	-	[26]
ATXN3	MJD	CAG repeat	siRNA	j	-	[29]
CACNA1A	SCA6	Partial exon inclusion	siRNA/shRNA	a,c	-	[25]
COL1A1	OI	2-nucleotide substitution	siRNA/shRNA	a,h	-	[18]
COL6A3	UCMD	Exon exclusion	siRNA	j	yes	[24]
COL7A1	DDEB	Exon exclusion	siRNA	c,j	-	[23]
EGFR	Cancer	Exon exclusion	siRNA	c,i,j	yes	[14]
EGFR	Astrocytoma	Exon exclusion	siRNA	a	yes	[22]
FGFR2	CS	2-nucleotide substitution	shRNA	k	yes	[20]
HTT	HD	3-nucleotide deletion	siRNA	b,j	yes	[13]
HTT	HD	CAG repeat	RNA duplex	j	-	[27]
HTT	HD	CAG repeat	RNA duplex	j	-	[28]
RYR1	MH	3-nucleotide substitution	siRNA	k	yes	[19]
RYR1	CCD	2-nucleotide substitution	siRNA	k	yes	[19]

TOR1A	DYT1	3-nucleotide deletion	siRNA	a,b	-	[7]
TOR1A	DYT1	3-nucleotide deletion	shRNA	a,b	yes	[12]

Model: a: cells transfected with WT or mutated transcripts, b: co-transfection of WT and mutants *in vitro*, c: cell-free assay or reporter assay *in vitro*, e: Transgenic mice expressing WT or mutated transcripts, h: cell lines endogenously expressing WT or mutant, i: overexpression of mutated transcript *in vivo*, j: patient-derived cells, k: knock-in mouse model. TOR1A: Torsin A, DYT1: Dystonia. HTT: Huntingtin, HD: Huntington's disease, EGFR: Epidermal growth factor receptor, APP: Amyloid precursor protein, AD: Alzheimer's disease, COL1A1: α 1 chain of type I collagen, OI: Osteogenesis imperfecta, RYR1: Ryanodine receptor type 1, MH: Malignant hyperthermia, CCD: Central core disease, FGFR2: Fibroblast growth factor receptor 2, CS: Craniosynostosis, COL7A1: α 1 chain of type VII collagen, DDEB: Dominant dystrophic epidermolys bullosa, COL6A3: α 3 chain of type VI collagen, UCMD: Ullrich congenital muscular dystrophy, CACNA1A: Cav2.1 voltage-gated calcium channel, SCA6: Spinocerebellar ataxia type 6, ATXN3: Ataxin 3, MJD: Machado-Joseph disease.