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Splice isoform-specific suppression of the Ca_v2.1 variant underlying Spinocerebellar ataxia type 6

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Abstract

Spinocerebellar ataxia type 6 (SCA6) is an inherited neurodegenerative disease caused by a polyglutamine (polyQ) expansion in the Ca_v2.1 voltage-gated calcium channel subunit (CACNA1A). There is currently no treatment for this debilitating disorder and thus a pressing need to develop preventative therapies. RNA interference (RNAi) has proven effective at halting disease progression in several models of spinocerebellar ataxia (SCA), including SCA types 1 and 3. However, in SCA6 and other dominantly inherited neurodegenerative disorders, RNAi-based strategies that selectively suppress expression of mutant alleles may be required. Using a Ca_v2.1 mini-gene reporter system, we found that pathogenic CAG expansions in Ca_v2.1 enhance splicing activity at the 3' end of the transcript, leading to a CAG repeat length-dependent increase in the levels of a polyQ-encoding Ca_v2.1 mRNA splice isoform and the resultant disease protein. Taking advantage of this molecular phenomenon, we developed a novel splice isoform-specific (SIS)-RNAi strategy that selectively targets the polyQ-encoding Ca_v2.1 splice variant. Selective suppression of transiently expressed and endogenous polyQ-encoding Ca_v2.1 splice variants was achieved in a variety of cell-based models including a human neuronal cell line, using a new artificial miRNA-like delivery system. Moreover, the efficacy of gene silencing correlated with effective intracellular recognition and processing of SIS-RNAi miRNA mimics. These results lend support to the preclinical development of SIS-RNAi as a potential therapy for SCA6 and other dominantly inherited diseases.

Introduction

Spinocerebellar ataxia type 6 (SCA6) is a debilitating form of ataxia in which cerebellar Purkinje neurons preferentially degenerate (Kordasiewicz and Gomez, 2007). It is one of the most common forms of autosomal dominant cerebellar ataxia and one of nine inherited

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neurodegenerative diseases caused by a polyglutamine (polyQ)-encoding CAG repeat expansion (Williams and Paulson, 2008). In SCA6, the expansion resides near the 3' end of the voltage-gated P/Q-type calcium channel gene, *Ca_v2.1* (*CACNA1A*). Despite a wealth of data describing *Ca_v2.1* channel function (Pietrobon, 2010), it remains unclear how the polyQ expansion in *Ca_v2.1* leads to SCA6. Consequently, efforts to design effective therapies for this currently untreatable disease have met with difficulty.

SCA6 shares pathological signatures with other polyQ disorders, such as intraneuronal protein aggregates and nuclear translocation of polyQ fragments (Ishiguro et al., 2009; Kordasiewicz et al., 2006; Kubodera et al., 2003; Seidel et al., 2009). Two important features of SCA6, however, make it unique among the polyQ diseases. First, SCA6 is caused by a relatively small polyQ expansion in *Ca_v2.1*, only 20–33 glutamines in length (Kordasiewicz and Gomez, 2007; Zhuchenko et al., 1997). In contrast, expansions of at least 35–38 glutamines are needed to cause disease in the other polyQ disorders. Second, unlike other polyQ disease proteins, only a subset of *Ca_v2.1* isoforms contain the polyQ tract (Bourinet et al., 1999; Tsunemi et al., 2006); alternative splicing near the 3' end of *Ca_v2.1* leads to the production of two distinct channel isoforms that either lack or contain the C-terminal polyQ tract (Supplementary Fig. 1). These two variants are abundant in adult cerebellar Purkinje neurons where they are expressed at roughly equivalent levels and are thought to be functionally redundant (Kanumilli et al., 2006; Tsunemi et al., 2008; Watase et al., 2008).

The clinical severity of polyQ disorders, including SCA6, is linked both to the size of the polyQ expansion and to the expression level of the protein harboring the expansion (Williams and Paulson, 2008). Several groups including ours have used RNA interference (RNAi) (Scholefield and Wood, 2010) to target the expression of polyQ proteins. For several polyQ disorders, however, the use of RNAi as a clinical therapy will require preferential targeting of expanded polyQ proteins that minimally disrupts levels of wild-type, non-expanded polyQ protein. This is the case for SCA6 in which global (i.e. nonselective) RNAi targeting of the *Ca_v2.1* channel in Purkinje neurons would likely lead to cerebellar dysfunction (Fletcher et al., 2001; Saito et al., 2009).

Allele-specific RNAi strategies for polyQ diseases have relied primarily on targeting single nucleotide polymorphisms (SNPs) in tight linkage disequilibrium with the CAG expanded alleles (Alves et al., 2008; Lombardi et al., 2009; Miller et al., 2003; Pfister et al., 2009). Though this is a promising approach, its potential is hindered by the limited prevalence of the targeted SNPs in the disease population. In SCA6, the existence of functionally redundant C-terminal splice variants offers the alternative possibility of targeting only those splice variants that encode polyQ-containing *Ca_v2.1* isoforms. Here we investigate alternative *Ca_v2.1* splicing and develop an RNAi molecule that selectively targets the polyQ-encoding splice variants underlying SCA6. Unlike SNP-based RNAi approaches to polyQ disease, this splice isoform-specific (SIS) RNAi molecule targets a sequence present in all individuals afflicted with SCA6. In addition, we describe a novel, artificial microRNA (miRNA) delivery scaffold based on a miRNA enriched in human brain, miRNA-124. Our results provide support for the preclinical development of SIS-RNAi as a novel therapeutic approach to SCA6.

Materials and Methods

siRNA Synthesis

Oligonucleotide sequences are listed in Supplementary Fig. 2. siRNA duplexes were formed by adding 500 picomoles of sense and antisense RNA to 10X annealing buffer (1M Potassium acetate, 0.3M HEPES-KOH pH7.4, 20mM Magnesium acetate), heating the

mixture to 95°C for 1 min and cooling it down at 37°C for 1 hr. RNA molecules were synthesized as previously described (Miller et al., 2003).

shRNA and miRNA Vectors

shRNA and miRNA vectors were generated using the BLOCK-iT U6 RNAi Entry Vector Kit (Invitrogen). For shRNAs, sense (top) and antisense (bottom) DNA oligonucleotides were designed, annealed and cloned into the pENTR/U6 vector following the manufacturer's recommendations (Invitrogen). The artificial miRNA expression cassette was built by embedding targeting sequences in a miRNA backbone composed of the basal stem (52 nucleotides) and loop (17 nucleotides) sequences of the human miR124-1 miRNA (<http://rfam.sanger.ac.uk/family?entry=RF00239>). The resultant long DNA oligonucleotides were annealed and cloned into the pENTR/U6 vector following the manufacturer's recommendations (Invitrogen).

Ca_v2.1 expression plasmids

The plasmid pEGFPCa_v2.1_V2 (a gift from Dr. Christopher Gomez, University of Chicago) was previously described (Restituito et al., 2000) and the pEGFPCa_v2.1_V1 plasmid was generated by deleting one of the two GGCAG pentamers using a site-directed mutagenesis approach (QuikChange XL, Stratagene). Primers used in the reaction were: 5'-CACATGGCGCACCGGCAGTAGTTCCG-3' and 5'-CGGAACTACTGCCGGTGCGCCATGTG-3'. The mCherry-*t*Ca_v2.1_V2 expression plasmid was generated by excising an XhoI-EcoRI fragment from a human *Ca_v2.1* cDNA (nucleotides 5579 and 7769, including exons 35 through 47; accession no.: AF004883.1) and ligating it into the XhoI and EcoRI sites of the mCherry plasmid (Clontech). The mCherry-*t*Ca_v2.1_V1 expression plasmid was generated following the same site-directed mutagenesis approach described above. The procedure used to generate the human Ca_v2.1 mini-genes is described graphically in Supplementary Fig. 3.

Cell Culture, Transfections and Western Blot Analysis

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum. Transfection reactions were performed in 12-well plates using 4μl Lipofectamine 2000 Reagent (Invitrogen) following manufacturer's recommendations. Twenty-four or forty-eight hours post-transfection, EGFP-Ca_v2.1 protein levels were analyzed using a mouse polyclonal antibody against GFP (Roche) at a 1:1,000 dilution. mCherry-*t*Ca_v2.1 mini-gene protein levels were analyzed using a goat polyclonal antibody against Ca_v2.1 (D-20, Santa Cruz Biotechnology) at a 1:200 dilution. α-tubulin levels were detected using a mouse polyclonal antibody (Sigma) at a 1:10,000 dilution.

Northern Blot

Small RNAs were isolated (mirVana miRNA Isolation kit, Ambion), loaded (4μg) on a 15% acrylamide/bis-acrylamide (19:1) gel containing 8M urea and electrotransferred (200mA for 30 min in 0.5X TBE buffer) onto Hybond N+ nylon membranes (Amersham). Membranes were prehybridized overnight in ULTRAhyb-Oligo hybridization buffer (Ambion) at 34°C and hybridized for 48-hrs with a ³²P-end-labeled probe (mirVana™ Probe & Marker Kit, Ambion) directed against the WE1 guide strand, WE1 passenger strand or the human 5S RNA. Following hybridization, membranes were washed and exposed to film (Blue Autoradiography Film BX, MidSci, St. Louis, MO). Probe sequences: 5'-GGCAGGGCAGTAGTTCCGTAA-3' (guide strand), 5'-TTACGGACTACTGCCCTGCC-3' (passenger strand), and 5'-GATCGGGCGCGTTCAGGGTGGTAT-3' (5S rRNA as a loading control).

3'RACE

Small RNAs (1µg) were polyA-tailed (Poly(A) Polymerase Tailing Kit, Epicentre Biotechnologies), purified and immediately added to a FirstChoice RLM-RACE Kit (Ambion) first-strand cDNA synthesis reaction. The reaction contained 250ng poly(A)-tailed small RNA, 4µl dNTP mix, 1µl 3'RACE adaptor, 2µl 10X RT buffer, 4µl RNase inhibitor, and 1µl M-MLV-RTase. We used WE1-specific primers (guide strand, 5'-TTACGGAAGTACTGCCCTGCC-3'; passenger strand, 5'-GGCAGGGCAGTAGTTCCGTAA-3') and 3'RACE inner or outer primers to amplify the cDNA library. The resultant PCR products were separated on a 2% agarose gel and specific bands of ~100bp in size were excised, purified and subcloned. The small cDNA library was sequenced at The University of Michigan's DNA Sequencing Core using the Sanger sequencing method.

Ca_v2.1_V2-specific quantitative PCR assay

Levels of Ca_v2.1_V2 mRNA were quantified in a real-time quantitative PCR reaction (MyiQ2 Two-Color Real-Time PCR Detection System, BioRad) containing 2µl of cDNA, 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 50-picograms of 5' primer (5'-CACCACCACCACCACCACCACCAC-3') and 33-picograms of Ca_v2.1_V2-specific 3' primer (5'-CACTAACGGAACTACTGCCCTGCCGG-3'). Cycling conditions were: 95°C for 10 sec, 63°C for 6 sec and 72°C for 6 sec (40 cycles). Quantification analysis was performed using the iQ5 optical system analysis software (Bio-Rad). Because the mCherry sequence is present in all Ca_v2.1 mini-gene-derived transcripts, Ca_v2.1_V2 levels were normalized to and calculated as a fraction of total mCherry levels.

Human Ca_v2.1 3'end splice variants population assay

SK-N-SH cells (Human neuroblastoma cell line) were grown in Dulbecco's Modified Eagle's Medium containing 10% Fetal Bovine Serum. Transfection reactions were performed in 6-well dishes using 2.5 µg of miRNA plasmid and 10 µl Lipofectamine 2000 Reagent (Invitrogen) following manufacturer's recommendations. Twenty-four hours after transfection, total RNA was isolated using TRIzol Reagent (Invitrogen). First-strand cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). PCR primers hEx46-F (5'-TCGCCCAGCGAGGGCCGAGAGCACA-3') and hEx47-R (5'-GTGCTGGTACCAGATGTTGAGGGG-3') were used to amplify across the human Ca_v2.1 exon 46/47 junction. PCR products were cloned into pCR2.1-TOPO (Invitrogen) and sequenced. Levels of total Ca_v2.1 mRNA were quantified in a real-time quantitative PCR reaction using the same primers, and normalized as described above.

Statistical analysis

Western blots were quantified using Quantity One software (Bio-Rad). Statistical analyses were performed using *GraphPad Prism* software. Results from 3 or 4 independent experiments were included in the analysis. Differences in mean protein levels were determined using a paired *t*-test analysis. For Ca_v2.1_V2-specific quantitative PCR, Ca_v2.1_V2 levels were normalized to mCherry and reported as fold expression over the mCherry-*t*Ca_v2.1_V2 with 11 CAG repeats. Unpaired *t*-test was used to examine the associations between 11 to 24 repeats and 11 to 72 repeats.

Results

CAG expansions enhance production of the polyQ-encoding Ca_v2.1 splice-isoform

Alternative splicing at the exon 46/47 junction of Ca_v2.1 results in the production of two major Ca_v2.1 3'end mRNA splice isoforms (Fig. 1A). One isoform, Ca_v2.1 variant-1

(Ca_v2.1_V1), contains a single GGCAG pentamer followed by a UAG stop codon. Because the stop codon is upstream of the CAG repeat, this isoform encodes functional Ca_v2.1 channels lacking the polyQ domain. The other isoform, Ca_v2.1 variant-2 (Ca_v2.1_V2), contains two adjacent GGCAG pentamers (2GGCAG) upstream of the UAG codon, which shifts the reading frame and leads to the translation of functional Ca_v2.1 channels with a longer, polyQ containing C-terminus (Fig. 1 and Supplementary Fig. 1).

Intriguingly, CAG repeat expansions in *Ca_v2.1* appear to cause an abnormal increase in polyQ-encoding Ca_v2.1 mRNA isoforms (Tsunemi et al., 2008; Watase et al., 2008). To gain further insight into this poorly understood phenomenon, we generated truncated Ca_v2.1 mini-genes containing the last 13 exons (exons 35–47) and the last intron (int46) of the human *Ca_v2.1* gene fused to the mCherry sequence (Fig. 1B, *tCa_v2.1_{int}*). Three *tCa_v2.1_{int}* constructs were made, differing only in CAG repeat length (11, 24 and 72 CAGs). Inclusion of int46 was expected to model alternative splicing at the exon-46/47 junction and produce Ca_v2.1 transcripts containing one or two GGCAG pentamers.

Expression of Ca_v2.1 mini-genes (*tCa_v2.1_{int}*) encoding 11, 24 or 72Qs resulted in the production of polyQ-containing Ca_v2.1_V2 isoforms detectable with an anti-Ca_v2.1 antibody. The molecular weight of Ca_v2.1_V2 isoforms derived from the mini-genes (Fig. 1C lanes 4–6) was indistinguishable from Ca_v2.1_V2 isoforms derived from control cDNA expression vectors (Fig. 1C, lanes 1–3). We also noticed, and confirmed by RT-PCR and sequencing, the production of unanticipated Ca_v2.1 splice variants (Supplementary Figures 4 and 5). These variants are not annotated in the human EST database and could be an artifact of the mini-gene reporter system.

Importantly, lengthening the polyQ stretch from 11 to 72Qs led to an incremental increase in Ca_v2.1_V2 protein levels. Using a Ca_v2.1_V2-specific quantitative PCR assay we asked whether this increase occurred as a result of changes in Ca_v2.1_V2 mRNA levels. Indeed, expanding the CAGs in the Ca_v2.1 mini-gene from 11 to 72 repeats correlated with an increase in polyQ-encoding Ca_v2.1_V2 mRNA production (Fig. 1C).

Ca_v2.1_V1 protein levels were undetectable following expression of Ca_v2.1 mini-genes, independent of CAG repeat length (Fig. 1C). Ca_v2.1_V1 mRNA could only be detected after gene-specific RT-PCR amplification (data not shown). In an effort to explain this finding, we analyzed the splice acceptor site sequences responsible for Ca_v2.1_V1 and Ca_v2.1_V2 production using the Human Splicing Finder (Desmet et al., 2009) software. This analysis revealed that a strong canonical AG-G splice acceptor site drives Ca_v2.1_V2 production whereas production of Ca_v2.1_V1 depends on the use of a less preferred, AG-U splice acceptor site (Fig. 1A and Supplementary Fig. 4). From this analysis, we predicted that eliminating the Ca_v2.1_V2 splice acceptor site would result in the use of the adjacent Ca_v2.1_V1 splice acceptor site (Supplementary Fig. 4). To test this hypothesis, we generated Ca_v2.1 mini-genes containing a mutated Ca_v2.1_V2 splice acceptor site (Fig. 1D). As expected, production of Ca_v2.1_V2 isoforms was abolished while Ca_v2.1_V1 protein was easily detected. Unexpectedly, expanding the CAG from 11 to 72 repeats in the mutated Ca_v2.1 mini-genes led to similar incremental increases in Ca_v2.1_V1 protein levels (Fig. 1E). In contrast, the levels of other isoforms detected with the anti-Ca_v2.1 antibody did not increase in response to a lengthening of the CAG repeats (Fig. 1E, higher molecular weight bands). In fact, CAG repeat expansions led to an overall reduction in the levels of these isoforms. Finally, because there is no polyQ stretch in Ca_v2.1_V1, this CAG repeat length-dependent effect cannot be due to a polyQ-dependent stabilization of the encoded protein. Rather, it likely reflects a CAG repeat length dependent enhancement of splicing activity at the exon-46/47 junction.

Splice-isoform-specific RNAi suppression of the polyQ-encoding Ca_v2.1 variant

Since the Ca_v2.1_V1 and Ca_v2.1_V2 protein isoforms are predicted to be functionally redundant in adult Purkinje neurons (Chaudhuri et al., 2004), we reasoned that splice-isoform-specific (SIS)-RNAi suppression of the polyQ-encoding Ca_v2.1_V2 mRNA would be of therapeutic benefit in SCA6. We previously showed that allele-specific gene silencing can be achieved *in vitro* and *in vivo* by placing nucleotide mismatches at the 10th nucleotide position of an siRNA-guide strand and an otherwise fully complementary RNA target (Miller et al., 2003; Rodriguez-Lebron et al., 2009; Rodriguez-Lebron and Paulson, 2006). Based on these findings, we sought to selectively suppress Ca_v2.1_V2 expression by placing the 2GGCAG target sequence near the 10th nucleotide position of five different, anti-Ca_v2.1_V2 RNAi guide strands (Supplementary Fig. 6). None of these short-hairpin RNAs, however, was capable of silencing Ca_v2.1_V2 expression (Supplementary Fig. 6C), which is likely due to their high guanosine-cytosine (GC) content (67–72%).

In vitro studies have also shown that, in addition to nucleotide mismatches at the 10th position of the siRNA/RNA-target duplex, mismatches at the 16th position of this duplex can also disrupt Argonaute-2 (Ago-2)-mediated cleavage of the RNA target sequence (Schwarz et al., 2006). Accordingly, we centered the 2GGCAG target sequence on or near the 16th position of the guide strand and generated three new siRNAs named WE1, WE2 and WE3 (Fig. 2A). The new siRNAs had a lower GC content (57–67%), but for each construct we also produced two additional siRNAs carrying one (m1) or two (m2) mismatched uridines near the 5' end of the passenger strand to further reduce the GC content in the siRNA duplex.

The activity of these nine new siRNAs was tested in transiently transfected HEK293 cells. Epifluorescence and western blot analyses revealed that WE1 and WE1m1 siRNAs effectively suppressed expression of an EGFP-Ca_v2.1_V2 fusion protein (Fig. 2B–C). In contrast, the negative controls (null or transfected with a 'missense' siRNA) and the remaining 7 siRNAs (WE1m2, WE2, WE2m1, WE2m2, WE3, WE3m1 and WE3m2) did not suppress EGFP-Ca_v2.1_V2 expression.

We next determined if the WE1, WE1m1 and WE1m2 targeting sequences could discriminate between Ca_v2.1 transcripts containing one or two GGCAG pentamers. The three anti-Ca_v2.1_V2 sequences and the three negative control sequences (missense, WE3 and WE3m1) were cloned into first-generation shRNAs and co-transfected with EGFP-Ca_v2.1_V1 (1GGCAG) or EGFP-Ca_v2.1_V2 (2GGCAG) expression vectors. Compared to negative control shRNAs, WE1, WE1m1 and WE1m2 shRNAs significantly suppressed expression of the polyQ-containing EGFP-Ca_v2.1_V2 (decreased to 46.5% ± 8.3, 47.0% ± 9.3 and 44.7% ± 9.8 of control levels, respectively) in co-transfected cells (Fig. 3A). In contrast, WE1, WE1m1 and WE1m2 shRNAs did not affect the expression of EGFP-Ca_v2.1_V1 (Fig. 3B). Thus, anti-SCA6 RNAi molecules carrying the 2GGCAG targeting sequence near the 16th nucleotide position can selectively suppress the polyQ-encoding Ca_v2.1 variant in cells.

miRNA mimics mediate potent silencing of the polyQ-encoding Ca_v2.1 variant

Because SCA6 is a slowly progressive neurodegenerative disease, preventive treatment will require chronic administration of therapeutic molecules. In contrast to the case with first-generation RNAi hairpins, long-term intracellular production of artificial miRNA-like hairpins is well tolerated in the mammalian brain (Boudreau et al., 2009; McBride et al., 2008). Thus, we used the primary sequence of human miRNA-124-1 to engineer artificial miRNA mimics from which to deliver therapeutic RNAi molecules.

Human miRNA-124 (miR124) is a brain enriched miRNA that regulates neuronal maturation (Cheng et al., 2009). The expression profile, recognition and processing of this

miRNA have been extensively characterized (Krichevsky et al., 2006; Lagos-Quintana et al., 2002; Makeyev et al., 2007). The artificial RNAi delivery shuttle contains the basal stem and loop sequences of human miR124-1, with one of the three WE1 targeting sequences (WE1, WE1m1 and WE1m2) or a negative control sequence (missense) replacing the endogenous miR (guide strand) and miR* (passenger strand) sequences of miR124-1 (Fig. 4A). The U6 small nucleolar non-coding RNA promoter was used to drive production of the miR124-based miRNAs.

Anti-Cav2.1_V2 miRNA mimic activity was first tested in co-transfection experiments. We consistently observed miR124-WE1-mediated suppression of EGFP-Cav2.1_V2 when compared to the miR124-Miss negative control (Fig. 4B). None of the miR124-based constructs interfered with EGFP-Cav2.1_V1 or α -tubulin expression, suggesting that all miR124-WE1 constructs specifically targeted expression of the polyQ-encoding EGFP-Cav2.1_V2. In dose-response experiments, WE1, WE1m1 and WE1m2 selectively suppressed expression of EGFP-Cav2.1_V2 in a dose-dependent manner, whether as shRNAs or miR124-based precursors (Fig. 4C). Surprisingly, at the lowest tested dose (2:1 ratio) only the miR124-WE1 and miR124-WE1m1 constructs significantly suppressed EGFP-Cav2.1_V2 expression ($74\% \pm 10$ and $73\% \pm 8$ of control, $p = 0.030$ and 0.045 respectively). We also noticed a reduction in miR124-WE1 activity when 1 or 2 cytosine-uridine mismatches (WE1m1 or WE1m2) were present in the shRNA or miR124-based duplex near the 5' end of the passenger strand (Fig. 4C).

At the highest tested dose (16:1 ratio), all of the WE1-based RNAi constructs, whether shRNA or miR124-based, significantly suppressed the EGFP-Cav2.1_V2 transcript without disrupting EGFP-Cav2.1_V1 expression (Fig. 4C). This remarkable specificity suggests that disrupting Watson-Crick base-pairing near the 16th nucleotide position of an otherwise fully complementary guide strand/RNA-target duplex can significantly impair RISC-mediated cleavage of the RNA target in cultured cells.

We next tested the ability of anti-Cav2.1 SIS-RNAi miRNA mimics to selectively silence endogenous Cav2.1_V2 expression in a human neural cell line. We chose the SK-N-SH neuroblastoma cell line because it expresses detectable levels of Cav2.1 mRNA and is routinely used for the study of neuronal apoptosis and neuroprotection (Chiou, 2006; Lee et al., 2006). Compared to negative controls (null and miR124-Miss), we observed a significant reduction in the levels of Cav2.1 mRNA in SK-N-SH cells expressing the miR124-WE1 miRNA mimic (down to 18.7%, $\pm 4\%$ of control, $n = 3$) (Fig. 5A). Expression of miR124-WE1 or miR124-Miss did not elicit overt cellular toxicity in SK-N-SH cells (Supplementary Fig. 7).

We characterized the specificity of SIS-RNAi against endogenously expressed Cav2.1 using a modification of a previously published method (Watase et al., 2008). Cav2.1 3' end-specific cDNA libraries generated from control or miR124-WE1-treated SK-N-SH cells were amplified and cloned into plasmids. The levels of Cav2.1 3' end splice variants were determined by sequencing individual clones from each library across the exon-46/47 junction. PolyQ-encoding Cav2.1 variants (Cav2.1_V2) were the predominant isoform in untreated SK-N-SH cells (~68% of isoforms compared to ~32% for Cav2.1_V1) and in SK-N-SH cells expressing the miR124-Miss RNAi control (~52% of isoforms compared to ~48% for Cav2.1_V1) (Fig. 5B). In contrast, expression of miR124-WE1 SIS-RNAi led to a marked increase in the percentage of Cav2.1_V1 isoforms (up to ~75% of all isoforms) with a corresponding decrease in the percentage of polyQ-encoding Cav2.1_V2 isoforms (down to ~25% of all isoforms) in SK-N-SH cells (Fig. 5B). This miR124-WE1-mediated change in endogenous Cav2.1 3' end splice-isoform ratio strongly supports the potency and specificity of miR124-WE1 SIS-RNAi.

Effective processing of miRNA mimics underlies the potency of Ca_v2.1 SIS-RNAi

In principle, therapeutic RNAi molecules may target transcripts other than the intended target, leading to serious adverse events. Such 'off-targeting' is thought to arise primarily from pairing of the guide strand 'seed' sequence (nucleotide positions 2–8) to the 3'-untranslated region (3'UTR) of transcripts (Birmingham et al., 2006; Grimson et al., 2007). We queried the RefSeq database and found that the 21-nucleotide-long WE1 target sequence only occurs in the Ca_v2.1_V2 transcript. In addition, 3'UTR target sites for the 'seed' sequence of the WE1-derived guide strand occur at a relatively low frequency in the human transcriptome (Anderson et al., 2008). While this analysis does not eliminate the possibility of off-targeting effects associated with miR124-WE1 expression in human cells, it does suggest that the potential for off-targeting would be limited to relatively few human transcripts.

Of critical importance to the specificity and safety of RNAi-based therapies is the preferential loading of the RNAi guide strand into the RISC. Loading one of the two RNAi strands into RISC is guided primarily by thermodynamic asymmetry between the two RNAi duplex ends. We characterized the processing and asymmetry of anti-Ca_v2.1_V2 RNAi duplexes by northern blot analysis. As shown in Fig. 6A, for each of the three effective RNAi constructs (WE1, WE1m1 and WE1m2) the levels of guide strand produced by the shRNA and miR124-based platforms were relatively similar (compare shRNA-WE1 and miR124-WE1). In contrast, expression of shRNAs resulted in a much greater accumulation of unprocessed precursors than occurred with the miR124-based constructs.

A strong bias towards preferred loading of the WE1 guide strand into RISC (Fig. 6) is suggested by the fact that we did not detect fully processed, mature passenger strands following expression of shRNA-WE1 and miR124-WE1 precursors. Intriguingly, a U-C mismatch near the 5' end of the passenger strand (WE1m1) led to a marked decrease in the levels of fully processed WE1 guide strand (Fig. 6A), which was even more pronounced when two mismatches were introduced (WE1m2). This decrease in mature guide strand levels was mirrored by an increase in the levels of mature passenger strands (Fig. 6B). Thus, adding mismatches to the 5' end of the passenger strand in the WE1 duplex altered the strand bias properties of the duplex, shifting it away from favoring guide strand loading toward increased incorporation of passenger strand into RISC.

We next mapped the sites of Drosha and Dicer cleavage on the WE1 shRNA and miRNA precursors using a modified 3'-rapid amplification of cDNA ends (3'RACE) approach (Boudreau et al., 2008). Confirming previous findings (Boudreau et al., 2008), we observed that shRNA platforms are processed to yield guide strands with highly reproducible, unmodified 3' end sequences (Table 1). In contrast, the 3' end sequence of miR124-WE1 guide strands varied considerably. In fact, only 22% of analyzed 3' end guide strand sequences matched the annotated Drosha cleavage site for the human miR124 precursor sequence. Approximately 60% of the analyzed guide strand sequences were post-transcriptionally modified by the addition of uridines to the 3' end (Table 1). These variations in nucleotide sequence were not observed at the 3' end of passenger strands.

From a therapeutic perspective, it is worth noting that modifications to the 3' end sequence of the guide strand do not appear to affect its activity or specificity against Ca_v2.1_V2 when compared to the unmodified shRNA-derived WE1 guide strand (Fig. 4C). Importantly, because similar post-transcriptional modifications are observed in some cellular miRNAs, including miR124 (Lagos-Quintana et al., 2002), our data suggest significant biological compatibility between anti-Ca_v2.1 SIS-RNAi miRNA-mimics and the endogenous cellular RNAi machinery.

Discussion

Several lines of evidence support the development of SIS-RNAi as a therapeutic strategy for SCA6. First, Cav2.1 function, initially thought to be impaired in SCA6, appears normal in mouse models of SCA6 (Saegusa et al., 2007; Tanabe et al., 2007; Watase et al., 2008). Thus, therapies designed to modulate Cav2.1 channel kinetics in SCA6 brain might be, at best, minimally beneficial. Second, although *Ca_v2.1* knockout mice are severely ataxic and die prematurely, mice with one functional *Ca_v2.1* allele (expressing 50% of normal Cav2.1 levels) are phenotypically normal (Fletcher et al., 2001). Moreover, in studies by Watase et al, mice with an estimated 90% reduction in polyQ-encoding Cav2.1 mRNA expression in cerebellar Purkinje neurons were indistinguishable from wild-type littermates. Based on this, we anticipate that SIS-RNAi-mediated suppression of polyQ-encoding Cav2.1 mRNA would be well tolerated in adult cerebellar Purkinje neurons. However, given the level of Cav2.1 knockdown observed in human SK-N-SH cells following SIS-RNAi, it will be important to assess, in vivo, the extent to which SIS-RNAi targeting of polyQ-encoding Cav2.1 mRNAs affects overall Cav2.1 levels. Finally, several studies support a model of SCA6 pathogenesis that includes the accumulation and aberrant nuclear translocation of an expanded polyQ-containing Cav2.1 fragment (Ishiguro et al., 2009; Kordasiewicz et al., 2006; Kubodera et al., 2003; Marqueze-Pouey et al., 2008). Thus, SIS-RNAi-mediated suppression of the polyQ-encoding Cav2.1 splice variant should also reduce levels of putative pathogenic Cav2.1 fragments and be of therapeutic benefit in SCA6.

CAG repeat expansions in *Ca_v2.1* cause an abnormal increase in alternatively spliced polyQ-encoding mRNA transcripts in both human and mouse Purkinje neurons (Tsunemi et al., 2008; Watase et al., 2008). We observed a similar phenomenon using a Cav2.1 mini-gene system. Extending the previous findings, we demonstrated that this rise in polyQ-encoding Cav2.1 mRNA levels leads to increased production of polyQ-containing Cav2.1 protein, which likely would contribute to disease pathogenesis. Analysis of the splice acceptor sites at the exon-46/47 junction of *Ca_v2.1* suggests that the selective increase in Cav2.1_V2 stems from two independent but complementary activities. First, expanded CAG repeats enhance splicing at the exon-46/47 junction of *Ca_v2.1*. How this is accomplished is unknown, but like others (Watase et al., 2008) we speculate that CAG repeats influence local recruitment of splicing factors in a repeat length-dependent manner. Consistent with this, CAG expansions are predicted to act as an exonic-splicing-enhancer binding motif, possibly through an interaction with the pre-mRNA splicing factor SRp55 (ESE database (Cartegni et al., 2003)). Second, production of Cav2.1_V2 transcripts is guided by a preferred AG-G splice acceptor site sequence. We found that mutating this sequence causes a shift towards the use of the much less preferred AG-U Cav2.1_V1 splice site. Importantly, in the absence of the Cav2.1_V2 splice site, expansion of the CAG repeats also enhanced production of Cav2.1_V1 transcripts. Based on these observations, we believe that CAG expansions in SCA6 enhance splicing activity at the exon-46/47 junction in a length-dependent manner. This increased activity is usually channeled through a canonical AG-G splice site, favoring Cav2.1_V2 production. The result would be a selective increase in the levels of expanded polyQ-containing Cav2.1_V2 channels in the disease state. Indeed, expanding the CAG repeat from 14 to 84 repeats increases the percentage of polyQ-encoding Cav2.1 splice isoforms found in Purkinje neurons of SCA6 knockin mice (Watase et al, 2008). Unfortunately, the genetic strategy used to generate these mice led to unintended alterations in Cav2.1 mRNA processing that were independent of CAG repeat length. Thus, although this model might be suitable to investigate some aspects of SIS-RNAi *in vivo*, new models may need to be developed in order to identify the mechanism(s) underlying CAG length-dependent alterations in Cav2.1 splicing and to validate the therapeutic potential of SIS-RNAi. In addition, it remains to be determined whether this CAG-length dependent effect on splicing is specific to the Cav2.1 transcript or is more

broadly true of CAG repeat-containing transcripts. A more complete understanding of the molecular events driving Cav2.1 3'end splicing may shed light on novel therapeutic routes.

Effective clinical use of SIS-RNAi for SCA6 may require the use of viral-based gene therapy. Toward that end, we developed a novel human miR124-based RNAi delivery platform. The miR124-based RNAi constructs were efficiently recognized and processed by the cellular RNAi machinery. Compared to first generation shRNA constructs, SIS-RNAi molecules within the miR124 scaffold more potently inhibited Cav2.1_V2 expression in cells. Sequence analysis of the processed miR124-WE1 guide strands unexpectedly revealed non-templated nucleotide additions to their 3'ends. Post-transcriptional modification of miRNAs is becoming increasingly appreciated through the use of high throughput sequencing analyses (Marti et al., 2010; Pantano et al., 2010). Previous reports established the existence of these modifications in endogenous miR124 sequences (Lagos-Quintana et al., 2002; Landgraf et al., 2007). Given that our artificial miR124-based shuttle lacks the endogenous miR124 guide strand, our results suggest that the enzymatic complexes responsible for post-transcriptional modifications of miRNAs likely recognize miRNA precursors and/or act proximal to their processing by the Dicer/TRBP/PACT complex. Curiously, post-transcriptional modifications were not observed in the sequenced miR124-WE1 passenger strands, hinting at a possible link between strand-bias and miRNA post-transcriptional modification. The biological implications of this process remain unresolved; importantly, 3'end nucleotide additions to miR124-WE1 guide strands do not appear to impair their selective silencing activity.

In conclusion, RNAi-mediated suppression of polyQ disease genes has the potential of being a preventive therapy in this important class of neurodegenerative disorders. Specific targeting of expanded polyQ-encoding alleles, however, continues to challenge the development of RNAi into an actual molecular therapy. Here we have developed RNAi molecules that successfully and selectively target the polyQ-encoding Cav2.1 splice isoforms underlying SCA6. Because the SIS-RNAi strategy does not interfere with functional Cav2.1 channels lacking the polyQ domain, it has a built-in safeguard against the anticipated detrimental effects of non-selectively disrupting Cav2.1 expression in the SCA6 cerebellum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research Highlights

- Pathogenic CAG expansions alter Ca_v2.1 3'end splicing activity
- Altered-splicing activity leads to an increase in levels of polyQ-Ca_v2.1 variants
- Selective targeting of polyQ-encoding Ca_v2.1 splice isoforms using RNAi
- The RNAi machinery effectively processes human miR124-based artificial miRNAs.

Fig. 1. CAG expansions in *Cav2.1* enhance splicing activity of its transcript at the exon 46/47 splice junction

A. Alternative splicing at the exon 46/47 junction of human *Cav2.1* produces two major *Cav2.1* 3' end splice variants that differ in having either 1 (*Cav2.1_V1*) or 2 (*Cav2.1_V2*) GGCAG pentamers (bold and underlined). Donor and acceptor sites are underlined and highlighted in red. *Cav2.1_V1* and *Cav2.1_V2* acceptor sites are marked with gray and black asterisks, respectively. The amino acid sequence encoded through the exon 46/47 junction is also shown. *Cav2.1_V1* splice variant contains a stop codon upstream of the (CAG)_n repeat whereas a frame shift in *Cav2.1_V2* mRNA leads to translation of the downstream (CAG)_n repeat as a polyQ stretch. **B.** Diagram depicting constructs used in **C**

including *Ca_v2.1* mini-genes (*tCa_v2.1_{+int}*) and control cDNAs (*tCa_v2.1_{+V1}* and *tCa_v2.1_{+V2}*) lacking intron-46. **C.** An anti-*Ca_v2.1* antibody detects the expression of *Ca_v2.1_V2* but not *Ca_v2.1_V1* protein isoforms in HEK293 cells transiently transfected with *tCa_v2.1_{+int}* mini-genes (top and bottom arrowheads respectively). There is a CAG repeat length-dependent increase in *Ca_v2.1_V2* protein levels (top arrowhead) upon expression of *Ca_v2.1* mini-genes. A non-annotated *Ca_v2.1* splice-isoform was also detected (arrow). Consistent with these results, quantitative PCR analysis (right panel) showed a direct correlation between CAG repeat length and *Ca_v2.1_V2* spliced mRNA levels. **D.** Bioinformatics revealed that the *Ca_v2.1_V2* splice site sequence (AG-G) is preferred over the adjacent *Ca_v2.1_V1* splice site sequence (AG-T). We tested this prediction by mutating the putative preferred splice site (AG-G to CT-G; underlined and arrowhead) in a series of *tCa_v2.1_{+int}* mini-gene constructs used in E. **E.** Mutating this splice site leads to the production of the *Ca_v2.1_V1* protein isoform (bottom arrowhead) but not the *Ca_v2.1_V2* protein isoform (top arrowhead), with a similar CAG repeat length-dependent increase in *Ca_v2.1_V1* isoform, as observed above for the V2 isoform in *tCa_v2.1_{+int}* mini-genes. The asterisk denotes $p < 0.05$ (paired *t*-test) and error bars are $\pm$ SEM.

Fig. 2. siRNAs mediate effective Cav2.1_V2 gene silencing

A. Passenger strand sequences for three siRNA targets (WE1, WE2 and WE3) with the targeted 2GGCAG pentamer sequence centered near the 16th nucleotide (nt) position of the RNAi duplex. The 2-nt 3' overhangs are highlighted in gray. The m1 or m2 variants for each of the three duplexes represent siRNAs in which 1 or 2 guanines in the passenger strand were mutated to uridines (black box). **B, C.** Co-transfection experiments in HEK293 cells with the EGFP-Cav2.1_V2 plasmid showed that WE1 and WE1m1 siRNAs effectively suppress expression of Cav2.1_V2, as measured by EGFP epifluorescence (panel **B**) and anti-GFP western blot analysis (panel **C**). Bright-field (BF) analysis of HEK293 transfected cells (GFP) did not reveal siRNA-induced cytotoxicity. Total protein levels were normalized to α-tubulin.

Fig. 3. Short-hairpin RNAs mediate splice isoform-specific RNAi suppression of the polyQ-encoding Cav2.1 variant

A. The active WE1, WE1m1, WE1m2 and inactive WE3, WE3m1 siRNA sequences, together with a missense negative control sequence, were cloned into first-generation shRNA plasmids (pshRNA). To assess activity against Cav2.1_V2, pshRNA constructs were co-transfected with EGFP-Cav2.1_V2 expression plasmid into HEK293 cells. Western blot analysis revealed that all active pshRNA plasmids (WE1, WE1m1 and WE1m2) significantly suppressed EGFP-Cav2.1_V2 expression. A representative western blot (left) and quantification of the results from four independent experiments (right) are shown. **B.** The specificity of the RNAi plasmids for the polyQ-encoding Cav2.1_V2 transcript was confirmed by co-transfecting each shRNA plasmid with an EGFP-Cav2.1_V1 expression plasmid. None of the RNAi plasmids mediated Cav2.1_V1 gene silencing. A representative western blot (left) and quantification of results from 4 independent experiments (right) are shown. For the analyses in **A** and **B**, western blots were carried out using anti-GFP and anti- α tubulin antibodies (internal control). The mean ratios of Cav2.1_V2 or _V1 were calculated from four independent experiments, normalized to the internal control and reported as % control levels (control = missense). Error bars indicate + SEM. * = $p < 0.0001$, paired t -test.

Fig. 4. WE1 miRNA mimics mediate potent and selective suppression of Cav2.1_V2

A. The human miR124-1 miRNA primary sequence was used to design a novel artificial miRNA-delivery shuttle. The basal-stem and loop sequences (green) of the pri-miR124 transcript were used to flank the WE1 (red/yellow), WE1m1, WE1m2 and missense targeting duplexes. Filled and empty arrowheads mark the predicted Drosha and Dicer cleavage sites, respectively. **B.** The activity and specificity of miRNA-like RNAi constructs were assessed in HEK293 cells transiently co-transfected with both pEGFP-Cav2.1_V1 and pEGFP-Cav2.1_V2 and the indicated miR124 RNAi plasmids. All three RNAi constructs potently suppressed Cav2.1_V2 expression compared to a miR124-missense negative control plasmid (Miss). **C.** Results of dosing experiments testing a wide range of RNAi to target plasmid molar ratios. The efficacy of the miR124-shuttle delivery system (circles) was compared to that of the first-generation, U6-driven shRNA platform (triangles) carrying the same WE1 targeting sequence. Dose-response curves depict the anti-GFP western blot signal intensity normalized to α -tubulin signal and expressed as percent of control levels. Analysis of the lowest tested dose (2:1) revealed miR124-WE1 and miR124-WE1m1 RNAi constructs to be the most potent inhibitors of Cav2.1_V2 expression (mean = $74\% \pm 10$ and $73\% \pm 8$ of control, respectively). Open symbols track the suppression of Cav2.1_V2 by

miR124-based (circles) or shRNA-based (triangles) RNAi constructs, whereas filled symbols show the absence of suppression of $\text{Ca}_v2.1_V1$ by miR124-based (circles) or shRNA-based (triangles) RNAi constructs. * $p=0.030$ for WE1 and $p=0.045$ for WE1m1, paired t -test. Error bars are $\pm$ SEM, $n=4$.

Fig. 5. WE1 miRNA mimics mediate potent SIS-RNAi of endogenous Cav2.1_V2 in human neuronal cells

A. The activity of miR124-WE1 against endogenously expressed Cav2.1 mRNA was assessed in a human neuronal cell line (SK-N-SH cells). Quantitative PCR analysis revealed a significant reduction ($= p < 0.05$, paired t -test) in the levels of total endogenous Cav2.1 mRNA in cells treated with miR124-WE1 (mean = 18.7%, $\pm$ 4% of control, $n = 3$). **B.** Semi-quantitative analysis of Cav2.1_V1 and Cav2.1_V2 levels, assessed by direct sequencing of clones obtained from a Cav2.1-specific cDNA library generated from untreated and miRNA-treated SK-N-SH cells. Data are presented as the percentage of each isoform (Cav2.1 isoform/Cav2.1 total) within a specific sequenced population (# clones sequenced were 127, 89 and 74 for null, miR124-Miss and miR124-WE1, respectively).

Fig. 6. Effective recognition and processing of WE1 miRNA mimics by the cellular RNAi machinery

Intracellular processing of shRNA and miR124-based RNAi platforms was assessed by Northern blot analysis. **A.** Probes for the WE1 guide strand recognized two prominent small RNA transcripts (precursor and processed) in samples obtained from cells expressing shRNAs WE1, WE1m1 and WE1m2 whereas three prominent transcripts (Pri-miRNA, Pre-miRNA and mature-miRNA) were detected in samples obtained from cells expressing the miRNA mimics miR124-WE1, -WE1m1 and -WE1m2. The levels of fully processed, mature WE1 guide strands differed between the three active constructs (WE1>WE1m1>WE1m2) with this difference correlating directly with silencing efficacy (Fig 3C). Compared to miR124-WE1 precursors (pri- and pre-miRNA bands), WE1-shRNA precursors (middle band, first 3 lanes) accumulate to a greater extent. A probe against 5S RNA was used to control for differences in gel loading. **B.** Anti-WE1 passenger strand probe revealed differences in 'strand-bias' between the WE1, WE1m1 and WE1m2 duplexes. The passenger strand was nearly undetectable in shRNA-WE1 and miR124-WE1 samples, but the addition of 1 (WE1m1) or 2 (WE1m2) mismatches to the WE1 duplex led to an incremental increase in detectable passenger strand. The far right panel (*) is an extended exposure of the same autoradiograph. The Ambion Decade Marker was used to estimate the size of the RNA molecules.

Table 1

Prevalence of 3'-RACE sequences for mapping Drosha and Dicer cleavage sites

Sequencing analysis of the WE1 guide and passenger strand 3'ends. We used a modified 3'RACE protocol to sequence the 3'ends of the miR124-WE1 and shRNA-WE1 guide and passenger strands. Shown are the most prevalent sequences recovered during this analysis. Post-transcriptional nucleotide modifications to the 3'end of the miR124-WE1 guide strand are underlined.

	3'RACE sequence	Percentage	Prevalence
Guide strand	5'-TTACGGAAGTACTGCCCTGCC-3'		
miR124	5'-TTACGGAAGTACTGCCCTGCC-3'	22%	4/18
	5'-TTACGGAAGTACTGCCCTGCC <u>T</u> -3'	22%	4/18
	5'-TTACGGAAGTACTGCCCTGCC <u>TT</u> -3'	17%	3/18
	5'-TTACGGAAGTACTGCCCTGCC <u>TC</u> -3'	6%	1/18
	5'-TTACGGAAGTACTGCCCTGCC <u>TTT</u> -3'	6%	1/18
	5'-TTACGGAAGTACTGCCCTGCC <u>TAG</u> -3'	6%	1/18
	5'-TTACGGAAGTACTGCCCTGCC <u>TTAT</u> -3'	6%	1/18
	5'-TTACGGAAGTACTGCCCTGCCA <u>T</u> -3'	11%	2/18
	5'-TTACGGAAGTACTGCCCTGCCA <u>AT</u> -3'	6%	1/18
shRNA	5'-TTACGGAAGTACTGCCCTGCC-3'	50%	4/8
	5'-TTACGGAAGTACTGCCCTGCC-3'	13%	1/8
	5'-TTACGGAAGTACTGCCCTGCC <u>TT</u> -3'	38%	3/8
Passenger strand	5'-GGCAGGGCAGTAGTCCGTAA-3'		
miR124	5'-GGCAGGGCAGTAGTCCGTAA-3'	25%	2/8
	5'-GGCAGGGCAGTAGTCCGTAAAT-3'	13%	1/8
	5'-GGCAGGGCAGTAGTCCGTAAATT-3'	25%	2/8
	5'-GGCAGGGCAGTAGTCCGTAAATTT-3'	38%	3/8
shRNA	5'-GGCAGGGCAGTAGTCCGTAA-3'	100%	7/7