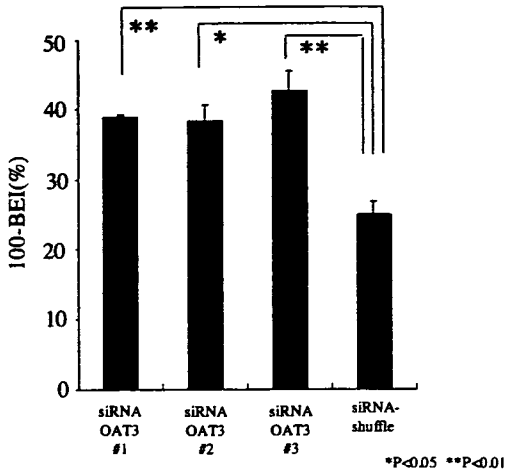

Fig. 4. Effect of siRNA on transport function of OAT3 by BEI. The 50 μg siRNA dissolved in the 5–10% volume PBS of mouse body weight was rapidly injected into the tail vein 36 h before the BEI assay. The residual radioactivity of OAT3 substrate, [^{3}H]benzylpenicillin in the brain, was measured at 40 min after intracerebral injection.

nous injection (Fig. 3A, right). These results indicate that hydrodynamic injection method is effective for delivery of siRNA to brain capillary endothelial cells.

In vivo effect of siRNA on transporter function of OAT3 in vivo

The in vivo inhibitory effect of siRNA OAT3 on the brain-to-blood efflux transport was examined with BEI method with intracerebral injection of OAT3 substrate, [^{3}H]benzylpenicillin. We intravenously injected siRNA OAT3 #2 to 11 mice, siRNA OAT3 #1 and #3 to 3 mice each, and siRNA-shuffle (control) to 7 mice with hydrodynamic method. Transport function of OAT3 was evaluated by BEI method at 36 h after the injection of siRNA. The results of 100-BEI, percentage of OAT3 substrate remaining in the brain after injection, are shown in Fig. 4. The value of 100-BEI of siRNA OAT3 #2 is significantly higher than that of siRNA-shuffle by 26.4%. Those of siRNA OAT3 #1 and #3 were also similarly higher than that of control. The results that plural different siRNAs to the OAT3 gene similarly increased 100-BEI value indicated that these siRNA influences were not "off-target effect" on molecules other than OAT3 in the brain. Taken together, these results suggested that in vivo applied-siRNA to OAT3 could suppress the brain-to-blood efflux function of OAT3.

Discussion

This is the first report of successful in vivo inhibition of endogenous gene in BCECs by systemic intravenous injection of siRNA. Furthermore, we demonstrated that our gene silencing effect was enough to suppress the transport function of OAT3 endogenously expressed in BCECs at BBB. We could deliver siRNA to BCECs by hydrodynamic injection method, but not by regular intravenous injection from the mouse tail vein. It has been thought that a rapid injection of a large bolus of solution develops a high pressure in the inferior vena cava, causing retrograde movement of the solution to the abdominal organs including liver and kidneys. Such a sharp increase in venous pressure enlarges the liver fenestrae and promotes membrane permeability of the hepatocytes, making siRNA enter the cells [16]. Since BCECs are circulated from the tail vein via lung capillary, the phasic hydrodynamic pressure in the inferior vena cava should decrease in the lung. However, rapid loading of extremely large volume of solution, 40–80% of circulating plasma volume should considerably increase hydrostatic pressure in the carotid artery due to volume overload. In addition, the rapid injection of large volume solution prevents the solution from being mixed with the serum containing RNase and keeps the concentration of siRNA extremely high when it is delivered to BCECs.

This in vivo knockdown method with siRNA to BCECs is expected to be a powerful tool for investigating function of BBB. The BBB is formed by the tight intercellular junctions of BCECs and regulates CNS homeostasis and drug delivery by restricting the transfer of substances between the circulating blood and the brain [17]. We have developed Brain Efflux Index as a reliable in vivo method of analyzing efflux transport at the BBB [18]. The efflux function of a transporter protein expressed in BCECs, such as OAT3, can be well evaluated by combining in vivo knockdown method with siRNA and BEI method.

Since synthetic siRNA does not work in the cells for no more than six days [19], long-term silencing of the target gene is necessary for investigating other functions of BCECs in the pathophysiology of atherosclerosis and Alzheimer's disease. Long-standing gene suppression can be achieved in vivo with adenovirus and adeno-associated virus (AAV) vectors expressing short hairpin RNA (shRNA) [20,21]. Actually, with the adenovirus expressing shRNA to SOD1 gene (2.0×10^9 pfu), we could efficiently suppress the endogenous SOD1 level of brain capillary-rich fraction by regular intravenous injection into mouse tail vein (unpublished data). For the evaluation of BCEC function, however, the AAV may be better than adenovirus, because BBB function should be less affected due to limited local immune response to the AAV capsid [22].

The hydrodynamic injection does not cause marked injury to organs in the animals [23], but it is hard to be clinically applied to patients because of its extremely high hydrostatic pressure and volume overload. Possible alternate is a regional delivery of large dose siRNA into carotid artery, but development of less invasive systemic delivery system in vivo is necessary for a therapeutic application of siRNA. Novel cationic liposomes have been reported to transduce efficiently siRNA into the liver [24] as well as tumor tissue [25]. These siRNAs formulated with cationic liposomes also induce interferons and cytokines in vivo through toll-like receptors [26,27] which should change the BBB function. Recently, the lipid-conjugated siRNA

at the 5′-end of the sense strand enhanced cellular uptake and gene silencing [28]. Combined with chemical modification of 2′-O-methylation and phosphorothioate to stabilize siRNA, substantial gene silencing in the liver and jejunum was achieved by a regular intravenous injection into the mouse tail vein [29]. Now, we are trying to use these new siRNA delivery methods to achieve more effective, stable, and safe gene suppression in BCECs for a clinical application.

Acknowledgments

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Transgenic Small Interfering RNA Halts Amyotrophic Lateral Sclerosis in a Mouse Model*[S]

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Many autosomal dominant diseases such as familial amyotrophic lateral sclerosis (ALS) with copper/zinc superoxide dismutase (SOD1) mutation may be induced by missense point mutations that result in the production of proteins with toxic properties. Reduction in the encoding of proteins from such mutated genes can therefore be expected to improve the disease phenotype. The duplex of 21-nucleotide RNA, known as small interfering RNA (siRNA), has recently emerged as a powerful gene silencing tool. We made transgenic (Tg) mice with modified siRNA, which had multiple mismatch alternations within the sense strand, to prevent the "shutdown phenomenon" of transgenic siRNA. Consequently, the *in vivo* knockdown effect of siRNA on SOD1 expression did not diminish over four generations. When we crossed these anti-SOD1 siRNA Tg mice with $SOD1^{G93A}$ Tg mice, a model for ALS, siRNA prevented the development of disease by inhibiting mutant G93A SOD1 production in the central nervous system. Our findings clearly proved the principle that siRNA-mediated gene silencing can stop the development of familial ALS with SOD1 mutation.

RNA interference is the process of sequence-specific, post-transcriptional gene silencing, initiated by double-stranded RNA. RNA interference is a multistep process that involves generation of 21–23-nucleotide small interfering RNA (siRNA),[3] resulting in degradation of homologous RNA. One rational approach to therapy using siRNA is to eliminate the aberrant protein encoded by mutant alleles in dominantly inherited diseases.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the degeneration of motor neurons in the central nervous system. Although most cases of ALS are sporadic, 5–10% of ALS cases are familial, and of these, ~20% are due to missense point mutations in the gene encoding copper/zinc superoxide dismutase (SOD1) (1). Recent studies using transgenic (Tg) mice and cell culture models of ALS with SOD1 mutations have indicated that SOD1 mutations induce the disease by their toxic properties, not by a loss of SOD1 activity (2). Therefore, inhibition of mutated allele expression is expected to provide a direct approach to therapy for this type of familial ALS. In cultured cells, siRNA can effectively inhibit the production of mutant proteins in various neurodegenerative diseases including ALS (3). Furthermore, virus-mediated siRNA delivered by direct injection of viral vectors to the brain or muscle delays phenotypic expression in Tg mice *in vivo* (4–7). However, it has not been proved in principle whether inhibition of mutant genes with siRNA can truly stop dominantly inherited diseases. The most difficult problem in *in vivo* therapy with siRNA is that there is no sophisticated method of delivering siRNA throughout the central nervous system. Therefore, to answer this question, as a first step, we tried to make siRNA Tg mice in which siRNA was ubiquitously expressed in the brain, and we then crossed these siRNA Tg mice with $SOD1^{G93A}$ Tg mice to efficiently deliver siRNA throughout the central nervous system.

Moreover, we utilized modified short hairpin RNA (shRNA), which has mismatch alternations within the sense strand, to make Tg mice. This method was able to enhance the genetic stability of the shRNA expression cassette in the genome over generations.

MATERIALS AND METHODS

Construction of Anti-SOD1 shRNA Expression Vector—We generated an anti-SOD1 shRNA cassette as reported previously (3). We inserted the anti-SOD1 shRNA cassette immediately downstream of the human U6 promoter in pUC19 (Takara, Tokyo, Japan), with a PGK-neo-poly(A) cassette (Fig. 1*A*). Three G → A alternations were introduced (denoted by asterisks below) in the sense strand: 5′-GGUGG*AAAUG*AAGAAAG*UAC-3′ (Fig. 1*B*). This sequence was a good and common siRNA target region in both human and mouse SOD1 mRNAs. To select this target site, we performed a BLAST similarity search to minimize off-target effects.

Generation of Anti-SOD1 siRNA Tg Mice—To produce anti-SOD1 siRNA Tg mice, the anti-SOD1 shRNA expression vector was introduced into 129/Sv embryonic stem (ES) cells (Chemicon, Temecula, CA) by electroporation, and individual stable integrants were tested for expression of SOD1 protein by Western blot analysis. ES cell clones that exhibited greatly decreased SOD1 expression were injected into C57BL/6 blastocysts (CLEA Japan, Tokyo, Japan), and the resulting chimeric male mice were mated with C57BL/6 females. The offspring, in which germline transmission was determined by the following PCR method, were referred to as anti-SOD1 siRNA Tg mice.

Double Tg mice were generated by crossing $SOD1^{G93A}$ Tg mice (G1H line from Jackson Laboratories, backcrossed to C57BL/6 mice) with anti-SOD1 siRNA Tg mice. Genotypes of these mice were determined

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental movie and a supplemental figure.

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[3] The abbreviations used are: siRNA, small interfering RNA; shRNA, short hairpin RNA; ALS, amyotrophic lateral sclerosis; SOD1, copper/zinc superoxide dismutase; Tg, transgenic; ES, embryonic stem.

by PCR analysis of tail DNA. PCR was carried out using the following primer sets: 5′-CTTGGGTAGTTTGCAG-3′ and 5′-CAGGAAACAGCTATGAC-3′ for anti-SOD1 siRNA Tg mice and 5′-CATCAGCCCTAATCCATCTGA-3′ and 5′-CGCGACTAACAATCAAAGTGA-3′ for $SOD1^{G93A}$ Tg mice. The mice were maintained under pathogen-free conditions and handled in accordance with the Guidelines for Animal Experimentation of the Institute for Advanced Technology of Kinki University and of Tokyo Medical and Dental University.

Northern Blot Analysis—Mice were deeply anesthetized with pentobarbital sodium, sacrificed, and perfused with cold phosphate-buffered saline. Total RNA was extracted from the brain and spinal cord by using TRIzol (Invitrogen). Total RNA (20 μg) was fractionated on a 1% formaldehyde agarose gel and transferred to a Nytran membrane (Schleicher & Schuell). The lower part of the membrane was hybridized with a purified PCR fragment, corresponding to mouse SOD1 cDNA (bases 15–495); it was labeled with fluorescein by using a Gene Images random-prime labeling kit (Amersham Biosciences). The upper part of the membrane was hybridized with a probe specific for β-actin. The signals were visualized with a Gene Images CDP-star detection kit (Amersham Biosciences). For detection of small RNA, total RNA (25 μg) was separated by electrophoresis on a 14% polyacrylamide-urea gel and transferred to a Hybond-N+ membrane (Amersham Biosciences). The blot was hybridized with a probe of the non-mutated sense sequence of shRNA, which was labeled with fluorescein by using a Gene Images 3′-Oligolabeling kit (Amersham Biosciences) and visualized as mentioned above.

Western Blot Analysis—ES cell lysates were prepared with radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Protein samples were extracted from tails, brains, and spinal cords and homogenized in buffer containing 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of extracted protein were mixed with Laemmli sample buffer, denatured, and separated on 15% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane (Bio-Rad), blots were probed with anti-SOD1 polyclonal antibody S-100 (1:7000, StressGen Biotechnologies, Victoria, British Columbia, Canada) or anti-β-tubulin monoclonal antibody (1:500, BD Biosciences) followed by the relevant horseradish peroxidase-conjugated immunoglobulin (Amersham Biosciences). Immunoblots were detected using ECL reagent (Amersham Biosciences).

Immunohistochemical and Histopathological Analyses—The lumbar segments of the spinal cords were removed and fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4. They were cryoprotected with sucrose solution and frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan). For immunohistochemistry, sections (10 μm thick) of the spinal cord at the level of the third lumbar (L3) vertebra from anti-SOD1 siRNA Tg mice and wild-type littermates were mounted onto the same gelatin-coated slide and incubated with anti-SOD1 polyclonal

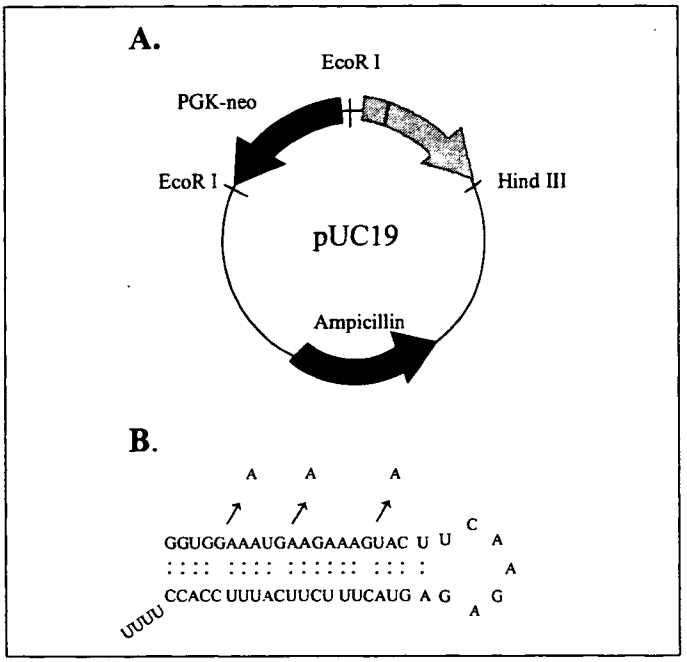

FIGURE 1. **Construction of anti-SOD1 shRNA expression vector.** *A*, the anti-SOD1 shRNA expression vector included an anti-SOD1 shRNA cassette with human U6 promoter and a PGK-neo-poly(A) cassette. *B*, predicted secondary structure of anti-SOD1 shRNA. Three G → A alternations were introduced, but only in the sense sequence of shRNA (*arrows*).

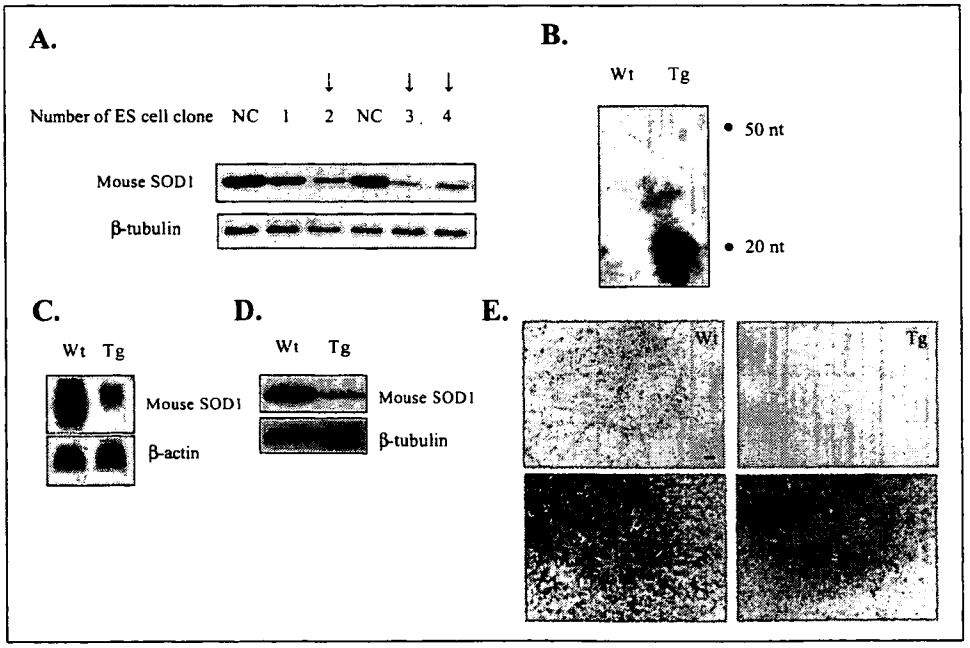

FIGURE 2. **Efficient knockdown of SOD1 in anti-SOD1 siRNA Tg mice.** *A*, production of SOD1 protein in four different ES cell clones on Western blot analysis. *NC* = negative control ES cells. ES cell clones indicated by *arrows* showed marked reduction in SOD1 production. *B*, detection of the antisense strand of siRNA in the brain on Northern blot analysis. *C*, SOD1 mRNA on Northern blot analysis in the brain. *D*, SOD1 protein on Western blot analysis in the brain. *E*, the presence of SOD1 in the spinal cord, as detected by immunohistochemistry (*upper panels*). *Lower panels*, the same sections stained with hematoxylin and eosin. *Scale bar*, 30 μm. *Tg*, anti-SOD1 siRNA Tg mouse; *Wt*, wild-type littermate.

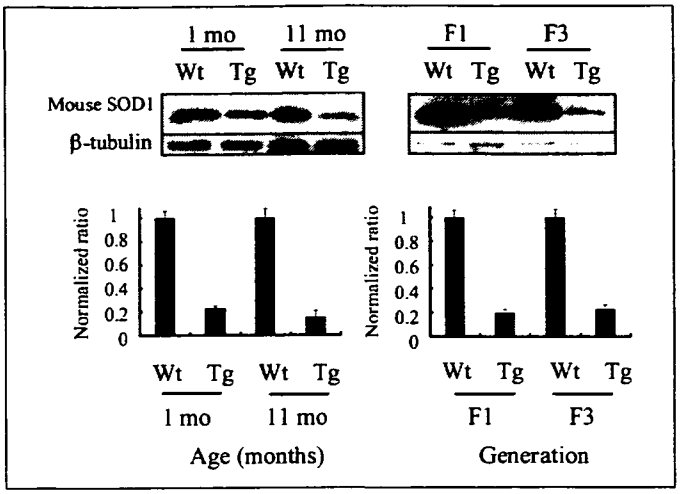

FIGURE 3. **The silencing effect of siRNA is stable.** SOD1 protein levels with aging (*left*) and from generation to generation (*right*) are shown. F1 and F3 mice were examined at 1 month (*mo*) of age. Values are the ratios to age-matched wild-type (*Wt*) littermates (mean and S.E.). $n = 3$ for each group. *ns* = not significant ($p > 0.05$, Student's *t* test).

antibody S-100 (1:1000, StressGen Biotechnologies). Staining was visualized by diaminobenzidine. For histopathological examination of the tissues of double Tg mice, SOD1^{G93A} Tg mice, and wild-type littermates, sections 10 μm thick were stained with hematoxylin and eosin.

L3 ventral roots were taken from the spinal cord and fixed in phosphate-buffered 2.5% glutaraldehyde, postfixed in 1% osmic acid, and then embedded in Epon. Toluidine blue-stained semi-thin transverse sections of these materials were used for evaluation of the density and size distribution of myelinated fibers.

Determination of Disease Onset and Progression—We compared the motor functions of double Tg mice with those of SOD1^{G93A} Tg mice and wild-type littermates by using a rotating rod apparatus (accelerating model, Ugo Basile Biological Research Apparatus, Varese, Italy). The mice were placed on the rod for four trials. Each trial lasted a maximum of 4 min, during which the rotating rod underwent linear acceleration from 4 to 33 rpm over 4 min. Disease onset was determined by the presence of hindlimb paresis on walking. Mortality was scored as date of death or inability of the mouse to right itself within 30 s of being placed on its side.

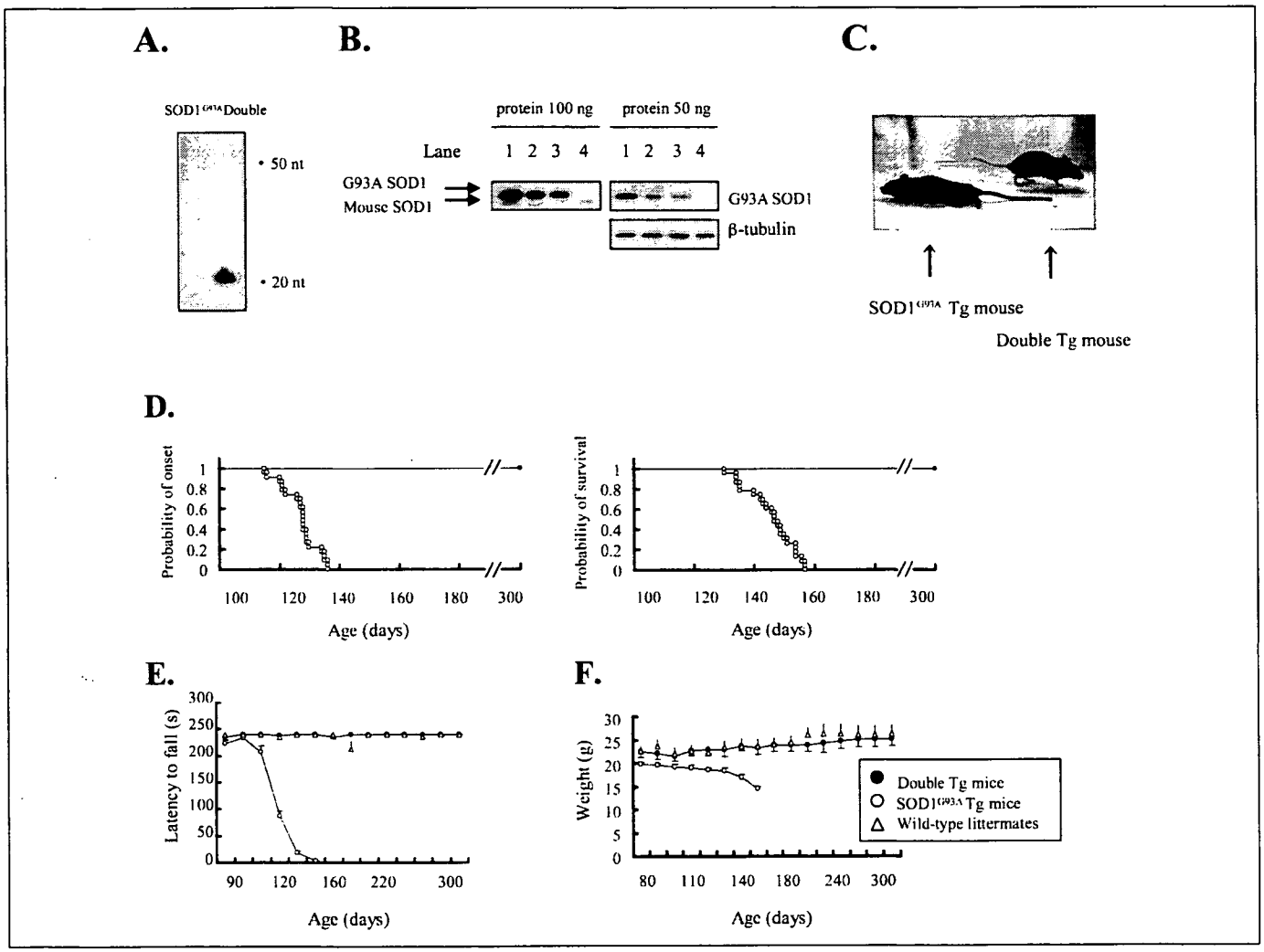

FIGURE 4. **Double Tg mice had a marked reduction in the amount of G93A SOD1 protein in the spinal cord and did not show the ALS phenotype.** *A*, detection of the antisense strand of siRNA in the spinal cord on Northern blot analysis. *nt*, nucleotides. *B*, levels of both mutant G93A SOD1 and mouse SOD1 proteins were similarly reduced in the spinal cords of double Tg mice on Western blot analysis. The level of G93A SOD1 protein in double Tg mice was lower than that of the low copy strain of SOD1^{G93A} Tg mice. *Lane 1*, SOD1^{G93A} Tg mouse; *lane 2*, low copy strain of SOD1^{G93A} Tg mouse; *lane 3*, double Tg mouse; *lane 4*, wild-type mouse. *C*, this SOD1^{G93A} Tg mouse at 130 days of age showed paralysis of both hindlimbs. In contrast, the double Tg mouse at the same age walked well. *D*, cumulative probabilities of onset of disease signs (*left*) and survival (*right*). There was a significant increase in the life span of the double Tg mice ($n = 6$; *closed circles*) compared with the SOD1^{G93A} Tg mice ($n = 23$; *open circles*). *E*, performances on the accelerating rotating rod apparatus. *F*, growth curves of female mice. Values are means and S.E.

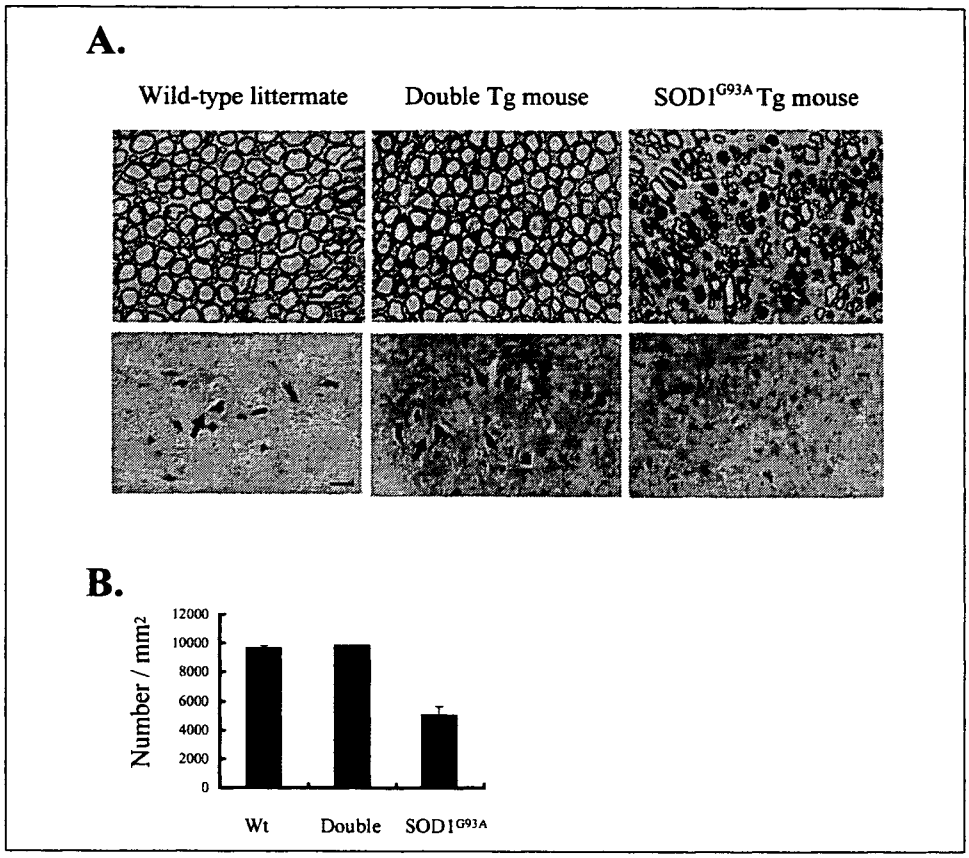

FIGURE 5. **Histological analysis of lumbar ventral root and spinal cord.** *A*, light micrographs of transverse L3 ventral root sections stained with toluidine blue (*upper panels*) and L3 spinal cords stained with hematoxylin and eosin (*lower panels*). The *arrows* indicate the anterior horn cells. *Scale bar*, 30 μm. *B*, the number of large motor fibers (≥5 μm) in L3 ventral roots. Values are means and S.E. $n = 3$ for wild-type littermates and SOD1^{G93A} Tg mice; $n = 1$ for double Tg mice.

Statistical Analysis—Statistical significance was assessed between groups by using Student's t test. Significance was set at $p < 0.05$.

RESULTS

Anti-SOD1 siRNA Tg Mice—We obtained 3 of 50 G418-resistant ES cell clones that showed an ~80% reduction in the level of endogenous SOD1 protein by Western blot analysis (Fig. 2*A*). Each ES cell clone was injected into C57BL/6 blastocysts, and chimeric male mice with high levels of ES cell descendants were obtained. These chimeras were outcrossed, and germline transmission of the shRNA was noted in numerous F1 progeny from one ES line (12/35) on PCR analysis. In the brains of anti-SOD1 siRNA Tg mice, expression of siRNA was clearly detected (Fig. 2*B*), and mouse SOD1 mRNA was strikingly reduced on Northern blot analysis (Fig. 2*C*). The level of SOD1 protein was also suppressed by about 80% on Western blot analysis (Fig. 2*D*). Anti-SOD1 siRNA Tg mice did not show any obvious phenotype such as growth retardation or motor signs, with the exception of infertility in females. In immunohistochemical analysis, the SOD1 immunoreactivity of both the gray and the white matter of the spinal cord in the anti-SOD1 siRNA Tg mice was much lower than that in the wild-type littermates. In the anterior horn of the spinal cord in anti-SOD1 siRNA Tg mice, the SOD1 immunoreactivity was reduced dominantly in the non-neuronal cells and neuropils (Fig. 2*E*).

The Silencing Effect of siRNA Is Stable with Age and through to the F3 Generation—To analyze changes in SOD1 protein levels with age and through the generations, we examined SOD1 protein levels in the tails of anti-SOD1 siRNA Tg mice and age-matched wild-type littermates by Western blot analysis. There was no obvious decrease in the effect of siRNA on knockdown of SOD1 production at 1 and 11 months old or in F1 and F3 mice (Fig. 3).

Mutant G93A SOD1 Protein Production Is Decreased in Double Tg Mice—By crossing anti-SOD1 siRNA Tg mice with SOD1^{G93A} Tg mice, we obtained six double Tg mice and 26 SOD1^{G93A} Tg mice. In the spinal cords of the double Tg mice, we clearly detected the expression of siRNA (Fig. 4*A*). Levels of both mutant human G93A SOD1 protein and mouse wild-type SOD1 protein in the spinal cords of the double Tg mice were similarly reduced. The percentage of reduction of mutant G93A SOD1 in the spinal cord was estimated to be about 80%. The level of mutant G93A SOD1 protein in the double Tg mice was about half that in the low copy strain of SOD1^{G93A} Tg mice (G1L/+ from Jackson Laboratories, backcrossed to C57BL/6 mice) in which disease onset occurred at 280 days of age (Fig. 4*B*).

Phenotype of Double Tg Mice Is Normal—SOD1^{G93A} Tg mice showed the first signs of motor deficits at a mean age of 127.3 ± 1.2 days. All of these mice then deteriorated progressively, showing a lack of mobility, failure to groom their fur, hindlimb dysfunction, breathing difficulties, and muscle atrophy. All SOD1^{G93A} Tg mice were dead by 157 days of age (Fig. 4, *C* and *D*).

In contrast, double Tg mice appeared normal and grew up similarly to wild-type littermates. Their motor performance on the rotating rod test did not differ from that of wild-type littermates over the entire 300-day duration of the experiment (Fig. 4*E*). The weights of SOD1^{G93A} Tg mice declined just before the onset of disease, but double Tg mice did not lose weight (Fig. 4*F*). The online supplemental movie dramatically shows that the transgenic anti-SOD1 siRNA completely prevented the development of the ALS phenotype seen in SOD1^{G93A} Tg mice (see Supplemental Movie).

Histological analysis of the spinal cord was performed at the end of disease in SOD1^{G93A} Tg mice and at 6 months of age in double Tg mice and the age-matched wild-type littermates. In the SOD1^{G93A} Tg mice,

the spinal cord at L3 showed a severe loss of motor neurons with an increase in the numbers of astrocytes (see Supplemental Figure), and myelinated axons in the L3 ventral root were atrophic and less dense. In contrast, the spinal motor neurons and axons in the double Tg mice appeared normal (Fig. 5, *A* and *B*).

DISCUSSION

One serious problem in using shRNA to generate Tg mice is that mutations can occur within the hairpin region of the shRNA sequence during replication, leading to a reduction in silencing efficiency with age and over generations. In fact, ~20% of our constructs without mismatch alternation were mutated within the hairpin region of the constructs upon introduction into *Escherichia coli* (8), and some anti-green fluorescent protein Tg mice lost the knockdown effect in the F1 generation, even with expression of siRNA (9). We previously showed that mismatch alternation of a few nucleotides in only the sense strand prevented mutation during replication without reducing the silencing effect (8). Thus, we introduced three mismatch alternations in the sense strand, and all of our anti-SOD1 Tg mice showed no decrease in siRNA effect over four generations. The mismatch alternations in the sense strand of our shRNA might have prevented a decrease in the siRNA effect *in vivo*. In view of these results, we think that crossing of siRNA Tg mice could be a useful strategy for analyzing the effect of knockdown of the gene of interest on the phenotype of the crossed mice.

Our results showed that development of the ALS phenotype in $SOD1^{G93A}$ Tg mice was completely suppressed by crossing with anti-SOD1 siRNA Tg mice. In our double Tg mice, siRNA overexpressed against the *SOD1* gene in anti-SOD1 siRNA Tg mice cleaved the mRNA of G93A SOD1 expressed in the crossed mice. We consider that prevention of development of the ALS phenotype in the double Tg mice was caused by the knockdown effect on SOD1 protein production. The mouse wild-type *SOD1* gene was similarly inhibited by the siRNA, but elimination of wild-type SOD1 has been reported to have no effect on the mutant SOD1-mediated ALS phenotype (10). An off-target effect of the siRNA on other unidentified mouse genes is also improbable. This is because 1) a BLAST search for our shRNA sequence showed no match in other areas of the mouse genome and 2) the infertility observed in female anti-SOD1 siRNA Tg mice has also been reported in mice with knock-out of the *SOD1* gene (11). Moreover, a close relationship between the copy number of G93A SOD1 and time of onset of the ALS phenotype is known to occur in $SOD1^{G93A}$ Tg mice (12).

More recently, direct injection of an siRNA-expressing viral vector into the spinal cord (5) or skeletal muscles (6, 7) is reported to reduce the severity of the ALS phenotype in $SOD1^{G93A}$ Tg mice. Although viral vector-mediated siRNA delayed the onset of disease or the decrease in grip strength, none of these vector-mediated siRNAs could prevent the disease. In contrast, our double Tg mice did not show any motor dysfunction at 300 days and were expected to remain free of signs of disease at 2 years of age, the time at which disease onset has been predicted from the rate of reduction in the amount of mutant SOD1 in the spinal cord (12). Most likely, this difference can be explained by the possibility that our transgenic siRNA had a greater knockdown effect than did the viral vector-mediated siRNA. Alternatively, reduction in the amount of mutant SOD1 in non-neuronal cells as well as neuronal cells in our double Tg mice might have contributed to the better outcome; the effects of siRNA were limited to the motoneurons when viral vectors were injected into the skeletal muscles (6, 7). There are several lines of evidence that production of mutant SOD1 in both neuronal and non-neuronal cells is critical in the mechanism of the disease (13–16).

Our findings clearly demonstrated that siRNA halted familial ALS by silencing the mutant gene. If a non-invasive method of delivery of siRNA to both neuronal and non-neuronal cells throughout the central nervous system can be developed, the concept of truly overcoming these autosomal dominantly inherited neurodegenerative diseases will no longer be an impossibility.

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Brief Communication

New RNAi Strategy for Selective Suppression of a Mutant Allele in Polyglutamine Disease

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ABSTRACT

In gene therapy of dominantly inherited diseases with small interfering RNA (siRNA), mutant allele-specific suppression may be necessary for diseases in which the defective gene normally has an important role. It is difficult, however, to design a mutant allele-specific siRNA for trinucleotide repeat diseases in which the difference of sequences is only repeat length. To overcome this problem, we use a new RNA interference (RNAi) strategy for selective suppression of mutant alleles. Both mutant and wild-type alleles are inhibited by the most effective siRNA, and wild-type protein is restored using the wild-type mRNA modified to be resistant to the siRNA. Here, we applied this method to spinocerebellar ataxia type 6 (SCA6). We discuss its feasibility and problems for future gene therapy.

INTRODUCTION

RNA INTERFERENCE (RNAi) is a powerful tool for posttranscriptional gene silencing. Small interfering RNA (siRNA) binds and cleaves the targeted RNA in a sequence-specific manner, thereby preventing translation of the encoded protein (Elbashir et al., 2001a). One possible therapeutic application of siRNA is the silencing of mutant genes that cause dominantly inherited diseases. We and others demonstrated that it is possible to design siRNA that selectively suppresses the expression of the mutant protein in amyotrophic lateral sclerosis (ALS), Alzheimer's disease, polyglutamine disease, and DYT1 dystonia (Gonzalez-Alegre et al., 2003; Miller et al., 2003, 2004; Li et al., 2004; Yokota et al., 2004). Recently, adeno-associated virus expressing siRNA injected into the cerebellum improved the polyglutamine-induced phenotype in a transgenic mouse model (Xia et al., 2004).

Although siRNA can discriminate even a single nucleotide alternation (Elbashir et al., 2001b), selectivity is not complete (Gonzalez-Alegre et al., 2003; Miller et al., 2003, 2004; Li et al., 2004; Yokota et al., 2004), and at a higher concentration of siRNA, the wild-type allele is more inhibited (Miller et al., 2004). In addition, the cleavage efficiency of the mutant allele is not necessarily excellent because selection of the mutant allele-specific siRNA has a restriction; that is, the siRNA target sequence should include the mismatch. In diseases caused by an expanded trinucleotide repeat, such as polyglutamine diseases, it is impossible to design siRNA that can recognize the expanded CAG repeat. In spinocerebellar ataxia type 3 (SCA3), a C/G polymorphism related to CAG repeat expansion has been used to design siRNA to discriminate the expanded allele (Miller et al., 2003; Li et al., 2004). We reported on siRNA with relative discrimination of the expanded allele of the SCA3 gene, which is possibly due to a

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change in the RNA secondary structure (Li et al., 2004). In SCA6, however, the CAG repeat length in the mutant allele is within the normal range of other polyglutamine diseases, so that the secondary structure of RNA does not alter greatly even in the mutant. Here, we show a new alternate method for allele-specific suppression by siRNA. After suppressing both mutant and wild-type proteins by the most effective siRNA, wild-type protein is returned by coexpression of siRNA-resistant wild-type mRNA. This new strategy can be applied to any mutation.

MATERIALS AND METHODS

Plasmid construction and siRNA synthesis

Construction of expression plasmids of the α1A calcium channel gene (CACNA1A) was reported previously (Kubodera et al., 2003). The CACNA1A cDNA in the plasmid was truncated at the 3′-terminal region containing 13 (normal, pQ13C) or 28 (expanded, pQ28C) CAG repeats. Each construct corresponds to nucleotide positions 6727–7521 and 6727–7566 of the full-length CACNA1A cDNA. Modified pQ13C, in which CACNA1A cDNA is mutated not to be cleaved by siRNA7493 but translated to the same amino acids as those of pQ13C, was made by PCR amplification using pQ13C as a template (Fig. 1). The reverse primer included the modified mutations (light gray in Fig. 1), and the PCR fragment was subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA).

Selection of an siRNA target site was made according to the reported protocol (Reynolds et al., 2004). Sense and antisense strands of siRNA oligonucleotides (ODNs) were synthesized and prepared as described previously (Yokota et al., 2003). siRNA for an unrelated target, hepatitis C virus (HCV) gene, was used as a negative control.

Transfection and Western blotting

To see the effect of siRNA for CACNA1A mRNA, we cotransfected both siRNA and expression plasmids of CACNA1A to human emboryonic kidney 293T cells. Transfection was done with Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol.

For Western blotting at 24 hours posttransfection, cells were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100), separated on a 10%–20% gradient of SDS-PAGE, immunoblotted with rabbit polyclonal antibody specific for the C-terminal portion of the α1A calcium channel protein (A6PRT-C) (Ishikawa et al., 1999), then made visible by enhanced chemiluminescence (ECL) (Amersham Bioscience, Buckinghamshire, England).

RESULTS

Using the synthetic siRNA (siRNA7493; 5′-ACAGCGAGAGUGACGAUGAdTdT-3′ in sense sequence), expressions of both wild-type (Q13C) and mutant (Q28C)

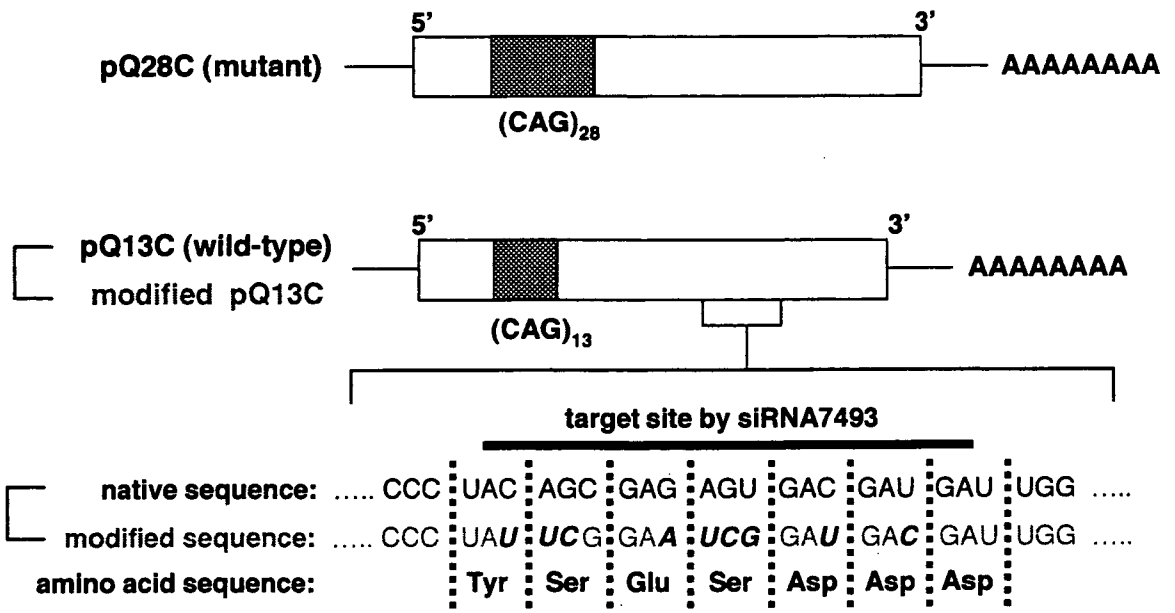

FIG. 1. Schema of mRNAs transcribed by expression plasmids of truncated CACNA1A. The RNA sequence around the target site of siRNA7493 is shown at bottom. The bold bar indicates the targeted sequence by siRNA7493. Characters in boldface italics are RNA nucleotides that are mutated from the wild-type. There is no change in amino acid sequences expressed by pQ13C or modified pQ13C.

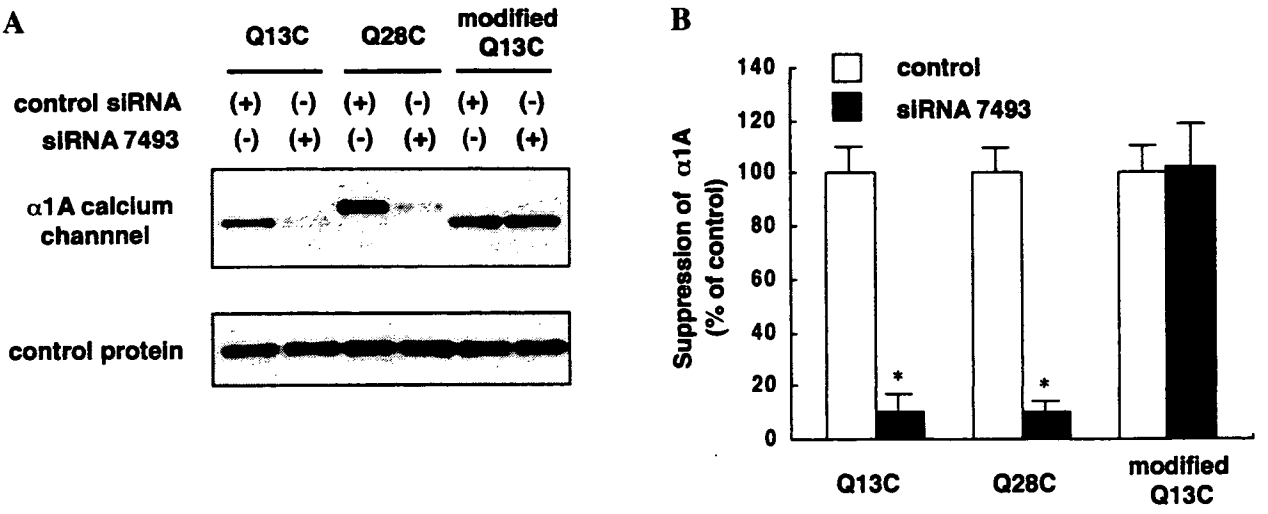

FIG. 2. Effect of siRNA7493 on expression of pQ13C, pQ28C, and modified pQ13C. (**A**) Western blot analysis of cells transfected with the indicated expression plasmids and control siRNA/siRNA7493 at 25 nM. The superoxide dismutase 1 (SOD1) protein staining was used as a loading control. (**B**) Quantitation of signal intensities. The suppression level of target protein was compared with transfection of control siRNA. siRNA7493 could markedly silence pQ13C and pQ28C but not modified pQ13C. Values are means ± SEM. $*p<0.001$ (Student's *t*-test).

α1A calcium channel proteins were markedly decreased by >90% on Western blot analysis (Fig. 2).

Because the siRNA7493 did not discriminate wild-type and mutant allele, we tried to restore the wild-type α1A calcium channel protein expressed by modified pQ13C, in which CACNA1A cDNA sequence is resistant to siRNA7493. We modified wild-type CACNA1A cDNA construct (modified pQ13C). The amino acid sequence encoded by modified pQ13C was the same as that of native pQ13C, but the nucleotide sequence targeted by siRNA was altered (Fig. 1). In fact, expression of modified pQ13C was not suppressed at all by siRNA7493 (Fig. 2).

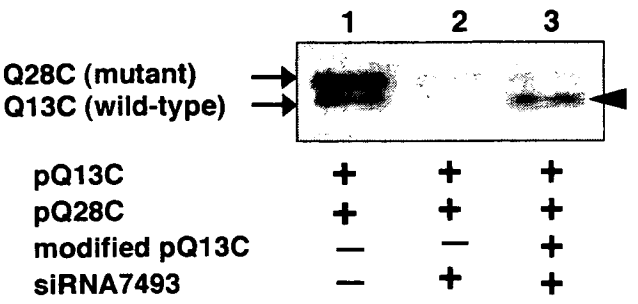

FIG. 3. Effect of siRNA7493 in cells cotransfected with pQ13C, pQ28C, or modified pQ13C (25 nM). Expressions of both pQ13C and pQ28C were markedly decreased by siRNA7493 (lane 2), but wild-type α1A calcium channel was restored to the same intensity level as that of lane 1 by expression of modified pQ13C (arrowhead) (lane 3). siRNA7493 (−) indicates no siRNA.

Next, we cotransfected modified pQ13C with pQ13C, pQ28C, and siRNA7493 to 293T cells. Modified pQ13C restored the expression of the wild-type α1A calcium channel that had been markedly inhibited by siRNA7493 (Fig. 3). Consequently, the mutant allele of CACNA1A was selectively silenced, whereas the wild-type protein was unchanged.

DISCUSSION

SCA6 is an autosomal dominant cerebellar ataxia characterized by late onset, pure cerebellar ataxia (Ishikawa et al., 1999). The causative gene for SCA6 has been identified as CAG repeat expansion in the α1A voltage-dependent calcium channel gene (Zhuchenko et al., 1997). The α1A subunit mediates Ca^{2+} influx across presynaptic and somatodendritic membranes, thereby triggering fast neurotransmitter release and other key neuronal responses. α1A-deficient mice develop ataxia and dystonia and die before 4 weeks of age (Jun et al., 1999), and the natural mutant mice of cacna1a, *leaner*, in which channel function is severely reduced, produce severe ataxia (Lorenzon et al., 1998). In gene therapy of SCA6 with siRNA, therefore, reduction of endogenous α1A calcium channel expression may produce an undesirable effect, and preservation of wild-type α1A calcium channel expression is necessary.

Our new method for allele-specific suppression by siRNA has the following advantages. (1) Any type of mutation can be applied by our method. (2) Only one set of the siRNA and wild-type protein-expressing vector in

our strategy works in many different mutants in a single gene; there are more than 100 mutations of superoxide dismutase 1 (SOD1) gene for familial ALS (*alsod1.iop.kcl.ac.uk/*) and presenilin 1 (PS1) gene for familial Alzheimer's disease (*www.molgen.ua.ac.be/ADMutations/*). (3) Greater than 90% suppression efficiency of mutant allele expression usually can be achieved using the recent prediction program of siRNA site (Reynolds et al., 2004) because the best siRNA site can be selected from the whole sequence of the target mRNA. In contrast, suppression efficiency of conventional siRNA should include the mutation, so that the target region is limited.

On the other hand, this method has the following important problems. (1) The expression level of the restored wild-type protein is difficult to control. If it is much greater than the endogenous level, it may produce an unexpected side effect. (2) Both siRNA and restored wild-type protein should be delivered to every cell, preferably by putting both expressing cassettes of short-hairpin RNA and siRNA-resistant wild-type cDNA into a single vector. To date, however, ODN is better than expression vectors for *in vivo* delivery of siRNA by systemic intravenous injection (Anton et al., 2002; Soutscheck et al., 2004). (3) There may be unknown differences of endogenous and exogenously expressed protein functions.

The efficacy of this strategy should be confirmed in an *in vivo* model, and the cited problems must be further addressed. Our new approach promotes the feasibility of using siRNA-based gene therapy for dominantly inherited diseases.

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