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Allele-specific Gene Silencing of Mutant mRNA Restores Cellular Function in Ullrich Congenital Muscular Dystrophy Fibroblasts

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Ullrich congenital muscular dystrophy (UCMD) is an inherited muscle disorder characterized clinically by muscle weakness, distal joint hyperlaxity, and proximal joint contractures. Sporadic and recessive mutations in the three collagen VI genes, *COL6A1*, *COL6A2*, and *COL6A3*, are reported to be causative. In the sporadic forms, a heterozygous point mutation causing glycine substitution in the triple helical domain has been identified in higher rate. In this study, we examined the efficacy of siRNAs, which target point mutation site, on specific knockdown toward transcripts from mutant allele and evaluated consequent cellular phenotype of UCMD fibroblasts. We evaluated the effect of siRNAs targeted to silence-specific *COL6A1* alleles in UCMD fibroblasts, where simultaneous expression of both wild-type and mutant collagen VI resulted in defective collagen localization. Addition of mutant-specific siRNAs allowed normal extracellular localization of collagen VI surrounding fibroblasts, suggesting selective inhibition of mutant collagen VI. Targeting the single-nucleotide *COL6A1* c.850G>A (p.G284R) mutation responsible a sporadic autosomal dominant form of UCMD can potently and selectively block expression of mutant collagen VI. These results suggest that allele-specific knockdown of the mutant mRNA can potentially be considered as a therapeutic procedure in UCMD due to *COL6A1* point mutations.

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Subject Category: siRNAs, shRNAs, and miRNAs Therapeutic proof-of-concept

Introduction

Ullrich congenital muscular dystrophy (UCMD) is clinically characterized by congenital muscle weakness, respiratory failure, and proximal joint contracture and scoliosis.[1] The prevalence of UCMD has been reported to be 1.3 per million in northern England.[2] In Japan, UCMD is the second most common congenital muscular dystrophy.[3,4] Autosomal recessive forms (recessive UCMD) are caused by homozygous or compound heterozygous null or frame-shift mutations in *COL6A1, COL6A2*, and *COL6A3* genes,[5] while the autosomal dominant or sporadic forms (dominant UCMD) are caused by heterozygous missense mutation for glycine substitution, in-frame exon deletion or in-frame splicing mutation in the region encoding triple helical domain in the genes.[6] Natural history studies on UCMD in Japan showed that sporadic cases account for 85% of the total UCMD cohort,[3] but both recessive and sporadic UCMD patients share similar clinical course and symptoms.[7] Up to this time, there is no approved pharmacological therapy for UCMD. However by the analyses in UCMD model mice, the impairment of mitochondrial function and autophagy process were reported and the pharmacological therapy has been suggested.[8,9]

Collagen VI consists of three polypeptide subunits, α1, α2, and α3, which are encoded by *COL6A1-3* genes.[10] All three subunits consist of an N-terminal globular domain, a triple helical domain, and a C-terminal globular domain homologous to von Willbrand factor A domain.[11] Triple helical domain of each subunit contains characteristic repetitive Gly-X-Y sequences which contribute to the heterotrimeric triple-helical structure formation of each subunit as other types of collagens.[12] The assembly of collagen VI starts with the intracellular association of the three subunits in the endoplasmic reticulum into a triple helical monomer, followed by formation of antiparallel dimers and alignment to form tetramers.[13] After being secreted into the extracellular space, collagen VI tetramers form microfibrils by end-to-end association.[14,15]

There are two pathological features of collagen VI deficiency in skeletal muscles of UCMD patients, collagen VI complete deficiency and sarcolemma-specific collagen VI deficiency.[16] CD is observed in recessive UCMD in which a truncated subunit with a null mutation is not incorporated into a collagen VI complex and residual subunits are retained within the cells. Sarcolemma-specific collagen VI deficiency, on the other hand, is observed in sporadic UCMD in which mutated collagen VI subunits are incorporated into collagen VI tetramers and cause the dominant-negative effect in collagen VI fibrils, seen as decreased sarcolemmal localization.[17]

Gene-specific therapy is a powerful and promising method for the correction of genetic disorders. It has been suggested that antisense oligonucleotide therapy, which attempt selective reduction in the expression of mutant transcripts, may have benefit for dominant UCMD, because the treatment needs to be carried within the cells before tetrameric formation and secretion of mutated collagen VI. A previous study used

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antisense oligonucleotides to induce selective out-of-frame exon skipping in a mutant transcript via a specific single-nucleotide polymorphism (SNP) recognition.[18] Since null mutations in *COL6A1-3* are attributed to recessive UCMD[5] and in-frame exon skipping of *COL6A1-3* transcripts causes dominant UCMD,[19,20] specific and selective methods for gene silencing of mutant transcript are required. Recently, allele-specific gene knockdown of a mutant transcript with small interference RNA (siRNA) has been reported.[21] Systematic methodology for design of siRNA allowed us to target point mutations of mutant allele in the disease. In this study, we aimed to establish the allele-specific gene silencing procedures in dominant UCMD. We targeted c.850G>A (p. G284R) point mutation in *COL6A1* gene which is the most common mutation in UCMD in Japan.[3] We designed and selected effective siRNAs using transfected cell line, and examined the efficiency of allele-specific knockdown and consequent effects on synthesis of collagen VI in UCMD primary fibroblasts.

Results

Differential inhibition of mutant COL6A1 versus wild type by specific siRNAs in cultured cells

We synthesized six kinds of siRNAs targeting c.850G>A of *COL6A1* with the point mutation at positions 7–12 as shown in **Figure 1a**. For the screening of siRNA, we made two kinds of artificial reporter constructs harboring *Renila* luciferase or *Photinus* luciferase and siRNA-targeted sequences with/without a G>A mutation in 3′UTR as shown in **Figure 1b**. All siRNA suppressed the luciferase activities of mutated constructs significantly compared to those of wild-type ones in NIH3T3 cells. Among them, siRNAs 8A and 11A most effectively suppressed the luciferase activities of mutated constructs (**Figure 1c**). We also analyzed the gene suppression efficacies of siRNAs on wild-type/mutated full-length *COL6A1* transcripts in NIH3T3 cells. siRNA 8A showed the specific reduction in mutant *COL6A1* transcript ($P < 0.005$) (**Figure 1d,e**), while 11A showed a

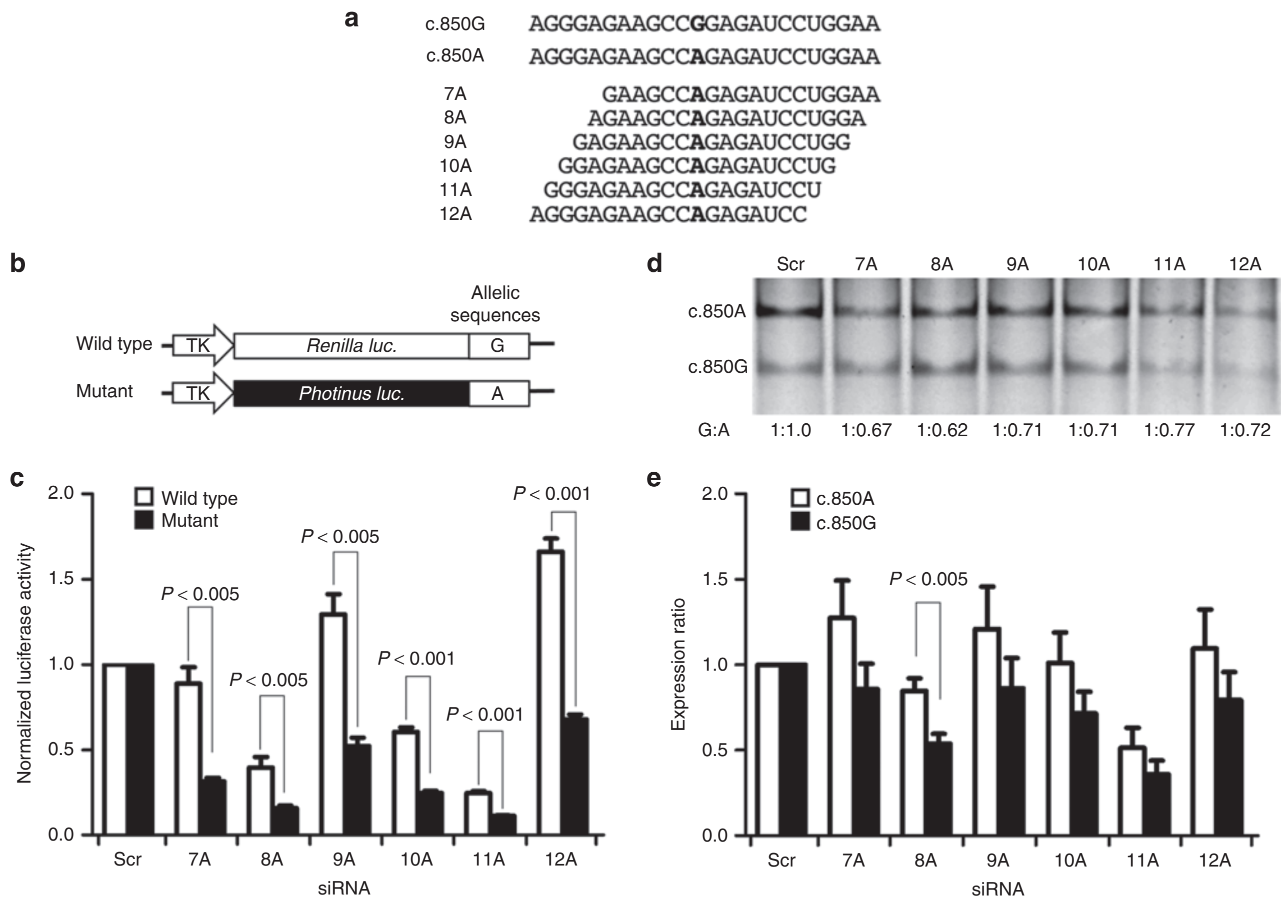

Figure 1 Screening of siRNA with point mutation. (**a**) Nucleotide sequences of wild-type and mutant *COL6A1* transcript and designed siRNAs. Wild-type (c.850G) and mutant (c.850A) *COL6A1* mRNA sequences around mutation site (bold) are shown. siRNAs are represented based on the sequence of the sense-strand siRNA. (**b**) Scematic drawing of reporter alleles. Reporter alleles were constructed based on *Photinus* and *Renilla luciferase* reporters driven by the same TK promoter. Wild type, *Renilla luciferase* reporter with allelic sequence of wild-type in 3′-UTR; Mutant, *Photinus luciferase* reporter with allelic sequence of mutant in 3′-UTR. (**c**) Gene silencing effects of siRNAs 7A to 12A to luciferase reporter genes. Normalized luciferase activities are plotted. Values are calculated relative to scramble siRNA using average of three to nine independent experiments. (**d**) Allele selectivity in gene silencing with siRNAs 7A to 12A. RT-PCR products for *COL6A1* transcript were digested with *Msp*I. The 46- and 28-bp bands represent mutant (c.850A) and wild-type (c.850G) fragments, respectively. G:A denotes ratios of normal (G) to mutated (A) transcripts in expression. Scr, scramble siRNA. (**e**) Effects of siRNA on gene silencing of wild-type and mutated *COL6A1* transcripts. Relative expression of *COL6A1* to *Neomycin* was plotted. Values are calculated relative to scramble siRNA using average of four to eight independent experiments. Error bars represent SEM.

remarkable knock-down effect toward total *COL6A1* transcripts without specificity toward the mutated transcript (**Figure 1d**,**e**) as seen in **Figure 1c**. siRNAs, 7A, 9A, 10A, and 11A showed significant suppression of a mutant construct in luciferase assay (**Figure 1b**), but such significant effects were not observed for full-length *COL6A1* transcripts (**Figure 1d**,**e**).

We attempted to increase the specificity of siRNAs to mutant transcripts by introducing a mismatch nucleotide (**Figure 2a**). By introducing a mismatch to 11A, all siRNAs, 11A-13U to 11A-17G reduced gene suppression efficacies on both luciferase constructs, but the gene suppression specificities to the mutants were rather remarkably elevated by reducing suppression rates on the wild-type constructs (**Figure 2b**). All siRNAs, however, did not have any significant improvement on specificity of gene suppression of full-length mutated *COL6A1* transcripts (**Figure 2c**,**d**).

Similarly, in introduction of mismatch nucleotides to 8A, some siRNAs (8A-12U, 8A-13A, 8A-15G, 8A-16A, and 8A-17C) enhanced the specificity in suppression of the mutant contracts on luciferase assay (**Figure 3a**,**b**). On full-length *COL6A1* transcripts, 8A-13A and 8A-16A showed similar specificity as 8A toward the mutated transcripts (**Figure 3c**). Notably, only 8A-13A showed the most robust knock-down effect on a mutant transcript ($P < 0.001$) as compared to the nonmismatch siRNA, 8A ($P < 0.005$, **Figure 3d**). Considering gene suppression effects on luciferase reporter constructs and full-length mutated *COL6A1* transcripts, we used siRNAs, 8A and 8A-13A for further studies.

siRNAs effectively knockdown mutant and wild-type *Col6A1* transcripts in UCMD fibroblasts

To verify the ability of the selected siRNA sequences, 8A and 8A-13A to target *COL6A1*, we transiently transfected them to UCMD primary fibroblasts and determined mRNA expression by quantitative RT-PCR (Q-PCR). With 8A siRNA, the expression of mutant *COL6A1* transcript was specifically suppressed to 24–67%, and 24–58% (in average, 40 and 42%) of original expression in fibroblasts from UCMD1 and UCMD2 patients, respectively, although expression of normal transcript was also decreased to 30–101 and 41–81% (in average, 59 and 61%) in UCMD1 and UCMD2 fibroblasts, respectively (**Figure 4a**). 8A-13A siRNA showed higher suppression of mutant transcript to 27–60% and 20–57% (in average, 45 and 41%) of original expression in fibroblasts from UCMD1 and UCMD2 patients, but the specificity to mutant transcript was similar to those with 8A. In UCMD cells, the selectivity to mutant transcript is noted, as there is a significant difference in expression of each transcript ($P < 0.01$–0.001, **Figure 4a**).

siRNAs restore collagen VI localization in UCMD fibroblasts

To check the effect of gene knockdown on protein translation, we checked collagen VI production in primary fibroblasts

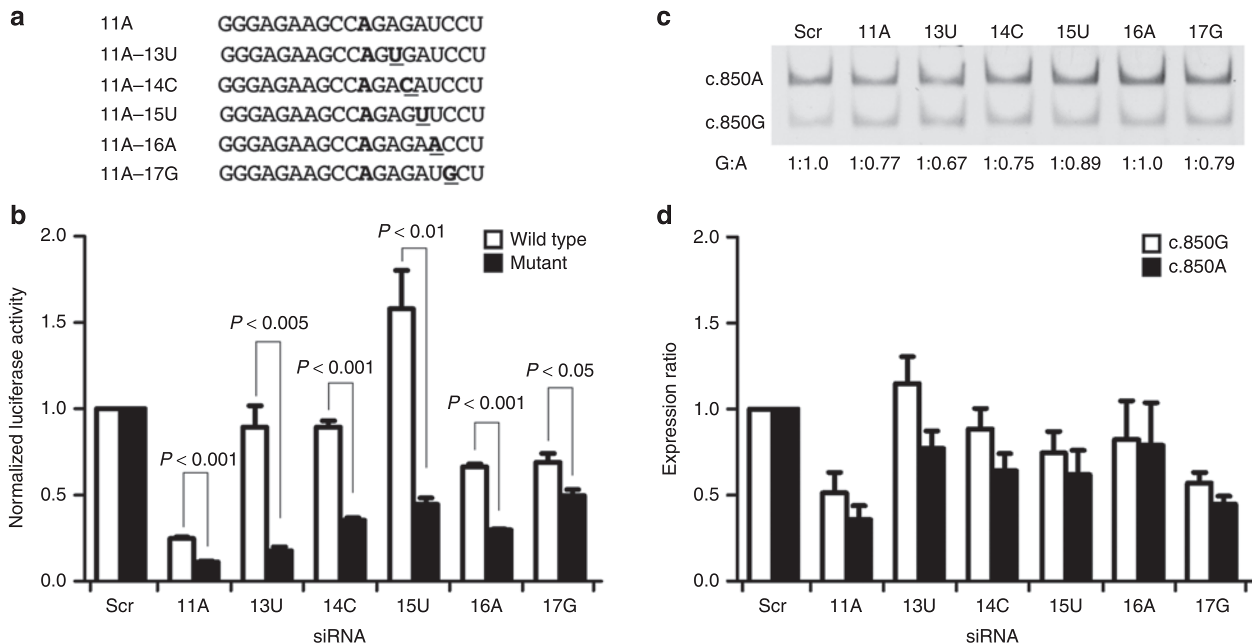

Figure 2 Screening of siRNA (11A) with point mutation. (**a**) Nucleotide sequences of siRNAs with a mismatch nucleotide (11A-13U to 11A-17G). siRNAs are represented based on the sequence of the sense-strand siRNA. Sequence of 11A is shown in top. Mutation sites (bold) and mismatch nucleotides (bold and underlined) are shown. (**b**) Gene silencing effects of a series of 11A siRNAs to luciferase reporter genes. Scr, scramble siRNA; 13U, 11A-13U; 14C, 11A-14C; 15U, 11A-15U; 16A, 11A-16A; 17G, 11A-17G. Values are calculated relative to scramble siRNA using average of three to nine independent experiments. (**c**) Allele selectivity in gene silencing of a series of 11A siRNAs. Mutant (c.850A) and wild-type (c.850G) transcripts were measured as shown in Figure 1. G:A denotes ratios of normal (G) to mutated (A) transcripts in expression. (**d**) Gene silencing effects of a series of 11A siRNAs to wild-type and mutated *COL6A1* transcripts. Relative expression of *COL6A1* to *Neomycin* is plotted. Values are calculated relative to scramble siRNA using average of four to eight independent experiments. Error bars represent SEM.

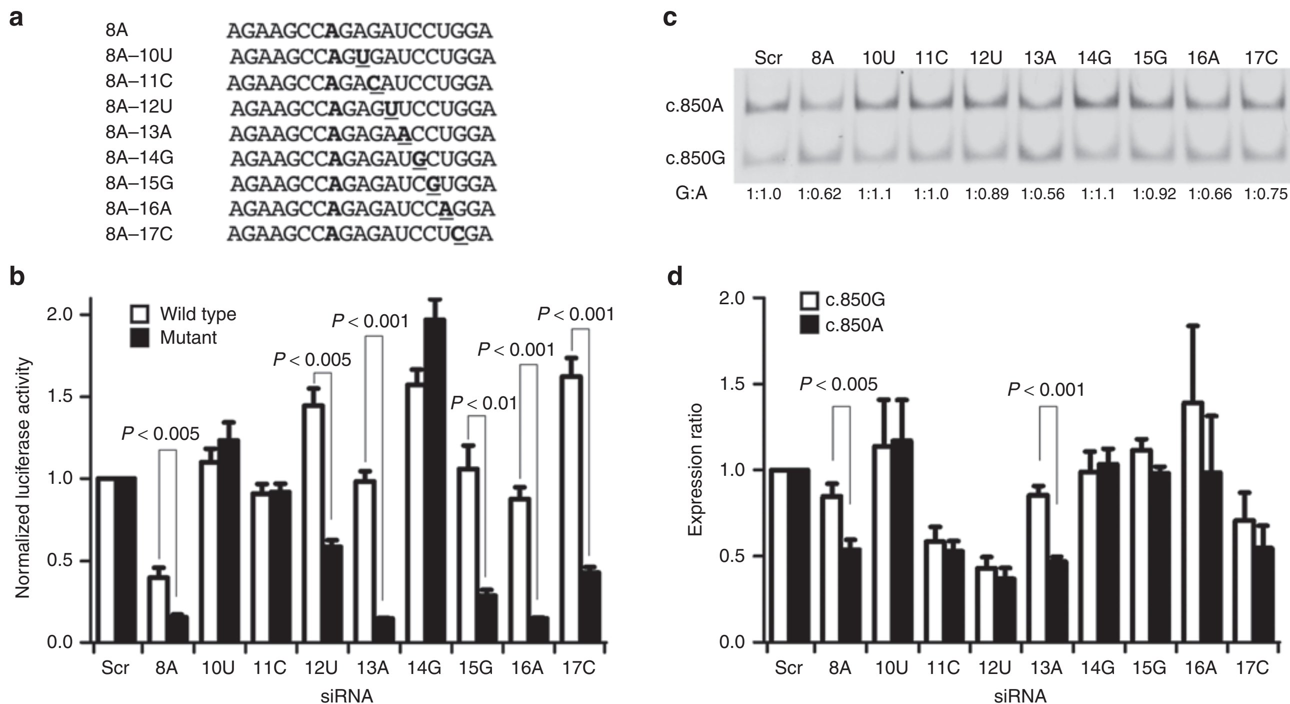

Figure 3 Screening of siRNA (8A) with point mutation. (**a**) Nucleotide sequences of designed siRNAs with a mismatch nucleotide (8A-10C to 8A-17C). Designed siRNAs are represented based on the sequence of the sense-strand siRNA. Sequence of 8A is shown in top. Mutation sites are shown in bold and mismatch nucleotides are shown in bold and underlined. (**b**) Gene silencing effects of a series of 8A siRNAs to luciferase reporter genes. Scr, scramble siRNA; 10U, 8A-10U; 11C, 8A-11C; 12U, 8A-12U; 13A, 8A-13A; 14G, 8A-14G; 15G, 8A-15G; 16A, 8A-16A; 17C, 8A-17C. Values are calculated relative to scramble siRNA using average of three to nine independent experiments. (**c**) Allele selectivity on gene silencing of a series of 8A siRNAs. Mutant (c.850A) and wild-type (c.850G) transcripts are measured as shown in **Figure 1**. G:A denotes ratios of normal (G) to mutated (A) transcripts in expression. (**d**) Gene silencing effects of a series of 11A siRNAs on wild-type and mutant *COL6A1* transcripts. Relative expression of *COL6A1* to *Neomycin* is plotted. Values are calculated relative to scramble siRNA using average of four to eight independent experiments. Error bars represent SEM.

after siRNA transfection. siRNAs 8A and 8A-13A improved the localization of collagen VI around cells (**Figure 4b**), suggesting that the production of mutant or defective collagen is reduced.

Discussion

In this study, we developed a method for allele-specific gene silencing with siRNA that targets a *COL6A1* c.850G>A, a mutation that is common in patients with autosomal dominant (sporadic) UCMD cases, which account for 85% of all UCMD patients. Several efforts to understand the mechanism of disease in sporadic UCMD have shown that the mutant collagen VI molecules in excreted collagen VI microfibrils results in a dominant-negative effect.[6,7] But because *COL6A1* encodes an essential protein, treatment would necessitate selective inhibition of mutant gene expression without affecting wild-type expression. Thus, specific suppression of mutant allele would be a promising strategy as the other dominant-inherited diseases. Several methods to modulate gene expression have been developed, including gene editing by zinc finger nuclease, TALEN, CRISPER/CaS, and modification of transcripts by exon skipping and gene knockdown, and protein inhibition by antibodies and chemicals. Among these methods, allele-specific gene knockdown and exon skipping may be the most reasonable therapeutic method for sporadic UCMD, because mutated molecules form a high-order complex with normal molecules by showing the dominant-negative effects within the cells. Recently, Gualandi *et al.*[18] reported antisense oligonucleotide-mediated messenger depletion by targeting common SNP. They induced out-of-frame exon skipping in COL6A2 mutant transcripts, which has resulted in depleting the mutated transcript via nonsense-mediated RNA decay. Mutant/wild-type ratio of COL6A2 transcripts was improved after antisense oligonucleotides treatment (82.7–73%; before and after the treatment). They also reported the recovery of collagen VI secretion and collagen-based interconnected microfilament network in the extracellular matrix. Indeed, they showed efficacy of antisense oligonucleotide-mediated specific exon skipping of a mutant transcript and that discriminate SNP sites from mutation sites in triple helical regions. Since almost of the patients with a heterozygous mutation are sporadic, the common SNPs cistronic with a dominant mutation would not be always found in different ethnic populations. Targeting specific mutation sites is a good alternative in designing siRNA. In our previous report,[3] among the 28 sporadic UCMD patients in Japan, 13 patients have a point mutation in *COL6A1-3* and 4 patients have c.850G>A in *COL6A1*. Since the other mutations are diverse, for the therapies to all of point mutations, we should design nine kinds of siRNAs, but once the concept and strategies are established, it would be easy to apply this method for another cases.

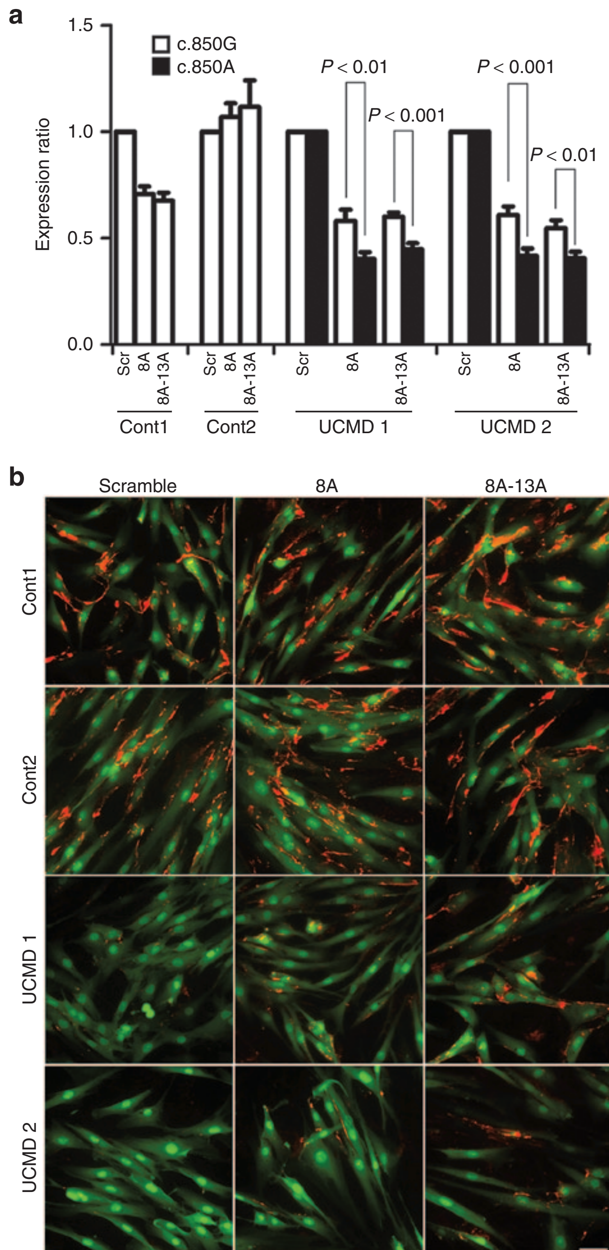

Figure 4 Gene silencing effects of siRNAs, 8A and 8A-13A to Ullrich congenital muscular dystrophy (UCMD) skin fibroblasts. (**a**) Gene silencing effects of siRNAs, 8A and 8A-13A on normal control (cont1 and cont2) and UCMD (UCMD1 and UCMD2) fibroblasts. The relative expression of mutant (c.850A) and wild-type (c.850G) *COL6A1* transcripts to GAPDH transcript is shown. Values are calculated relative to scramble siRNA in average of 10 and 14 independent experiments for normal and UCMD fibroblasts, respectively. Error bars represent the SEM. (**b**) Recovery of the collagen VI localization of UCMD fibroblasts by siRNA treatment. Red, collagen VI; green, whole cell stain. Scale bar denotes 100 μm.

Allele-specific siRNA therapies in models of neurodegenerative disorders and muscular dystrophies have demonstrated a robust and specific silencing of mutant transcripts by 75%.[21,22] In this study, we showed that designing siRNAs which can effectively discriminate between mutant and wild-type alleles is largely affected by the position of mutation site in siRNA, as seen in siRNA 8A. In addition, introduction of a nucleotide mismatch in the seed region enhanced the ability of siRNAs to discriminate between the mutant and wild-type transcripts.[23] The mismatch at c.855 site in 8A siRNA (8A-13A) improved the specificity to allelic knockdown. However, introduction of a mismatch at the other sites unexpectedly showed strong *COL6A1* gene silencing but diminished the specificity to the mutant transcript, suggesting that mismatch bases do not always work in improving allele specificity, especially on the full-length transcripts.

Relatively low gene silencing efficacy of siRNA to full-length *COL6A1* transcripts as compared to artificial reporter transcripts which only contain mutation sites, allows us to imagine there are another factors determining the gene silencing effect other than base-pairing between the siRNA and target RNA. In our experiments using artificial luciferase reporters inserted target sequences in 3′UTR, we found the specific and higher gene-silencing effects to the c.850A mutant construct by designed siRNAs. However, we did not find such specificities of gene silencing effects to c.850A mutant in the experiments with full-length *COL6A1* transcript. These results suggest that although base-pairing formation between the siRNA and target RNA would be important for the working of siRNA, the secondary structure of *COL6A1* mRNA around a mutation site also might be important. The structural prediction indicates that the region around c.850G>A mutation site of *COL6A1* mRNA is GC-rich and repetitive sequence, and has a specific secondary structure to be tightly compacted by hairpin loop formation as shown in **Supplementary Figure S1**. Interestingly, the effective siRNAs (8A and 11A) are aligned to match the end of base pairing, in which they would be inserted to all bases in a series of base-pairing and dissociate such a hairpin structure. Moreover, at position c.850, guanine in normal allele would make base pairing within a transcript, while adenine in mutated transcript does not. Thus, for the design of siRNAs against repetitive sequence, a secondary structure in transcript should be taken in consideration.

In this article, we employed primary fibroblasts for evaluating the effect of siRNAs. Although the nature of these phenotypes in UCMD fibroblasts has not been clarified, the mislocalization of collagen VI into the cell surface mimics the sarcolemma-specific collagen VI deficiency status in UCMD muscles, making this cell model an excellent tool to evaluate effect of therapeutic treatment as a primary screening. We previously reported that impairment of the retention of secreted collagen VI microfibrils on the cell surface in UCMD fibroblasts.[24] In this study, this cell phenotype was improved by the treatment with siRNAs, 8A and 8A-13A, suggesting that allele-specific gene silencing may be useful in recovering cell function in UCMD, even though the consequent expression rate of wild-type *COL6A1* transcript to mutant in UCMD fibroblast by siRNA treatment was 3 to 2. We should consider the dominant-negative effects of mutated COL6A1 molecules on construction of collagen VI microfilament in previous model by Pan *et al.*[20]: when the molecular stoichiometry of the normal COL6A1 subunits to mutants is 1:1 in UCMD, normal collagen VI tetramers would be present in $(1/2)^4 = 1/16$ (6.25%). When the molecular stoichiometry of the normal subunits to mutants shifts to 3:2 by siRNA treatment, normal collagen VI tetramers would be present in $(3/5)^4$

= 81/625 (13.0%). Such twice increase in normal collagen VI tetramers might contribute to at least partial improvement of cellular functions and collagen VI expression in UCMD fibroblasts. Previous report have suggested that only increase in twice of normal collagen VI tetramers would be enough for recovery of the function in UCMD fibroblasts.[18] These results also indicate that these selected siRNA may be ideal candidates for preclinical trials if efficient delivery can be achieved using appropriate animal models for sporadic UCMD. Allele-specific gene silencing with siRNA targeting point mutations is a potential viable approach for patients with dominant-negative disorders, including sporadic UCMD.

Materials and Methods

Patient fibroblasts. This study was performed in conformity with the Declaration of Helsinki for investigation involving human subjects and was approved by the ethics committee of the National Center of Neurology and Psychiatry. Primary fibroblasts used in this study were obtained by skin biopsy with informed consent from two patients with identified heterozygous c.850G>A (p.G284R) mutation in *COL6A1* gene and two diseased control patients without mutation in *COL6A1-3* genes. Mutated and normal *COL6A1* cDNA were amplified from total RNA isolated from the fibroblasts and cloned into pcDNA3.1 (Invitrogen, CA).

Design of siRNA. To achieve specific and effective knock down of a mutant transcript with c.850G>A by siRNA, we performed initial screening by selecting the siRNAs that resulted to efficient knockdown according to mutation position. Second and final screening was done by introducing mismatch nucleotides to the siRNA. For first screening, we examined six kinds of 19-mer siRNA with di-deoxyribothymidine at 3′-end in which mutation is placed at nucleotide position 7–12, respectively as shown in **Figure 1**. For second screening, a mismatch nucleotide was introduced at positions 2–9 3′-flanked to the mutation position as shown in **Figures 2** and **3**. Sense and antisense RNAs were synthesized in Nippon EGT (Toyama, Japan) and double-stranded RNA was made by annealing. For control experiments, scramble siRNAs were used.

Screening of siRNA. For verifying efficacy of siRNA, we used mouse NIH3T3 embryonic fibroblast cell line (35 mm diameter), in which endogenous *Col6a1* expression was quite low. The luciferase-based reporter alleles in phRL-TK (*Renila* luciferase) and pGL3-TK (*Photinus* luciferase) plasmids were generated according to previous report.[25] The wild-type or mutated 31-bp sequences which contain each upstream and downstream 15-bp sequences around a mutation site in *COL6A1* were inserted into 3′-untranslated region of luciferase genes (**Figure 1b**). Cotransfection of the luciferase-based reporter plasmids, pBI-β-galactosidase reporter (Clontech, CA), pTet-off plasmid (Clontech) and siRNAs (80 nmol/l) was performed using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the expression levels of luciferase and β-galactosidase were measured by Dual-luciferase reporter assay system and Beta-Glo assay system (Promega, WI).

For evaluation of gene silencing effects of siRNAs on full-length *COL6A1* transcripts, wild-type (c.850G>G) and mutant (c.850G>A) *COL6A1* cDNA were ligated into pcDNA3.1 (Invitrogen) under cytomegalovirus promoter and a phospho-glycero kinase-*Neomycin* cassette. Cotransfection of *COL6A1* plasmids with siRNAs (100 nmol/l) was performed using Lipofectamine 2000. The amounts of transfected plasmids were adjusted to achieve the same expression of both transcripts. Two days after transfection, total RNA was prepared by Trizol and PureLink RNA mini kit (Invitrogen) and cDNA was synthesized with random primer. Human *COL6A1* expression was measured by amplification of the fragment corresponding to nucleotide position 810–960 of *COL6A1* cDNA by Q-PCR with SYBR-green Quantitative PCR kit (Qiagen KK, Tokyo, Japan) and normalized with *Neomycin* expression. Primer sequences used for amplification were: *COL6A1*, 5′ACGAGGCAAGCCGGGGCTCCCA3′ and 5′TCCCTTGTAGCCCTTGGGTCCC3′; *neomycin*, 5′GGC ACAACAGACAATCGGC3′ and 5′GATCTCCTGTCATCTCA CCTTG3′.

To measure the proportion of normal and mutant *COL6A1* transcripts, the logarithmically amplified Q-PCR products were digested with *Msp* I and separated with SYBR gold (Invitrogen) on polyacrylamide gel electrophoresis. Intensities of 46- and 28-bp bands, which were derived from mutated and normal PCR products respectively, were measured by ImageQuant LAS 4000 mini and ImageQuant TL (GE Healthcare, Buckinghamshire, UK). The amount of each transcript was calculated by correction of band intensities with band size.

Primary cell analyses. Cell culture, immunocytochemistry and cell detachment test of patients' fibroblasts were done essentially by following previous methods,[17] with some modification in which the staining of secreted collagen VI was performed without cell permeabilization with detergent of the fixed cells. Cell shapes were visualized with Cellomics whole cell stain Green (Thermo, CA).

Statistics. Graphics and statistical analyses were performed with Graphpad Prism (GraphPad Software, CA). Data are presented as mean ± SEM. All data were analyzed using unpaired *t*-test in comparison between analyzing sample and control. *P* values were shown in graphs, when there was significance.

Supplementary material

Figure S1. Secondary structure model for COL6A1 mRNAs (c.850G and c.850A) around a mutation site.

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