厚生労働科学研究費補助金

障害者対策総合研究事業

「アンチセンスによる筋強直性ジストロフィーの治療の最適化」に関する研究

平成25年度　総括研究報告書

研究代表者　石浦　章一

平成26（2014）年4月

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総括研究報告書

アンチセンスによる筋強直性ジストロフィーの治療の最適化

総括研究者　　石浦章一　東京大学大学院 総合文化研究科　教授

研究要旨

筋強直性ジストロフィー１型(DM1)は、CTG トリプレットの伸長によって多くの遺伝子のスプライシング異常が起こる全身性の疾患である。多様な症状には、筋強直、白内障、耐糖能異常、精神遅滞、精巣萎縮などがある。私たちはモデルマウスを用いて、アンチセンス投与により筋強直症状を治療することに成功した。また、低分子化合物や CTG に対するアンチセンスも効果があった。これらの結果から、アンチセンス治療に低分子化合物を加えた複合療法が最も効率の良い治療法であることが示唆された。

分担研究者：西野一三（国立精神・神経医療研究センター神経研究所・部長）

A．研究目的

筋強直性ジストロフィー１型(DM1)の症状は多様で、筋強直、白内障、耐糖能異常、精巣萎縮、などが典型的なものである。これらの症状は、スプライシングが異常になって出現すると考えられている。DM1は、我が国の筋ジストロフィーの中では一番多く、QOLの観点から筋力低下やミオトニアなどの治療法開発が望まれている。

本症の責任遺伝子は第19染色体にあるDMPKであり、その3’非翻訳領域にあるCTGリピートの伸長が病気の直接の原因である。また、筋強直性ジストロフィー２型(DM2)も発見されたが、これは第3染色体にあるZNF9遺伝子中のイントロン１にあるCCTGリピートの伸長である。また海外の研究結果によれば、伸長したリピートだけを発現させたHSA-LRマウスでも同じ症状が見られることもわかった。その結果、現在では、本症が、伸長したRNAに特定のスプライシング因子が結合し、スプライシング因子本来の機能が果たせないことで起こるという「RNA機能獲得説」が唱えられている。これは、私たちの以前の結果「スプライシング因子MBNL1がDM1とDM2の2つの異なる型の塩基リピートに結合することの発見」が証拠の１つとなっている。

以前より、バブル・リポソームを用いた導入効率の高いアンチセンス法によって疾患モデル動物のミオトニアを軽減することができていたが、最近、低分子化合物を用いてスプライシングを正常化することも可能になった。最終年度の平成25年度は、アンチセンス治療の最適化に的を絞り、モデル動物を用いた究極の治療法を開発することを目的とした。この目的のために、CTGに対するアンチセンスを用いることにした。

B．研究方法

マウス塩素チャネルミニ遺伝子と CTG480 コンストラクトを発現させたマウス細胞株 Neuro2a に、CTG に対するモルフォリノアンチセンス CAG を、長さを変えて発現させた。この場合、アンチセンスの塩基数は、15、20、25 とした。

次に、バブル・リポソームを用いて HSA-LR マウス TA 筋にモルフォリノアンチセンス 10 μg を筋注し（1 週間おきに 3 回）、最後の筋注後 1 週間で TA 筋を摘出した。

最後に、vivo-モルフォリノ修飾を施した CAG15 を同様に、バブル・リポソームとともに HSA-LR マウス TA 筋に 10 μg を筋注して効果を調べた。投与回数は 3 回とした。

(倫理面への配慮)

今回の実験は、東京大学・動物実験委員会の指

針通りに行った。

C．研究結果

Neuro2a 細胞を用いたアンチセンス実験では、アンチセンスの塩基数が短いほど、塩素チャネル遺伝子のスプライシング正常化効果が認められた。また、HSA-LR マウスに投与する実験でも、CAG15 に一番良好な効果が認められた。

そこで、生体内への浸透性が高いと考えられる CAG15 vivo-モルフォリノを 3 回、バブル・リポソームとともに筋注した結果、塩素チャネル遺伝子のみならず、SERCA 1 遺伝子エクソン 22 のスキップを減少させ正常化する効果が認められた。

D．考察

本年度は、長く伸びた CTG に対するアンチセンスの効果を調べた。その結果、アンチセンスの長さが短いほど、細胞とマウス組織で塩素チャネル遺伝子のスプライシングを正常化する効果が認められた。

本症は、全身性に多くの遺伝子のスプライシングが異常になる病気である。正常化することが必要なのは塩素チャネルのみならず、多くの遺伝子である。私たちは、典型的なスプライシング異常が見られる SERCA 1 を調べたところ、この遺伝子のスプライシングも正常化することが分かった。

本研究で用いた CTG に対するアンチセンス CAG は、抜本的に本症の症状を改善する可能性がある。また、前年度までに明らかにした低分子化合物マニュマイシン A と併用することにより、スプライシング正常化効果が増進する可能性があることが示唆された。

E．結論

筋強直性ジストロフィーのモデルである CTG リピートを 300 含むトランスジェニックマウス HSA-LR に対して、CAG アンチセンス vivo-モルフォリノオリゴは、バブル・リポソームと併用することで効果的な治療法になることがわかった。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（障害者対策総合研究事業）
分担研究報告書

アンチセンスによる筋強直性ジストロフィーの治療の最適化

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研究要旨

先天性筋強直性ジストロフィー（CDM）は、先天性ミオパチーの一種であるX連鎖性ミオチュブラーミオパチー（XLMTM）と臨床的、病理学的に類似の所見を呈することがある。従って、XLMTMとの鑑別診断を十分に検討することが、CDMの正確な診断に繋がる。本年度、遺伝学的にCDMもしくはXLMTMと確定した例において、病理学的に鑑別可能であるかどうかを検討した。その結果、タイプ2C線維数とperipheral halo線維数の比率で両疾患が鑑別可能であることを明らかにした。

A．研究目的

先天性筋強直性ジストロフィー（CDM）とX連鎖性ミオチュブラーミオパチー（XLMTM）とは遺伝学的に全く異なる疾患である。CDMは、染色体19q13上の*DMPK*遺伝子の3’非翻訳領域にある三塩基（CTG）反復配列が伸長（1000リピート以上）することで発症し、XLMTMは染色体Xq28上の*MTM1*遺伝子の機能喪失型変異により発症する。しかしながら、両疾患はしばしば臨床的、筋病理学的に極めて類似しており、鑑別が困難なことがある。ともに、臨床的には出生前に胎動減少、羊水過多を、出生後は重度の筋緊張低下、呼吸不全を呈し、筋病理学的には中心核とperipheral haloを呈することが知られている。

筋疾患が疑われる際には伝統的に筋生検が考慮されることに加えて、本邦では一部に遺伝子解析に対する慎重な考え方があることなどもあり、遺伝子診断に選考して筋生検が行われることが少なからずある。従って、筋病理から両疾患を鑑別する必要性は少なくない。そこで、CDMとXLMTMの病理学的鑑別方法を十分に検討することによってCDMおよびXLMTMの正確な診断に寄与したいと考え、本年度は、両疾患の筋病理学的鑑別に有用なマーカーを探索するため各種組織学的検討を行った。

B．研究方法

国立精神・神経医療研究センター骨格筋レポジトリーに登録されている症例のうち、遺伝学的に*DMPK*遺伝子の三塩基配列伸長が示唆された31例（CDM群）、及び*MTM1*変異の同定されている37例（XLMTM群）を対象とし、タイプ2C線維とperipheral haloを有する線維の割合を計算し、生検時年齢との関係を比較検討した。

（倫理面への配慮）

今回の実験は、DM患者生検筋からのmRNAスプライシング異常から派生した研究である。国立精神・神経医療研究センター倫理委員会で承認を得た書式を用いて、検体の研究利用に対するインフォームドコンセントを取得している。

C．研究結果

CDM、XLMTM両疾患において、タイプ2C線維とperipheral haloを有する筋線維の割合を計算し、筋生検時の年齢と比較した。CDMにおいてはタイプ2C線維、peripheral halo線維共に、出生直後は高い割合を占めた。この割合は、筋生検時の年齢が高くなるに従い低下した。一方、XLMTMにおいては、出生直後はタイプ2C線維、peripheral halo線維共に割合は少ないが、年齢が高くなるにつれて、peripheral halo線維のみ顕著な増加を認めた。これらの結果は、両疾患におけるperipheral haloの成因の違いを示唆している。

D．考察

CDMにおいて、特に出生直後に多数のタイプ2C線維を認めたのは、近年指摘されている筋線維の成熟遅延、すなわち未熟性の遷延を反映したためと推測された。一方、XLMTMにおいてはタイプ2C線維は少なく、peripheral haloが筋未熟性によるものではなく、近年の研究で明らかにされている筋構造維持の欠陥により筋障害が起こるという説を支持する結果であると考えられた。

E．結論

CDMとXLMTMは病理学的にタイプ2C線維とperipheral haloを有する筋線維の割合から鑑別可能であると考えられる。この結果は両疾患の疾患発症メカニズムの違いを反映していると考えられる。

F．健康危険情報

なし

G．研究発表

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2．学会発表

なし

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2．実用新案登録

なし

3．その他

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研究成果の刊行に関する一覧表

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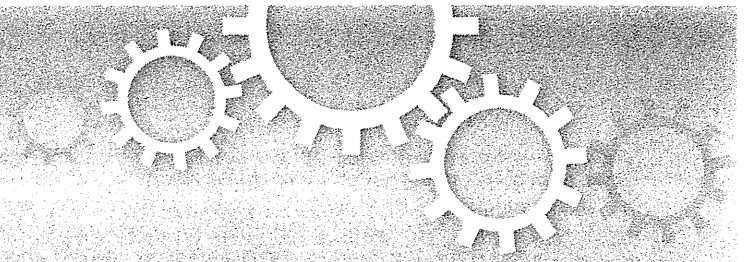

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SUBJECT AREAS:
RNA SPLICING
RNA
HIGH-THROUGHPUT SCREENING
DRUG DISCOVERY

Received
8 February 2013

Accepted
17 May 2013

Published
5 July 2013

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Manumycin A corrects aberrant splicing of *Clcn1* in myotonic dystrophy type 1 (DM1) mice

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Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults and as yet no cure for DM1. Here, we report the potential of manumycin A for a novel DM1 therapeutic reagent. DM1 is caused by expansion of CTG repeat. Mutant transcripts containing expanded CUG repeats lead to aberrant regulation of alternative splicing. Myotonia (delayed muscle relaxation) is the most commonly observed symptom in DM1 patients and is caused by aberrant splicing of the skeletal muscle chloride channel (*CLCN1*) gene. Identification of small-molecule compounds that correct aberrant splicing in DM1 is attracting much attention as a way of improving understanding of the mechanism of DM1 pathology and improving treatment of DM1 patients. In this study, we generated a reporter screening system and searched for small-molecule compounds. We found that manumycin A corrects aberrant splicing of *Clcn1* in cell and mouse models of DM1.

Myotonic dystrophy (DM), a genetic disorder, is the most common type of muscular dystrophy in adults[1]. The types of DM disease[2], DM types 1 (DM1) and 2 (DM2), differ genetically but are similar clinically. DM1 is caused by expansion of a CTG repeat in the 3′-untranslated region (UTR) of the DM protein kinase (*DMPK*) gene, whereas DM2 is caused by expansion of a CCTG repeat in intron 1 of the zinc finger 9 (*ZNF9*) gene[3–5]. DM1 presents with a variety of symptoms, such as myotonia, progressive muscle wasting, cataracts, insulin resistance, and intellectual deficits[1,6]. Identification of the DM2 mutation[5] and studies using DM1 mice that express the expanded CUG repeat[7] suggest that RNA gain-of-function causes the DM1 phenotype.

How does nucleotide expansion within a non-coding region cause DM1? An investigation of the molecular mechanism of DM1 proposed that expanded repeat RNA transcripts form hairpin structures that retain nuclear foci in DM1 cells[8], and affect the function of RNA-binding proteins[9–12]. 'Muscleblind-like' (MBNL) and 'CUGBP and ETR-3 like factor' (CELF) proteins are well-studied RNA-binding proteins. Sequestration of MBNL1 by toxic expanded RNA transcripts[11,13] and up-regulation of CUGBP-1[14] result in aberrant regulation of alternative splicing events in DM1[2]. Our previous studies indicated aberrant regulation of *MYOM1* and *PDLIM3* in DM1 patients[15,16]; more than 25 genes have been found to be misregulated in patients with DM[17]. Missplicing of the following genes has been associated with DM1 symptoms: *CLCN1*, which results in myotonia[7,18], *BIN1*, which is associated with T-tubule alterations and muscle weakness[19], and *INSR*, which contributes to insulin resistance[20]. However, there is no evident relationship between additional misspliced genes and DM1 symptoms. Therefore, further studies are needed to elucidate this association.

Myotonia is one of the features observed most commonly in individuals with DM1. People with myotonia are unable to relax certain muscles after use. For example, a person may not be able to release their grip on a doorknob or handle. In DM1 patients, the inclusion of alternative exons 6B and/or 7A, and retention of intron 2 of *CLCN1*, are observed due to aberrant regulation of alternative splicing[18]. Abnormal splicing of *CLCN1* results in a frameshift and produces premature termination codons in transcripts, leading to nonsense-mediated mRNA decay (NMD) or the production of a truncated protein with a dominant-negative effect[21]. A mouse model of DM1 expressing an expanded CUG repeat (*HSA*^{LR}) also shows increased inclusion of *Clcn1* exon 7A and displays

myotonia[7]. Our previous study using a *Clcn1* minigene identified regulation of exon 7A by MBNL and CELF proteins[22].

The identification of small-molecule compounds that correct missplicing events in DM1 would benefit both our understanding of novel aspects of DM1 pathogenesis and DM1 therapy. In DM1, there may be other key players in addition to MBNL and CELF. There are several approaches to DM1 therapy, such as overexpression of MBNL1[23], RNA interference targeting CUG repeat transcripts[24], inhibition of MBNL1 sequestration through use of a CAG oligonucleotide that binds to the CUG repeats[25] or a small molecule[26], and degradation of expanded CUG repeat transcripts through the RNase H pathway, which occurs through induction of 2′ methoxyethyl (MOE) gapmers[27]. Although use of an antisense oligonucleotide showed remarkable effects, there was a difficulty with body-wide delivery while small-molecule compounds have the advantage of oral formulation.

In this study, we established a Clcn1-L minigene reporter assay and found that manumycin A corrects abnormal splicing of *Clcn1*. Furthermore, we confirmed that injection of manumycin A corrects missplicing of *Clcn1* in a mouse model of DM1 via H-Ras pathway.

Results

Generation of the Clcn1-L reporter assay system. To identify small chemical compounds effective against aberrant splicing of *CLCN1* in DM1, we generated a minigene reporter vector containing the mouse *Clcn1* gene from exons 6 to 7 and the firefly luciferase gene (Fig. 1a). As shown in Fig. 1b, luciferase expression was obtained upon exclusion of exon 7A; however, inclusion of exon 7A produced a termination codon, resulting in a lack of luciferase expression. Next, we confirmed the co-transfection of Clcn1-L and DMPK constructs harboring either CTG18 (DM18) or interrupted CTG480 (DM480) repeats. RT-PCR analysis revealed that inclusion of exon 7A was significantly increased upon co-expression of Clcn1-L and DM480 compared to co-expression with DM18 (Fig. 1c,d). According to the luciferase analysis, co-expression of Clcn1-L and DM18 displayed a higher activity than did co-expression with DM480 (Fig. 1e). Note that we did not delete the initiation codon of luciferase gene. It is possible that an extra ATG codon in the Clcn1-L might induce translation of luciferase irrespective of exon 7A exclusion. However, in our experiments, the luciferase activity decreased when DM480 was transfected (Fig. 1e). Therefore, we concluded that this reporter system was properly working.

Identification of small-molecule compounds that correct aberrant splicing of Clcn1 under the expression of expanded CUG repeats in vitro. The ICCB Known Bioactives Library is a collection of compounds with defined biological activities. The library was screened to identify compounds that corrected aberrant splicing of *Clcn1* using our luciferase reporter assay. Because expanded CUG repeat expression causes aberrant regulation of alternative splicing in DM1, we transfected C2C12 cells with both the Clcn1-L luciferase reporter vector and DM480, which expresses the expanded CUG repeat. Although most compounds showed little effect compared to that of DMSO treatment (control, Table S1), some of the compounds showed high luciferase activity (Fig. S1), and Ro 31-8220, AGC, and manumycin A caused little toxicity to cells (Fig. S2). Since Ro 31-8220 was well studied with DM1[28] and AGC caused large SEM, manumycin A was chosen in this study for further analysis. Manumycin A (20 μM) showed high luciferase activity in the presence of the expanded CUG repeat (Fig. 2a and Fig. S1).

Next, we performed RT-PCR analysis to investigate whether manumycin A corrects aberrant *Clcn1* splicing caused by expression of the expanded CUG repeats. Results from RT-PCR showed that the addition of manumycin A effectively corrects aberrant splicing of *Clcn1* in the presence of the expanded CUG repeat (Fig. 2b,c). Percentages of *Clcn1* exon 7A inclusion showed that manumycin A treatment rescued abnormal exon 7A inclusion levels caused by expression of the expanded CUG repeat to levels similar to those for the normal CUG repeat (Fig. 2c). Additionally, we tested the dosage of manumycin A (~10-40 μM), and found that the effects of manumycin A were concentration-dependent (Fig. S3). High-dosage manumycin A rescued aberrant splicing in the presence of the expanded CUG repeat and showed skipping of exon 7A (Fig. S3) compared to the control (Fig. 2c).

Manumycin A corrects aberrant splicing of Clcn1 in a mouse model of DM1. Next, we examined the ability of manumycin A to rescue aberrant splicing of *Clcn1* in a mouse model (HSA^{LR}) of DM1, in which 250 CUG repeats are expressed under the control of the actin promoter. RT-PCR analysis showed elevated inclusion of *Clcn1* exon 7A in the HSA^{LR} mice compared with the wild-type mice (Fig. 3a,b). Injection of manumycin A induced a remarkable reduction in *Clcn1* exon 7A inclusion (Fig. 3c,d). However, manumycin A did not rescue aberrant splicing of *Serca1* and m-Titin (Fig. S4).

H-Ras regulates alternative splicing of Clcn1 exon 7A. Manumycin A is an antibiotic generated by *Streptomyces parvulus*[29]. It acts as a selective and vigorous inhibitor of Ras farnesyltransferase[30]. After translation, Ras protein requires several modifications: isoprenylation, proteolysis, methylation and palmitoylation[31–33]. Isoprenylation by the enzyme farnesyltransferase (FTase) or geranylgeranyltransferase I (GGTase I) is the first step in the post-translational modification of Ras. Farnesylation or geranylgeranylation is necessary for Ras to attach to the inner side of the plasma membrane. Without attachment to the cell membrane, Ras is unable to be activated[34]. H-Ras, K-Ras and N-Ras are the members of the Ras family. It is important that H-Ras is only farnesylated, whereas K-Ras and N-Ras can be farnesylated and geranylgeranylated. Thus, inhibitors of farnesyltransferase are effective in reducing the activity of H-Ras but not that of K-Ras and N-Ras[35]. Correction of *Clcn1* splicing by manumycin A may therefore be due to the inhibition of H-Ras activity. To test this possibility, we knocked down endogenous H-Ras expression using small interfering RNA (siRNA) and examined the effect of H-Ras inhibition on *Clcn1* splicing. We confirmed the efficacy of the siRNA in modulating the expression of the H-Ras by Western blot analysis (Fig. 4a). RT-PCR analysis showed reduced inclusion of *Clcn1* exon 7A in the presence of expanded CUG repeats (Fig. 4b,c). We also examined the effects of K-Ras and N-Ras knockdown and found that N-Ras knockdown reduced the inclusion of *Clcn1* exon 7A, whereas K-Ras knockdown did not alter *Clcn1* splicing (Fig. S5). Additionally, we tested whether expanded CUG repeats altered the expression levels of H-Ras. Although there was no significant difference between DM18- and DM480-transfected C2C12 cells, an upward trend was observed when cells were transfected with DM480 (Fig. S6).

Discussion

Misregulation of alternative splicing is a characteristic feature of DM1. Although the number of missplicing events is over 25, few genes have been reported to play a role in disease manifestations. Abnormal regulation of alternative splicing in the *CLCN1* gene is one of the events that can account for myotonia, which is often recognized in DM1. In this investigation, we generated a Clcn1-L reporter assay system and searched for small-molecule compounds that affect abnormal splicing of *Clcn1* in the presence of expanded CUG repeats. We found that manumycin A corrects aberrant splicing of *Clcn1*, which was confirmed *in vivo*. We also revealed that H-Ras was involved in the regulation of alternative splicing of *Clcn1* exon 7A.

Injection of DM1 model mice with manumucin A corrected aberrant splicing of *Clcn1*; however, splicing of *Serca1* and m-Titin, which

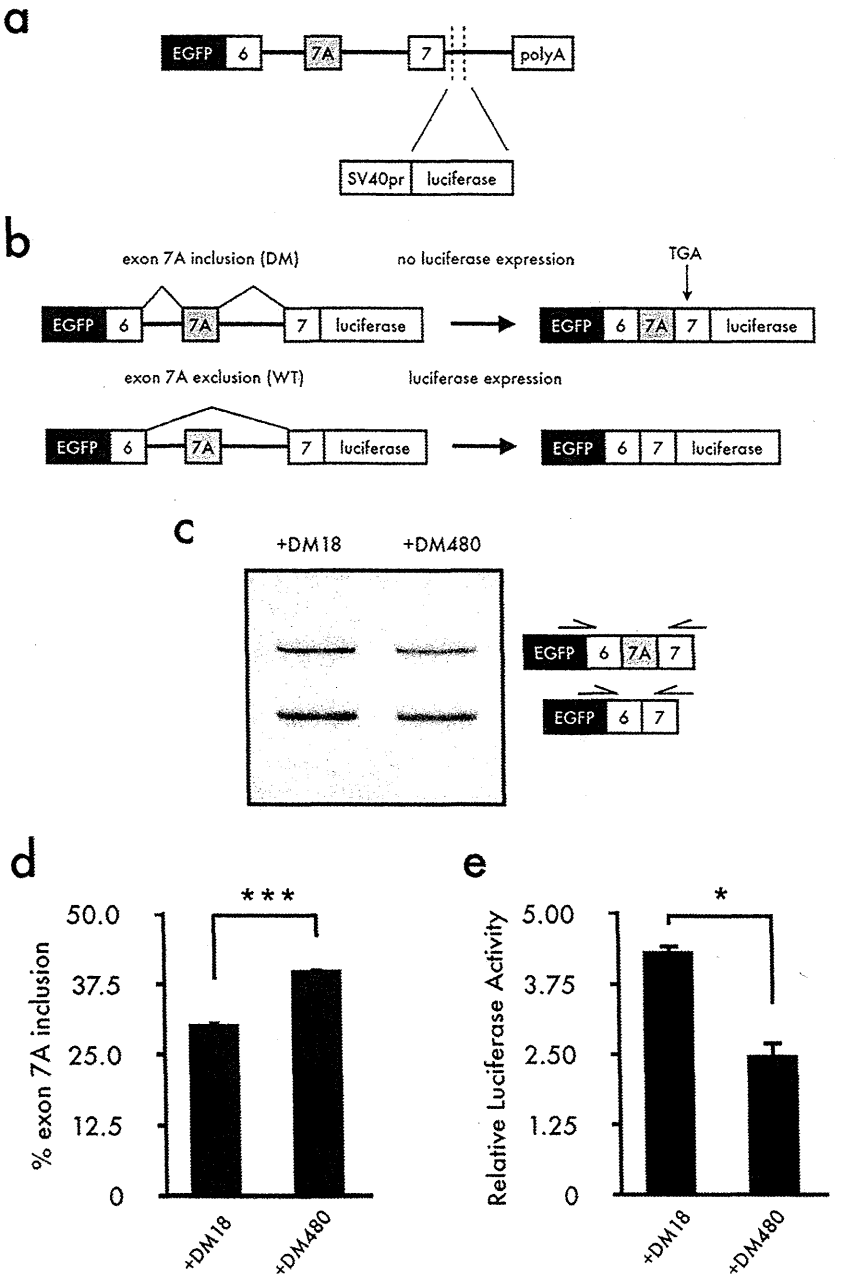

Figure 1 | Construction of the Clcn1-L reporter minigene and effect of triplet repeat expansion on the reporter vector Clcn1-L. (a) Schematic structure of the Clcn1-L minigene reporter. A genomic segment of mouse *Clcn1* containing exons 6 to 7 (including the intron) was sub-cloned downstream of EGFP in the pEGFP-C1 plasmid. Firefly luciferase was inserted in-frame with a correct *Clcn1* splicing pattern. (b) Schematic of how Clcn1-L functions. Exon 7A exclusion results in luciferase expression to detect correct *Clcn1* splicing. (c) Inclusion of *Clcn1* exon 7A increased upon expression of the expanded CUG repeat. (d) Bar charts show the quantified percentages of exon 7A inclusion (mean + SEM, n = 4). (e) Luciferase analysis showed that relative luciferase activity decreased upon expression of the expanded CUG repeat (mean + SEM, n = 3). The gel image was cropped around the region of interest and the samples (n = 4) were resolved in the same gel. Statistical significances were determined using *t*-tests ($*p < 0.05$, $***p < 0.001$).

is also abnormally regulated in DM1[36,37], was not changed. It has been reported that *Serca1* is regulated by MBNL1[37,38] and CUGBP1[39] and that m-Titin is also regulated by MBNL1[37]. Furthermore, the expression of MBNL1 and CUGBP1 was not altered by treatment with manumycin A (Fig. S7). For these reasons, we conclude that manumycin A did not alter *Clcn1* splicing through effects on MBNL1 and CUGBP1. How, then, does manumycin A correct *Clcn1* missplicing? Manumycin A is an inhibitor of Ras farnesyltransferase, and it could inhibit Ras activity. In this study, we demonstrated that H-Ras knockdown reduced the inclusion of *Clcn1* exon7A, indicating that H-Ras is involved in a regulation of *Clcn1* splicing.

Ras proteins perform functional roles in a large number of biological processes, leading to changes in cell morphology, survival, apoptosis, and gene expression[40]. Because Ras is positioned as the central molecular switch of these biological outcomes, it must interact with a variety of downstream targets. Recent studies implicated the Ras signaling pathway in alternative splicing regulation[41]. Activation of the Ras-PI3-kinase-PKB/Akt pathway alters the

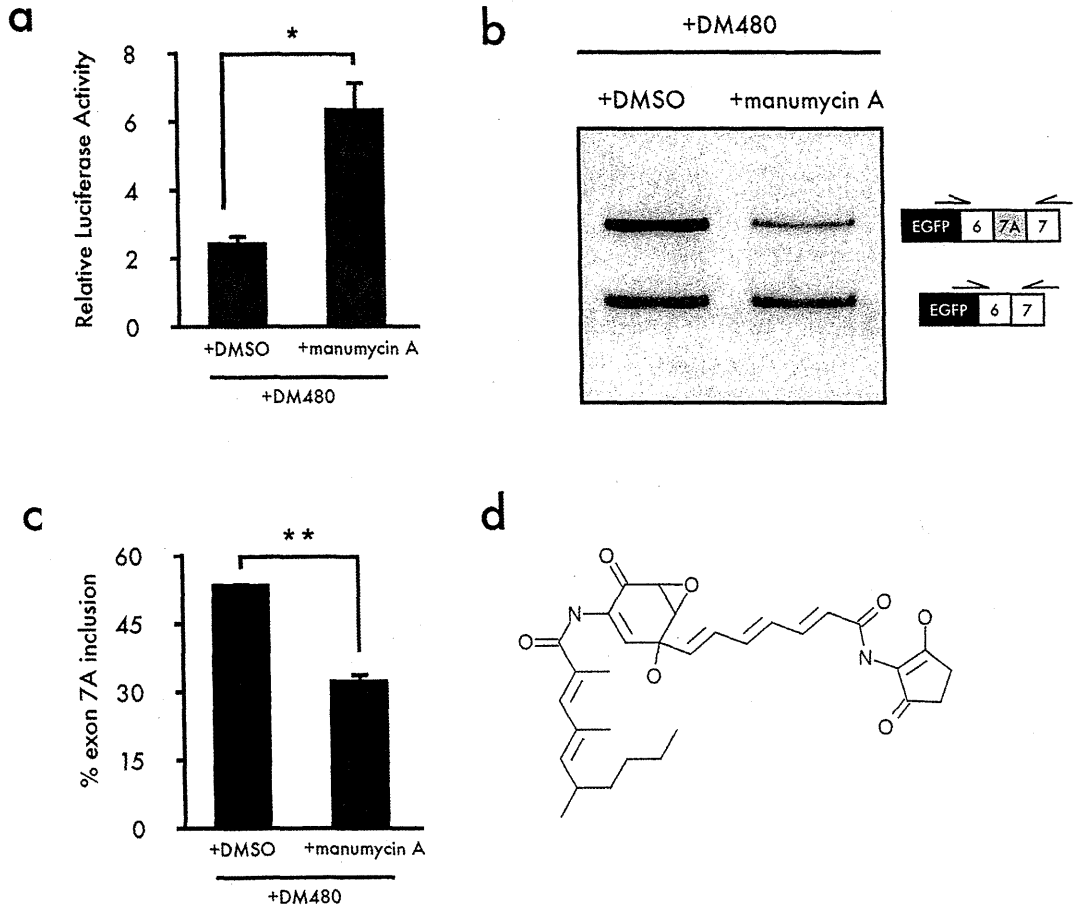

Figure 2 | Identification of small-molecule compounds that correct aberrant splicing of *Clcn1*. (a) A luciferase reporter assay showed that manumycin A corrected aberrant splicing in the presence of the expanded CUG repeat (mean + SEM, n = 3). (b) Cellular splicing analysis showed that manumycin A corrects aberrant splicing of *Clcn1*. (c) Quantification of the results shown in (b) (mean + SEM, n = 3). (d) Structure of manumycin A. The gel image was cropped around the region of interest and the samples (n = 3) were resolved in the same gel. Statistical significance was determined using *t*-tests ($*p < 0.05$, $**p < 0.01$).

phosphorylation level of the serine/arginine-rich (SR) proteins SF2/ASF and 9G8[42]. SR proteins are the best-characterized splicing regulatory factors, and it is well known that the activity of SR proteins is dependent on their phosphorylation level. Furthermore, the Ras-Raf-MEK-ERK pathway, another Ras pathway, is known to modulate alternative splicing[41,43].

Considering these studies, it is possible that manumycin A inhibits H-Ras activity and alters the H-Ras signaling pathway, resulting in correction of *Clcn1* splicing. Therefore, modulation of H-Ras signaling may influence a *trans*-acting factor other than MBNL1 and CUGBP1 and thereby contribute to *Clcn1* splicing. The effect of manumycin A is illustrated in Figure 5, although the question remains as to what kind of *trans*-acting factor is influenced by manumycin A and H-Ras. Importantly, manumycin A treatment showed reduced total amount of *Clcn1* mRNAs (with and without exon 7A) (Fig. 2b and Fig. 3c). Therefore, it is possible that manumycin A has another effect on either transcription or stability of *Clcn1* pre-mRNA.

A recent study revealed that oncogenic mutated H-Ras G12V inhibits muscle differentiation[44]. If H-Ras is involved in DM pathology, it may help us to better understand DM1. Interestingly, manumycin A only slightly changes the splicing of *Clcn1* in the absence of DM480 expression (Fig. S8). Furthermore, transfection of C2C12 cells with the expanded CUG repeat tended to increase H-Ras expression (Fig. S6). Considering the transfection efficiency of C2C12 cells, it is likely that the expanded CUG repeat could alter H-Ras expression. However, involvement of Ras in DM1 has not been reported thus far, and further studies are needed in the future.

DM1 is the most common muscular dystrophy in adults, affecting approximately one in every 8,000 individuals. However, there is as yet no cure for DM. In this study, we showed that manumycin A corrects aberrant splicing of *Clcn1* and revealed that H-Ras is involved in regulation of *Clcn1* splicing. We hope that further studies on manumycin A and H-Ras will lead to a novel therapy for DM1.

Methods

Plasmid construction. Clcn1-L is a luciferase reporter vector containing a genomic segment of mouse *Clcn1* (exon 6 to exon 7) and the firefly luciferase gene. Firefly luciferase (F-Luc) was amplified from the downstream of SV40 promoter of the pGL-3 promoter vector (Promega, Madison, WI, USA) by PCR using *PfuUltra* High-Fidelity DNA polymerase (STRATAGENE, La Jolla, CA, USA), generating a 1.7-kb product with the addition of a restriction site for *Sal*I and *Bam*HI. Therefore, this fragment did not contain SV40 promoter. The following primer pair was used: F-Luc forward, 5′-AAAGTCGACCCATGAAGACGCC-3′; and F-Luc reverse, 5′-CCGGATCCTTACACGGCGATCTT-3′. The fragment was inserted into the *Sal*I-*Bam*HI site of the Clcn1 minigene. The Clcn1 minigene contains PCR-amplified fragments of mouse *Clcn1* (exon 6 to exon 7) and has been described previously[22]. The nucleotide sequences of the DNA inserts and reading frame were confirmed to be correct by sequencing. DM18 and DM480 contain a fragment of the 3′ region of *DMPK* with CTG18 or interrupted CTG480 repeats, respectively, and have been described previously[23].

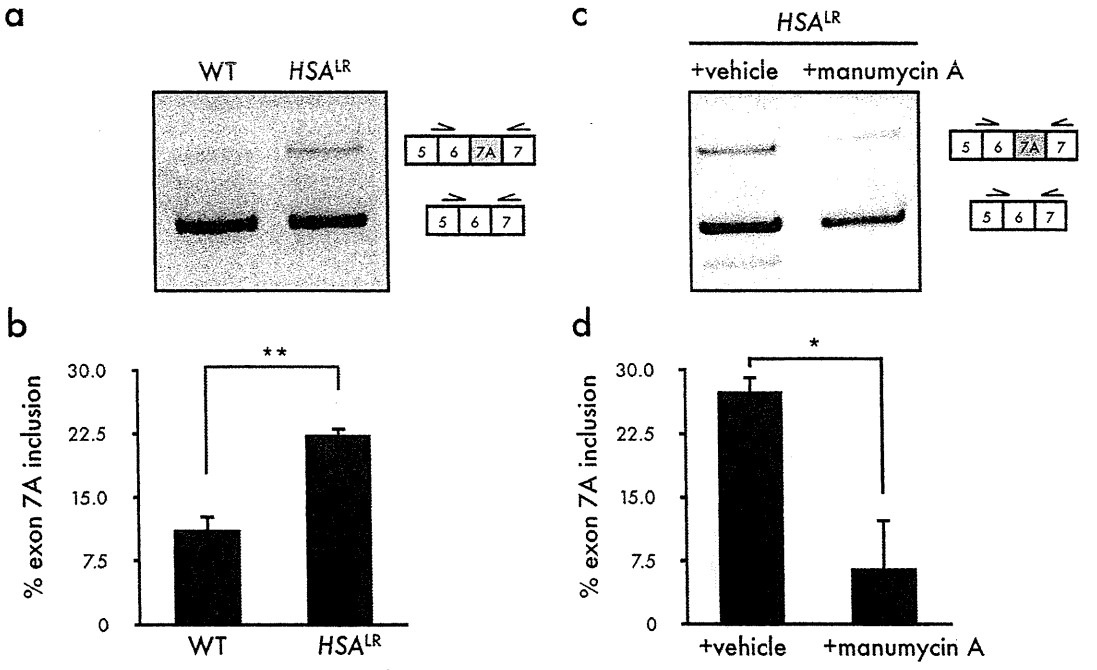

Figure 3 | Manumycin A corrects aberrant splicing of *Clcn1* in *HSA*^{LR} DM1 model mice. (a) RT-PCR analysis showed increased inclusion of *Clcn1* exon 7A in *HSA*^{LR} mouse. (b) Quantification of the results shown in (a) (mean + SEM, n = 3). (c) RT-PCR analysis showed reduced inclusion of *Clcn1* exon 7A 5 days after injection of manumycin A into TA muscles. (d) Quantification of the results shown in (a) (Mean + SEM, n = 3). The gel image was cropped around the region of interest and the samples (n = 3) were resolved in the same gel. Statistical significance was determined using *t*-tests (*$p < 0.05$, **$p < 0.01$).

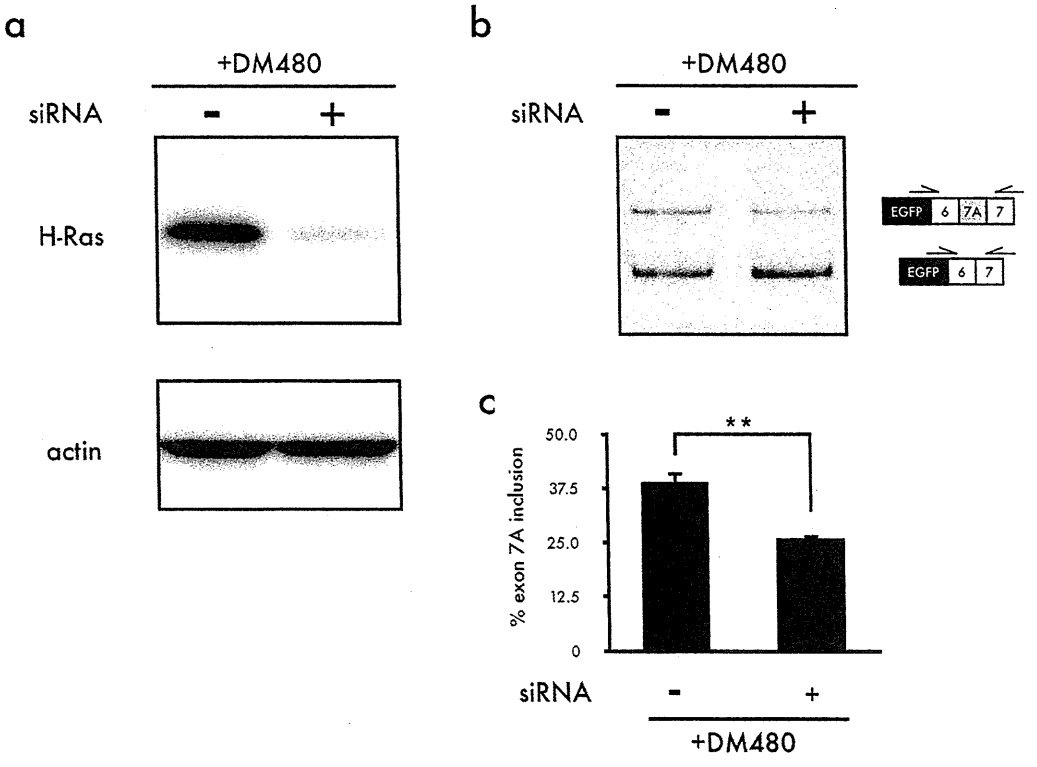

Figure 4 | The knockdown of H-Ras corrects aberrant splicing of *Clcn1* in the presence of the expanded CUG repeat. (a) Representative result of Western blot analysis of H-Ras in C2C12 cells. (b) Results of cellular splicing assays using Clcn1-L minigene, DM480 and siRNA. (c) Quantification of the results shown in (b) (mean + SEM, n = 3). The gel and blot image were cropped around the region of interest and the samples (n = 3) were resolved in the same gel or blot. Statistical significance was determined using *t*-tests (**$p < 0.01$).

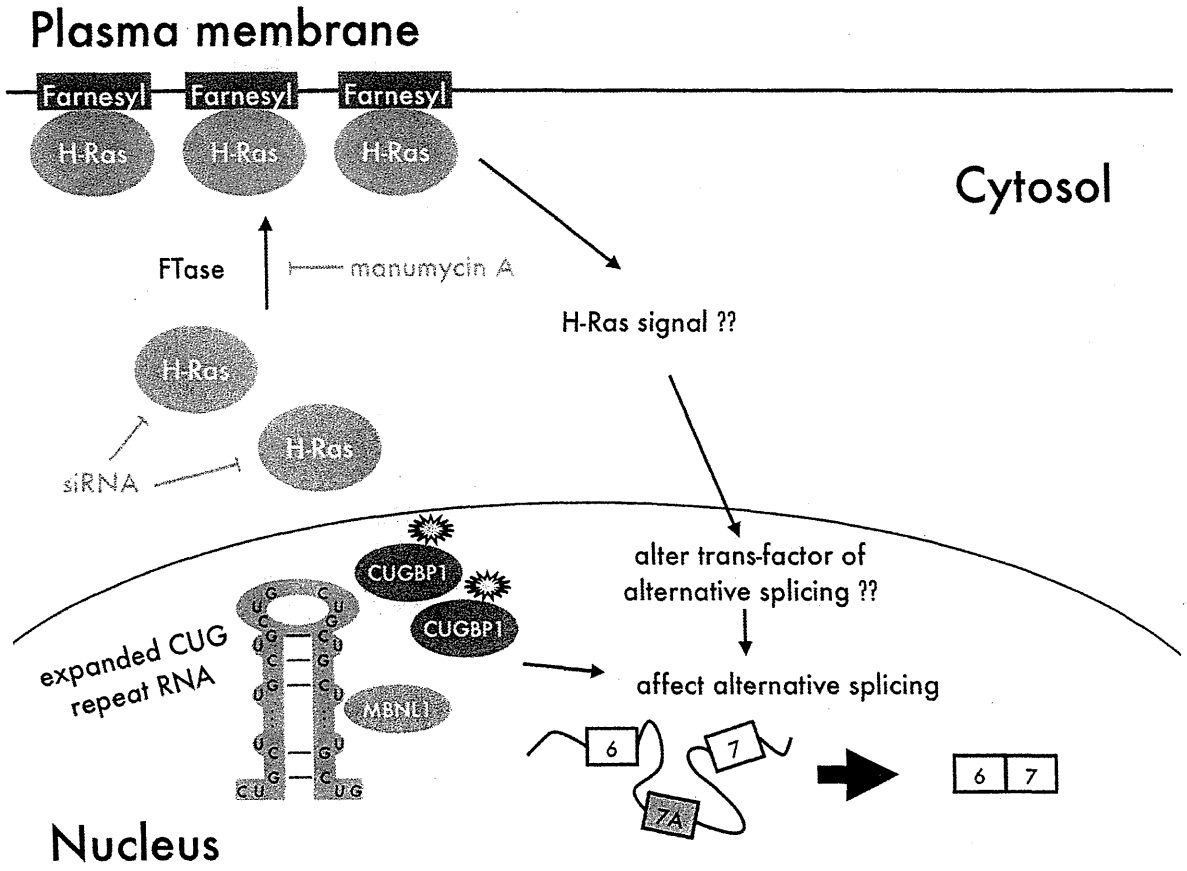

Figure 5 | Model of how manumycin A affects *Clcn1* splicing. In DM1, it is known that expanded CUG repeat RNA transcripts trap MBNL1 and up-regulate CUGBP1, resulting in aberrant regulation of alternative splicing. However, manumycin A does not affect the expression of these proteins. Manumycin A might act as a Ras farnesyltransferase inhibitor, thereby altering H-Ras signaling. This could in turn result in alteration of a *trans*-acting factor involved in alternative splicing other than MBNL1 and CUGBP1.

Cell culture and transfection. C2C12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% (v/v) fetal bovine serum and then incubated at 37°C with 5% CO_2. For the minigene and luciferase reporter assays, C2C12 cells were transfected with plasmids for expression of a minigene with toxic RNA transcripts using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection was conducted according to the manufacturer's protocol. Cells were cultured in 24- and 96-well plates for the minigene and luciferase reporter assays, respectively.

Luciferase reporter assay and drug screening. Culture, transfection, cell harvesting, and luciferase activity measurements were performed according to the standard methods of the Dual-Glo Luciferase Assay System (Promega). Clcn1-L and DM480 were co-expressed in cells. Plasmid pRL (Promega), which contains the sea pansy luciferase gene, was co-transfected as an internal control for normalization of transfection efficiency. In all experiments, luciferase activity was measured 48 h after transfection and was assayed using the Dual-Glo Luciferase Assay System (Promega). Firefly and sea pansy luciferase activities were measured using the Centro LB 960 (Berthold, Bad Wildbad, Germany), and the value of each sample was calculated as light units of firefly luciferase per light unit of sea pansy. The ICCB Known Bioactives Library (Enzo Life Sciences, Farmingdale, NY, USA) was referenced for drug screening. Chemical compounds were added 24 h after transfection at 0.1% (v/v), and luciferase activity was measured 24 h after the addition of chemical compounds.

Administration of manumycin A to DM mice. *HSA*^{LR} transgenic mice were used for animal experiments and have been described previously[45]. These mice express human skeletal actin mRNA, with approximately 250 CUG repeats in the 3′-UTR. Manumycin A was diluted to a final concentration of 75 ng/μl in saline containig 0.1% DMSO. Manumycin A (40 μl, 3.0 μg) or vehicle (0.1% DMSO in saline) was injected into the TA muscles of opposite limbs. Mice were killed 5 days after injection, and TA muscle was obtained for splicing analysis. All experiments were conducted according to the Regulations for Animal Experimentation at the University of Tokyo (Tokyo, Japan).

Identification of Clcn1 splice variants. Total RNA was isolated using a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA). cDNA synthesis was performed using a Prime-Script First Strand cDNA Synthesis Kit (TAKARA BIO, Otsu, Japan) with an oligo dT primer. The Clcn1-L minigene fragments were amplified by PCR (24–27 cycles) with the following primer pair: Clcn1-L forward, 5′-CATGGTCCTGCTGGAGTTCGTG-3′; and Clcn-L reverse, 5′-CTCCAAGTGGTGTTCCAAAACAGC-3′. To detect endogenous *Clcn1* fragments, PCR amplification (32-34 cycles) was carried out with the following primer pair: *Clcn1* forward, 5′-GCTGCTGTCCTCAGCAAGTT-3′; and *Clcn1* reverse, 5′-CTGAATGTGGCTGCAAAGAA-3′. PCR products were resolved on 8% polyacrylamide gels and stained with ethidium bromide. Band intensities were quantified using an LAS-3000 instrument and Multigauge software (FUJIFILM, Tokyo, Japan). The ratio of exon 7A inclusion in *Clcn1* was calculated as (7A inclusion)/(7A inclusion + 7A exclusion) × 100.

RNA interference. An siRNA specific for H-Ras (H-Ras siRNA) and a negative control siRNA (MISSION siRNA Universal Negative Control) were purchased from Sigma-Aldrich. The siRNA target sequences were as follows: mouse H-Ras siRNA sense, 5′- GUUGCAUCACAGUAAAUUAdTdT-3′; and mouse H-Ras siRNA antisense, 5′- UAAUUUACUGUGAUGCAACdTdT-3′. The efficacy of the RNAi-mediated knockdown of endogenous H-Ras and actin expression was determined by Western blot analysis. Antibodies specific for H-Ras (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) and actin (A2066; Sigma-Aldrich) were used.

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Acknowledgements

We thank Prof. Charles A. Thornton (University of Rochester) for providing us *HSA*^{LR} mice. We also thank Dr. Yoshihiro Kino for providing us the Clcn1 minigenes, DM18 and DM480 constructs. This work was supported by an intramural research grants (23–5) for Neurological and Psychiatric Disorders of NCNP from the Ministry of Health, Labour and Welfare, Japan, and by the Uesugi Foundation.

Author contributions

K.O., Y.O. and S.I. designed the experiments; K.O. performed all experiments; Y.O. assisted with the construction of the reporter vector; S.S. assisted with the interpretation of the results; M.P.T. prepared the mice; S.T. prepared the small chemical compound library; N.I. supported the project; S.I. supervised the work; and K.O. analyzed the data and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Oana, K. *et al.* Manumycin A corrects aberrant splicing of *Clcn1* in myotonic dystrophy type 1 (DM1) mice. *Sci. Rep.* 3, 2142; DOI:10.1038/srep02142 (2013).

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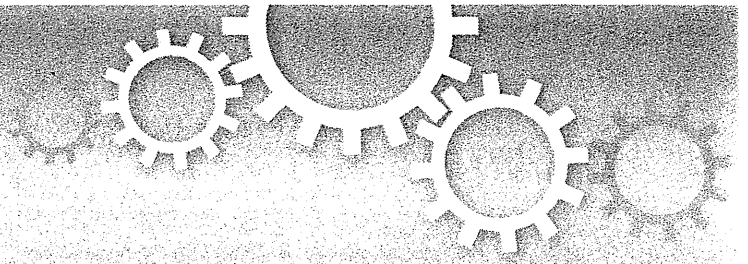

OPEN

SUBJECT AREAS:
MECHANISMS OF DISEASE
ANTISENSE OLIGO
DRUG DELIVERY
RNA SPLICING

Received
18 March 2013

Accepted
21 June 2013

Published
22 July 2013

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Ultrasound-enhanced delivery of Morpholino with Bubble liposomes ameliorates the myotonia of myotonic dystrophy model mice

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Phosphorodiamidate morpholino oligonucleotide (PMO)-mediated control of the alternative splicing of the chloride channel 1 (*CLCN1*) gene is a promising treatment for myotonic dystrophy type 1 (DM1) because the abnormal splicing of this gene causes myotonia in patients with DM1. In this study, we optimised a PMO sequence to correct *Clcn1* alternative splicing and successfully remedied the myotonic phenotype of a DM1 mouse model, the *HSA*^{LR} mouse. To enhance the efficiency of delivery of PMO into *HSA*^{LR} mouse muscles, Bubble liposomes, which have been used as a gene delivery tool, were applied with ultrasound exposure. Effective delivery of PMO led to increased expression of Clcn1 protein in skeletal muscle and the amelioration of myotonia. Thus, PMO-mediated control of the alternative splicing of the *Clcn1* gene must be important target of antisense therapy of DM1.

Myotonic dystrophy type 1 (DM1) is caused by expansion of the CTG repeat in the 3′ untranslated region (UTR) of the *DMPK* gene[1–4]. Patients with DM1 show multi-systemic symptoms, including muscle wasting, muscle weakness, myotonia, cardiac conduction defect, cataracts, mental retardation and insulin resistance[5]. A patient, however, does not always present with all of these symptoms and the severity of the disease varies among individuals. Among the symptoms, myotonia is the most prominent and common phenotype of DM1: most patients feel muscle stiffness and difficulty in relaxing muscles soon after developing the disease.

The characteristic feature of the pathology of DM1 is the aberrant regulation of dozens of alternative splicing events, and some of the abnormal splicing events have been suggested to be involved in some of the symptoms[6–10]. Myotonic discharge is thought to be caused by the aberrant alternative splicing of the chloride channel 1 (*CLCN1*) gene[11,12]. In patients with DM1, extra exons from intron 6 are spliced into the *CLCN1* mRNA, leading to the appearance of a premature termination codon in the subsequent exon, degradation of the mRNA by nonsense-mediated decay and decreased expression of CLCN1 protein[11]. The idea that abnormal splicing of the *CLCN1* gene causes myotonia is strongly supported by the fact that the *CLCN1* gene is the only gene responsible for congenital myotonia, and the identification of multiple mutations in patients with the disease and their families[13]. Wheeler and his colleagues corrected the abnormal splicing of the *Clcn1* gene in a DM1 mouse model, the *HSA*^{LR} mouse, by using an antisense oligonucleotide (AON) and successfully alleviated the myotonic phenotype[14]. Thus, correction of the abnormally regulated splicing is a promising treatment for DM1.

An AON is a short, synthetic nucleic acid molecule with a sequence complementary to a target transcript. It can be used to manipulate an alternative splicing event: the AON that binds to the region around the target exon, specifically splice sites or splicing enhancer domains, physically blocks assembly of the spliceosome on the exon and induces exon skipping[15]. The efficacy of an AON is dependent on its half-life, affinity for its target RNA and *in*

vivo kinetics. A variety of AON molecules with 2′-O modifications and/or unnatural backbones have been developed to improve nuclease resistance and affinity for RNA[15]. Among them, Morpholino (also referred as phosphorodiamidate morpholino oligonucleotide [PMO]) is one of the most hopeful AONs. PMO has morpholine rings linked with phosphorodiamidate linkages in its backbone instead of deoxyribose and phosphodiester bonds. Due to its completely unnatural chemistry, PMO is hardly recognised by cellular nucleases. It has higher affinity for RNA than for DNA; the T_m value of a hybrid of PMO and RNA is much higher than that of DNA and RNA[16]. Several papers have reported the local and systemic administration of PMO to mice and dogs. For example, when a high dose of PMO (3 g/kg) was administrated intravenously into the *mdx* mouse, a mouse model of Duchenne muscular dystrophy, the PMO entered skeletal muscle without any assistive delivery reagent[17]. However, unlike mdx mice, muscle penetration of Evans Blue dye did not increase in HSA^{LR} and wild-type mice[18], which indicated a physical barrier to PMO uptake should be grater in HSA^{LR} than in *mdx*. In preceding reports on PMO treatment in HSA^{LR}, intravenous administration of CAG25 PMO led no detectable improvements in *Serca1* splicing in HSA^{LR} mice[19], and even when PMO was injected intramuscularly its uptake was limited to the needle track[14]. In these studies, they used electroporation to administer unmodified PMO intramuscularly, so we investigated a less invasive PMO delivery method to develop PMO treatment for DM1.

Recently, ultrasound exposure has been used for the intracellular delivery of molecules such as dextran, plasmid DNA and siRNA. If ultrasound is sufficiently strong, it will generate microscopic vacuum bubbles in a solution by a process known as inertial cavitation. The bubbles immediately collapse, producing a shock wave, which is believed to transiently increase the permeability of cell membranes in the vicinity. Inertial cavitation is enhanced by using micro bubbles of echo-contrast gas. This method has been applied to gene delivery into various mouse tissues, including skeletal muscle, liver and tumour tissues[20–22]; however, introducing genes into deep tissues with microbubbles is difficult because of their size and instability. To overcome these problems, we previously developed a novel drug delivery reagent coined "Bubble liposomes", polyethylene glycol-modified liposomes (PEG liposomes) encapsulating echo-contrast gas[23]. Owing to the stability in serum and uniform microscopic size of PEG liposomes, we successfully delivered genes and siRNA into several tissues[24–28]. However, does the Bubble liposome-ultrasound delivery system efficiently deliver PMO into skeletal muscles in the HSA^{LR} mice? In this study, we examined the ability of the Bubble liposome-ultrasound system to deliver PMO into skeletal muscles of HSA^{LR} mice as a treatment for abnormal splicing.

We newly designed antisense PMOs targeting exon 7A of the *Clcn1* gene and delivered them into HSA^{LR} mice. The PMOs were successfully delivered into skeletal muscles by the Bubble liposome-ultrasound system, which decreased the inclusion of exon 7A *in vivo*. Furthermore, the injection of PMO ameliorated the myotonic phenotype of the model mice. Our results suggest that Bubble liposomes should be effective for delivering PMOs into muscle tissues and can be applied to PMO treatment of DM1.

Results

We first determined the optimal target sequence of the *Clcn1* pre-mRNA to promote skipping of exon 7A. To achieve this, we used a *Clcn1* minigene and examined its alternative splicing using a cell culture-based assay. The minigene contains the genomic region from exon 6 to exon 7 of the murine *Clcn1* gene. When it was transfected into COS-7 cells, approximately 50% of transcripts contained exon 7A (Fig. 1b, minigene only). To screen for an optimal AON sequence, we used 25-mer phosphorothioate 2′ *O*-methyl (PS2OMe) RNA, which can regulate alternative splicing by sterically preventing spliceosomal assembly, just like PMO. We examined PS2OMe RNA molecules that covered the whole of exon 7A (1–25, 26–50, 51–75 and 76–90) and the boundary of intron 6 and exon 7A (−10–15). We transfected the minigene together with the PS2OMe RNA into COS-7 cells and analysed the alternative splicing of the minigene. We found that −10–15 and 1–25 PS2OMe significantly reduced the rate of inclusion of exon 7A, with 1–25 PS2OMe being the most effective molecule (Fig. 1b).

Previously, we identified the 8 nt at the 5′ end of exon 7A as an exonic splicing enhancer (ESE) essential for basal inclusion of the exon[29]. Given that both −10–15 and 1–25 PS2OMe covered the ESE, and that 1–25 PS2OMe seemed to be more effective at excluding exon 7A, we speculated that another ESE (16–25) would be located in the region +16 to +25, and that 1–25 PS2OMe would not share it with −10–15 PS2OMe. To examine this possibility, we tested whether 16–40 PS2OMe enhanced normal splicing. 16–40 PS2OMe markedly reduced the rate of inclusion of exon 7A of the *Clcn1* minigene (Supplementary Fig. S1). As 26–50 PS2OMe did not change the alternative splicing, we conclude that the other ESE (16–25) is important for exon 7A inclusion. Thus, we used 1–25 AON, which targeted both ESEs, in subsequent experiments.

PS2OMe is highly resistant to nuclease-mediated degradation owing to its phosphorothioate linkages; however, it is still degraded slowly and releases monomers that have a toxic, free phosphorothioate group. In contrast, PMO is remarkably resistant to degradation and is not noxious. Therefore, we next investigated whether a PMO with the same sequence as 1–25 PS2OMe also improved the alternative splicing of exon 7A by using the cell culture-based splicing assay (Fig. 1c). The −11–14 PMO we used here had the same sequence as that Wheeler and his colleagues used in a previous study[14]. RT-PCR analysis showed that both 1–25 and −11–14 PMOs significantly reduced exon 7A inclusion. Although the effect of 1–25 PMO was greater than that of −11–14 PMO, no statistically significant difference was observed between them.

1–25 PMO was so effective at improving alternative splicing of the *Clcn1* minigene in cultured cells that we expected it to work well *in vivo*. To test whether 1–25 PMO could work *in vivo*, we administrated 60 μg of 1–25 PMO intramuscularly four times at weekly intervals into the *tibialis anterior* (TA) muscles of HSA^{LR} mice. The alternative splicing of the *Clcn1* gene was moderately improved, with an approximately 30% decrease in exon 7A inclusion. Electromyography (EMG) with a single needle electrode, however, revealed that the occurrence of myotonia was not altered by PMO injection (Supplementary Fig. S2). Because 60 μg of PMO was quite a high dose for administration into a single muscle, we assumed that an effective delivery system would be required to introduce 1–25 PMO into muscle tissues. We therefore examined the usefulness of ultrasound-enhanced delivery with Bubble liposomes for PMO delivery. We administrated 20 μg of 1–25 PMO three times at weekly intervals into the TA muscles of HSA^{LR} mice with or without Bubble liposomes and ultrasound (Fig. 2). RT-PCR analysis revealed that the rate of inclusion of exon 7A decreased to its lowest level when both Bubble liposomes and ultrasound were applied, indicating that use of the combination of Bubble liposomes and ultrasound could enhance PMO delivery efficiency.

We next investigated whether 1–25 PMO could cure myotonic symptoms in HSA^{LR} mice when delivered using the Bubble liposome-ultrasound system. We administrated 1–25 PMO as described above. Three weeks later, we harvested the injected muscles and conducted RT-PCR and immunohistological analyses. RT-PCR showed that 1–25 PMO decreased the inclusion of exon 7A to a level comparable to that in wild-type FVB/n mice (Fig. 3a). We checked four other alternative splicing events, *Cypher* (*Ldb3*) exon 11, *Mbnl1* exon 5, *Ryr1* exon 70 and *Serca1* exon 22, which are known to be abnormally regulated in patients with DM1and HSA^{LR} mice[30]. We found that the alternative splicing of none of them was changed by

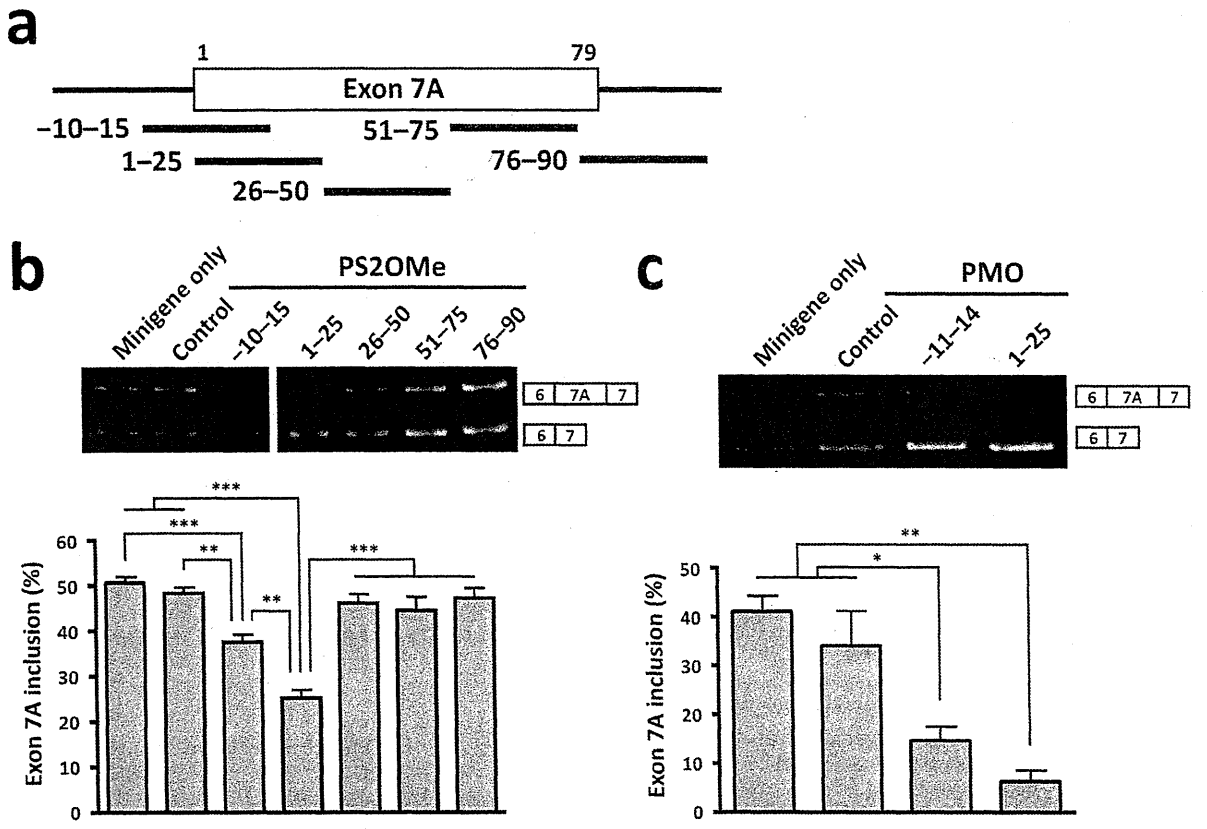

Figure 1 | AON-mediated exclusion of *Clcn1* exon 7A in COS-7 cells. (a) Locations of the target sites of AONs (thick black lines) along the *Clcn1* pre-mRNA. The numbers over exon 7A (rectangle) indicate the positions of nucleotides. (b) Cellular splicing assay to detect the exclusion of exon 7A of the *Clcn1* minigene by PS2OMe RNA in COS-7 cells. 1–25 was the most successful AON. A representative result is shown above and the bars indicate mean and s.e.m. ($n = 6$). (c) The same assay as in (b) except that PMOs were used. 1–25 PMO decreased the inclusion of exon 7A effectively ($n = 3$). Statistical significance was analysed by Tukey's multi-comparison test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

the injection of 1–25 PMO (Fig. 3b); thus, the effect of the PMO was specific to the alternative splicing of the *Clcn1* gene.

The abnormal splicing of *Clcn1* is believed to cause myotonia by introducing a premature termination codon into the subsequent exon and by decreasing the expression of the Clcn1 protein via nonsense-mediated mRNA decay. Thus, we examined whether 1–25 PMO restored the expression of Clcn1 protein in HSA^{LR} mice. Immunofluorescence analysis of TA muscles showed the sarcolemmal localisation of Clcn1 protein in wild-type muscle, but such a pattern was not detected in saline-injected HSA^{LR} mice. The injection of 1–25 PMO clearly restored the sarcolemmal distribution, demonstrating that correction of the abnormal splicing of the *Clcn1* gene led to the normal expression of its protein (Fig. 3c).

Finally, we investigated whether injection of 1–25 PMO improved the myotonic phenotype of HSA^{LR} mice. Electromyographic analyses showed bursts of action potentials after electrical stimulation (4–8 V) in HSA^{LR} mice, but not in wild-type mice (Fig. 4a). Myotonia occurred even in denervated muscles (data not shown), which indicates that it was not caused by hyperactivation of motor neurones, but by increased excitability of the sarcolemma. Myotonic EMG activities continued for 1 to 3 s and their average duration was 1.27 s in saline-treated TA muscles. The injection of 1–25 PMO decreased their duration, but the change was not statistically significant; the integrated EMG (iEMG) was reduced by the PMO administration ($P < 0.05$). The decreased iEMG indicated that 1–25 PMO mitigated the hyperexcitability of the HSA^{LR} muscles.

These results suggest that 1–25 PMO could improve the function of the *Clcn1* gene in the DM1 model mouse at the RNA, protein and

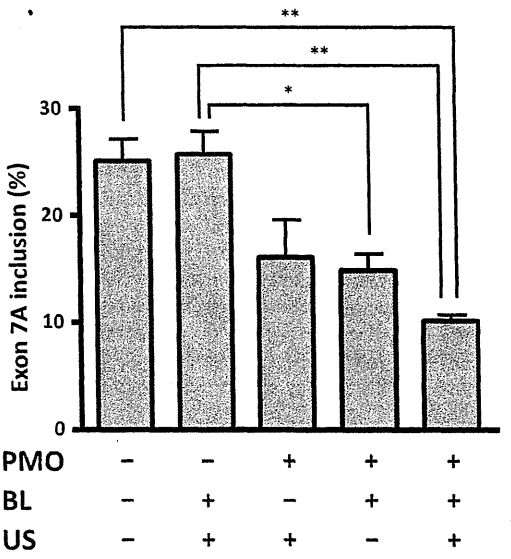

Figure 2 | PMO delivery by the combination of Bubble liposomes and ultrasound. 1–25 PMO (20 μg) or saline was locally administrated into TA muscles of HSA^{LR} mice with/without Bubble liposomes (BLs) and ultrasound (US). The inclusion rate of exon 7A decreased most when both Bubble liposomes and ultrasound were applied. ($n = 3$). The bars indicate mean and s.e.m., and statistical significance was analysed by Tukey's multi-comparison test (* $P < 0.05$, ** $P < 0.01$).

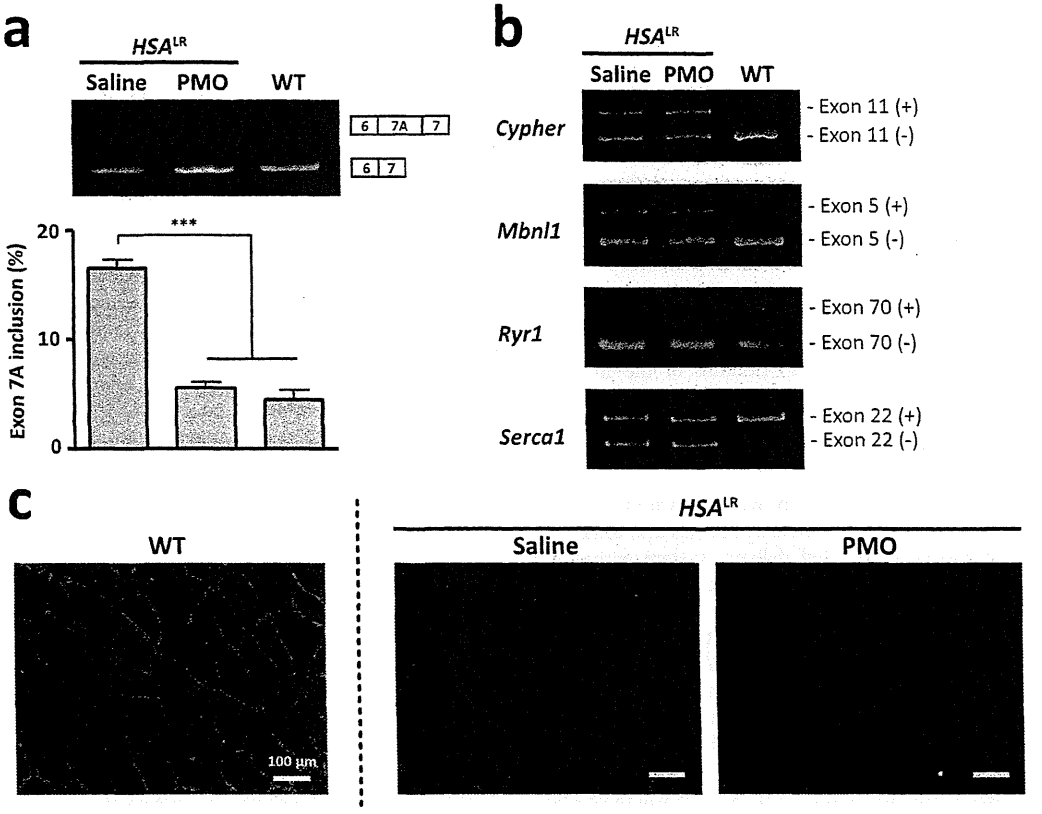

Figure 3 | *In vivo* improvement of aberrant splicing of the *Clcn1* gene by 1–25 PMO. (a) The alternative splicing of *Clcn1* exon 7A in HSA^{LR} and WT mice. 1–25 PMO (20 μg) or saline was locally administrated into TA muscles of HSA^{LR} mice. The ratio of the splicing variant containing exon 7A in thevPMO-injected muscles decreased to a level comparable with that in WT muscle ($n = 5$). The bars indicate mean and s.e.m., and statistical significance was analysed by Tukey's multi-comparison test (*** $P < 0.001$). (b) Abnormal splicing of other genes in HSA^{LR} mice was not affected by 1–25 PMO. (c) Immunofluorescence analysis of transverse sections of TA muscle with an anti-Clcn1 antibody. Injection of 1–25 PMO restored the sarcolemmal localisation of Clcn1 protein in TA muscles of HSA^{LR} mice.

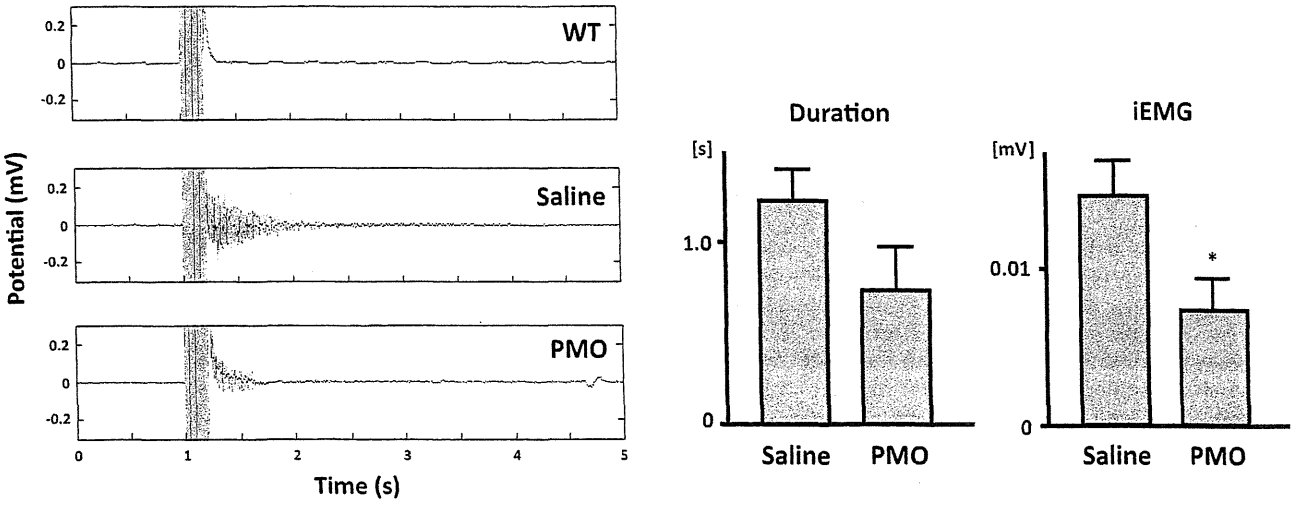

Figure 4 | Reduction of myotonic discharge by 1–25 PMO in HSA^{LR} mice. (a) Representative EMG signals of TA muscle. Tibialis muscle was electrically stimulated 1 s after the EMG recording started. In the HSA^{LR} muscle, repetitive discharges that were absent in the WT muscle could be seen. (b)vDuration of myotonic discharge and integrated EMG (iEMG). 1–25 PMO significantly reduced iEMG in HSA^{LR} muscle (saline, $n = 6$; 1–25 PMO, $n = 7$).The bars indicate mean and s.d., and statistical significance was analysed by Student's *t*-test (* $P < 0.05$).

phenotypic levels, and that the Bubble liposome-ultrasound system should be capable of delivering enough PMO to ameliorate myotonia.

Discussion

The greatest advantage of PMO is its remarkable innocuity. According to a report from Gene Tools, administration of a single 700 mg/kg dose of PMO to a mouse did not cause any obvious acute toxicity[31], while the 50% lethal dose of phosphorothioate DNA, a first-generation antisense oligonucleotide, was estimated to be 750 mg/kg in mice[32]. The high pharmaceutical potential of PMO is also supported by the fact that safety issues were not raised following its administration to mice and humans; however, PMO must overcome the low permeability of the cell membranes to achieve a significant effect on alternative splicing. Because of the inefficient cellular uptake of PMO, systemic delivery and functional splicing modification were not successful in a mouse model[33]. Therefore, for the clinical application of PMO, establishing an effective delivery method is essential.

The major achievement in this study was that we increased the efficiency of PMO delivery using Bubble liposomes and ultrasound. The use of the ultrasound-mediated delivery system with Bubble liposomes improved the alternative splicing of the *Clcn1* gene in HSA^{LR} mice to a level comparable to that of wild-type mice and decreased myotonic discharges, indicating that the delivery system increased introduction of 1–25 PMO into skeletal muscle. Our results show that the intramuscular injection itself delivered the PMO into muscle to some extent, and so the intramuscular injection might have contributed to the relief of the pathology. However, the Bubble liposome- and ultrasound-mediated enhancement of delivery efficiency suggests that the new delivery system will have a beneficial effect over much less invasive injection, such as intravenous injection, which cannot by itself be expected to promote the entry of PMO into skeletal muscle[19].

A fair amount of the *Clcn1* splicing variant without exon 7A was expressed even in the HSA^{LR} mouse. Because the myotonic discharge of a HSA^{LR} mouse was remedied by correcting *Clcn1* alternative splicing in a previous study[14], abnormal splicing of the gene must be the primary cause of myotonia. The expression levels of the "normal" splicing variant in saline-injected muscles were 57% of that in the wild-type muscles. PMO injection increased the expression about 1.4-fold to 78% of that in wild-type mice. As *Clcn1* heterozygous mutant mice did not show a myotonic phenotype[34], the myotonia in HSA^{LR} mice was unlikely to have been caused by haploinsufficiency of full-length Clcn1 protein. Instead, the truncated protein translated from the exon 7A-countaining mRNA may have dominant-negative activity, since full-length Clcn1 protein functions in a dimeric form. Berg *et al.* showed that the truncated Clcn1 protein did not function as a chloride channel, but rather disturbed the channel activity of full-length Clcn1 protein[35]. In this study, expression of the splicing variant containing exon 7A was decreased by 40% in the PMO-administered group. Thus, this may have contributed to the improvement of the pathology, as well as the increased expression of the exon 7A skipping variant. The dominant-negative hypothesis suggests that the AON therapy should completely prevent exon 7A inclusion when used to treat myotonia, in contrast to Duchenne muscular dystrophy, in which the partial restoration of dystrophin expression could lead to the improvement of muscle strength.

In the course of our search for the optimal PMO sequence to correct *Clcn1* splicing, we found that 1–25 and 16–40 PMOs suppressed the inclusion of exon 7A well, but that 26–50 PMO did not. The fact that steric blocking of the 16–25 region promoted exon skipping indicates that proteins that bind to this region are essential for exon 7A recognition. Previously, we showed that the 8 nt at the 5′ end of exon 7A serve as an ESE and that an RNA-binding protein, Mbnl1, prevented the inclusion of exon 7A by binding to the ESE[29]. Unlike the ESE, the sequence of the 16–25 region was pyrimidine-rich and did not contain the Mbnl1-recognition motif, YGCY. It remains to be determined which proteins bind to the region to regulate exon 7A splicing.

In this study, we tried to cure DM1 model mice using a PMO targeted to *Clcn1*. However, considering that dozens of genes are abnormally spliced in patients with DM1, it might be impractical to treat all symptoms due to mis-splicing of such genes with AONs at the same time. DM1 is caused by expansion of the CTG repeats in the 3′ UTR region of the DMPK gene. Transcripts with these expanded repeats sequester Mbnl proteins, which regulate alternative splicing, leading to global alternative splicing dysfunction[36]. Thus, expanded CUG repeat-containing RNA must be the most important target of antisense therapy for DM1, and many groups have studied the use of CAG repeat-containing AONs to dissociate Mbnl1 proteins from CUG repeat-containing RNA. Some trials to treat DM1 model mice with CAG AONs were successful[19,37], but here again the obstacle to clinical application of the AONs was the lack of an efficient delivery method. Our ultrasound-mediated delivery system with Bubble liposomes must have a beneficial effect on the delivery of CAG-containing AONs.

Methods

AONs. Phosphorothioate 2′ *O*-methyl RNA oligonucleotides and phosphorodiamidate morpholino oligonucleotides were purchased from IDT (Coralville, IA, USA) and Gene Tools (Philomath, OR, USA), respectively. The sequences of the oligonucleotides are listed in Supplementary Table ST1. Both AONs were dissolved in water.

Construct. The *Clcn1* minigene has been described previously[29]. Briefly, a *Clcn1* minigene fragment covering exons 6 to 7 was amplified from mouse genomic DNA by PCR and inserted into the *BglII-SalI* sites of pEGFP-C1 (Clontech Laboratories, Mountain View, CA, USA).

Cellular splicing assay. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Foster City, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37°C.

For the splicing assay, COS-7 cells were cultured in 12-well plates and transfected with 0.1 μg of the *Clcn1* minigene and AONs (0.1 μmol) at 60–80% confluence. Polyethylenimine and Endo-Porter (Gene Tools) were used for the transfection of PS2OMe RNA and PMOs, respectively. Forty-eight hours later, total RNA was extracted from the transfected cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich).

Animals. HSA^{LR} mice are FVB/n-background transgenic mice that express expanded CTG repeats under the control of the human skeletal actin promoter in skeletal muscle[38]. Compared with the first established line, the number of the repeat was reduced: the mice used in this study carried 180–200 repeats. All the mutant mice showed persistent contraction of gluteal muscles after they bucked. We used FVB/nJcl mice (Clea Japan, Tokyo, Japan) as wild-type controls.

The present study was approved by the Ethical Committee for Animal Experiments at the University of Tokyo, and was carried out in accordance with the Guidelines for Research with Experimental Animals of the University of Tokyo and the NIH Guide for the Care and Use of Laboratory Animals (NIH Guide, revised 1996).

Bubble liposomes. Bubble liposomes were prepared by previously described methods[24]. Briefly, PEG liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (NOF Corporation, Tokyo, Japan) and 1,2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine-polyethyleneglycol (DSPE-PEG2000-OMe) (NOF Corporation) at a molar ratio of 94 : 6 were prepared by a reverse phase evaporation method. Briefly, all reagents were dissolved in 1 : 1 (v/v) chloroform/diisopropyl ether. Phosphate-buffered saline was added to the lipid solution, and the mixture was sonicated and then evaporated at 47°C. The organic solvent was completely removed, and the size of the liposomes was adjusted to less than 200 nm using extruding equipment and a sizing filter (pore size: 200 nm) (Nuclepore Track-Etch Membrane; Whatman Plc, Maidstone, Kent, UK). The lipid concentration was measured using a Phospholipid C test (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Bubble liposomes were prepared from liposomes and perfluoropropane gas (Takachio Chemical Ind. Co. Ltd, Tokyo, Japan). First, 2-ml sterilised vials containing 0.8 ml of liposome suspension (lipid concentration: 1 mg/ml) were filled with perfluoropropane gas, capped and then pressurised with a further 3 ml of perfluoropropane gas. The vial was placed in a bath-type sonicator (38 kHz, 250 W) (SONO-CLEANER CA-4481L; Kaijo Denki, Tokyo, Japan) for 1 min to form Bubble liposomes.