厚生労働科学研究費補助金
(障害者対策総合研究事業（神経・筋疾患分野))
分担研究報告書

患者由来細胞を用いた *in vitro* でのエクソン・スキップ評価手法の確立

研究分担者　木村　　円
国立精神・神経医療研究センター
トランスレーショナル・メディカルセンター
臨床研究支援部　早期・探索的臨床試験室長

研究要旨

Duchenne 型筋ジストロフィー（DMD）に対するアンチセンス核酸を用いた臨床試験の実施に際しては、被験者への投与前に被験者由来細胞を用いて、被験薬の有効性（mRNA における標的エクソンのスキップ、およびジストロフィンタンパク質の発現）を確認することが必要である。細胞としては、皮膚由来線維芽細胞または筋由来筋芽細胞を、筋管細胞に分化させて用いる。筋管への分化手法にはウイルスによる *MYOD* 遺伝子の導入で行う。本手法による検討を、複数の患者由来細胞を用いて行い、mRNA でのスキップとジストロフィンの発現を確認した。臨床試験における被験者の適格性確認として、*in vitro* でアッセイを実施する評価手法が確立できた。

A. 研究目的

Duchenne 型筋ジストロフィー（DMD）のエクソン・スキップの臨床試験を実施するにあたり、特にファースト・イン・ヒューマン試験などの早期相においては、投与するアンチセンス核酸で目的とするエクソンのスキップ、およびジストロフィンタンパク質の発現が、投与前に確認されることが重要である。これは一般的な医薬品と異なり、エクソン・スキップを目的としたアンチセンス核酸は、適応となる変異形式を有する被験者に対してのみ有効性をもたらすが、健常人または適応とならない変異形式を有する DMD 患者に対しては、mRNA はフレームシフトのままであり、特に健常人にとっては正常なジストロフィンの発現を阻害し、有害な作用をもたらすこととなる。そのため、被験者の細胞を用いて *in vitro* でアンチセンス核酸の有効性を確認することは、有効性の観点のみならず、被験者の適格性判定の観点からも重要である。

In vitro アッセイに用いられる細胞は、ジストロフィン mRNA が十分に発現していることが必要であり、筋生検で得られる筋芽細胞が適している。しかしながら、筋組織から初代細胞を分離する際には高い割合で線維芽細胞も含まれ、筋芽細胞を純化する過程で十分量の筋芽細胞が得られないことも多い。一方、線維芽細胞は、生検骨格筋、あるいは生検皮膚からも得ることが可能であり、増殖も活発であることから確保は比較的容易である。線維芽細胞はそのままではジストロフィン mRNA の発現は少なく、アッセイに用いることは困難であるが、*MYOD* 遺伝子をウイルスで導入することにより、筋分化を誘導してジストロフィン mRNA の発現を増加させることが可能である。変異 mRNA がアンチセンス核酸によりインフレームに修正されると、短縮形ジストロフィンタンパク質としても検出される。このように、被験薬の投与前に *in vitro* アッセイ系により、エクソン・スキップを評

価する手法を構築することを目的として本研究を実施した。

B. 研究方法

国立精神・神経医療研究センター神経・筋疾患研究資源リポジトリー（NCNP リポジトリー）、またはCoriell cell repositoryに保存されている複数の DMD 患者由来を用いた。NCNP リポジトリーの細胞使用にあたっては、同センター倫理委員会の承認を得た。*MYOD* 遺伝子の導入はレトロウイルス（pRetro-X expression system, Clontech）またはレンチウイルス（pLenti-X expression system, Clontech）を用いて行った[1]。いずれの発現ベクターにも、*MYOD* 遺伝子とともに IRES 配列を挟んで ZsGreen1 蛍光タンパク質をマーカーとして組み込んだ。レトロウイルスによる系ではウイルス導入5日後にFACSを用いてZsGreen1陽性細胞をMyoD発現細胞として回収し、これを分化培地で培養して筋分化を誘導した。レンチウイルスによる系では、24時間ウイルス導入を行ったあと、FACS による選択は行わず分化培地に交換して筋分化を誘導した。いずれも筋分化誘導開始7日目にアンチセンス核酸を最大 10μM の濃度で48時間添加し、その後分化開始 14 日目まで培養して回収した。回収した細胞はRT-PCRおよびウェスタンブロット（WB）で評価した。RT-PCR による活性の評価は自動電気泳動装置（Experion, Bio-rad）を用いて、目的のエクソンがスキップされたPCR産物が、全体のPCR産物に占める割合で評価した。

C. 研究成果

1. エクソン48－52欠失DMD患者由来線維芽細胞を用いた検討

本細胞はエクソン53スキップにより、エクソン47とエクソン54が接続しインフレームとなる。この線維芽細胞にレトロウイルスの系で筋分化を誘導した。FACS で回収後の細胞は全て ZsGreen1 陽性であり、*MYOD* が共発現しているものと考えられた。その後、多核で幅の広い筋管細胞に分化した。アンチセンス投与後、濃度依存性に RT－PCR 上でのエクソン・スキップ活性は上昇した。また WB でもジストロフィンのシグナルを検出した。

2. エクソン51－55欠失DMD患者由来線維芽細胞を用いた検討

本細胞はエクソン50スキップにより、エクソン49とエクソン56が接続してインフレームとなる。本細胞については、まず1.と同様にレトロウイルスの系において、濃度依存性のエクソン 50 スキップおよびジストロフィン発現を確認した。次に、本細胞をレンチウイルスの系において検討した。レンチウイルスを用いた系では、ウイルスのトランスフェクション後に FACS による選択を行わずに筋分化を誘導した。アッセイ開始時点で播種した線維芽細胞のうち、レンチウイルス感染後に ZsGreen1 が目視で確認できる細胞は 30-50% であったが、分化誘導後は長軸方向に伸長し、多核となり形態的には筋管細胞となった。回収した細胞では RT-PCR, WB でそれぞれスキップとジストロフィン発現が確認され、レトロウイルスの系と同様の結果が得られた。

D. 考　察

検討の結果、DMD 患者由来線維芽細胞にレトロウイルスおよびレンチウイルスを用いて *MYOD* 遺伝子を導入し、筋分化を誘導する手法により、アンチセンス核酸の有効性を *in vitro* で検証することが可能であった。実際の臨床試験における活用では、以下の点について検討する必要があると思われた。

1. DMD の臨床試験のスケジュールにおける細胞採取の時期

計画中のプロトコールでは、治験薬の投与開始前および投与終了後に筋生検を行い、筋切片のジストロフィン免疫蛍光染色および WB にて、投与前後のジストロフィン発現を評価

する予定となっている。そのため、投与開始前の筋生検が、細胞を採取するのに適切なタイミングと考えられる。しかし、もし当該被験者がすでに診断・研究目的で細胞を提供し、これがレポジトリーに保存されている場合にはこれを用いることも可能と思われる。その場合、採取組織から初代細胞の分離に要する期間（約１ヶ月）が省略できるため、臨床試験の実施期間を短縮できるメリットがある。

2. *MYOD* 遺伝子導入時のレトロウイルスとレンチウイルスの使い分けについて

一般的にレトロウイルスは分裂細胞に効率的に感染するため、増殖速度の早い線維芽細胞には高効率に導入されるが、増殖速度が遅い場合には効率が低下する傾向にある。そのため、アッセイに必要な細胞を確保することが困難な場合がある。レンチウイルスは非分裂細胞にも感染するとされているため、このような場合にはレトロウイルスの代替として用いることが可能である。しかし、我々のレンチウイルスベクターでは力価を確保するための濃縮が必要であり、ウイルスの収量としてはレトロウイルスよりも少なくなる傾向にあった。そのため、レンチウイルスは増殖速度の低い線維芽細胞に対して例外的に用いることが、ウイルスの収量を踏まえた効率的な使用法であると考えられた。

E. 結　論

DMD に対するアンチセンス核酸を用いた臨床試験の実施にあたっては、有効性確認および適格性確認の観点から、被験者由来線維芽細胞に *MYOD* 遺伝子を導入し、筋管細胞に分化させて *in vitro* でアンチセンスの評価を行うことが必要である。本手法によるエクソン・スキップの評価を患者由来細胞を用いて行ったところ、mRNA でのスキップとジストロフィンタンパク質の発現を確認することが出来た。

参考文献

1. Saito T, *et al.*, PLoS One. 5:e12239, 2010

F. 健康危険情報

なし

G. 研究発表

I 論文発表

＜英文＞

【欧文原著】

1) Yamashita S, Kimura E, Tawara N, Sakaguchi H, Nakama T, Maeda Y, Hirano T, Uchino M, Ando Y : Optineurin is potentially associated with TDP-43 and involved in the pathogenesis of inclusion body myositis. ***Neuropathol Appl Neurobiol.*** 39:406-16, 2013

2) Uchino M, Yamashita S, Uchino K, Mori A, Hara A, Suga T, Hirahara T, Koide T, Kimura E, Yamashita T, Ueda A, Kurisaki R, Suzuki J, Honda S, Maeda Y, Hirano T, Ando Y : Muscle biopsy findings predictive of malignancy in rare infiltrative dermatomyositis. ***Clin Neurol Neurosurg.*** 115:603-6, 2013

3) Tanaka A, Woltjen K, Miyake K, Hotta A, Ikeya M, Yamamoto T, Nishino T, Shoji E, Sehara-Fujisawa A, Manabe Y, Fujii N, Hanaoka K, Era T, Yamashita S, Isobe K, Kimura E, Sakurai H : Efficient and Reproducible Myogenic Differentiation from Human iPS Cells: Prospects for Modeling Miyoshi Myopathy In Vitro. ***PLoS One.*** 8:e61540, 2013

4) Nakamura H, Kimura E, Mori-Yoshimura M, Komaki H, Matsuda Y, Goto K, Hayashi YK, Nishino I, Takeda SI, Kawai M : Characteristics of Japanese Duchenne and Becker muscular dystrophy patients in a novel Japanese national registry of muscular dystrophy (Remudy). ***Orphanet J Rare Dis.*** 8:60, 2013

5) Yamashita S, Uchida Y, Kojima S, Sakaguchi H, Kimura E, Maeda Y, Uchino M : Heatstroke in patients with Parkinson's disease. ***Neurol Sci***. 33:685-687, 2012
6) Yamashita S, Sakaguchi H, Mori A, Kimura E, Maeda Y, Hirano T, Uchino M : Significant CMAP decrement by repetitive nerve stimulation is more frequent in median than ulnar nerves of patients with amyotrophic lateral sclerosis. ***Muscle & nerve***. 45:426-428, 2012
7) Sakaguchi H, Yamashita S, Hirano T, Nakajima M, Kimura E, Maeda Y, Uchino M : Myasthenic crisis patients who require intensive care unit management. ***Muscle & nerve***. 46:440-442, 2012
8) Ikeda T, Kimura E, Hirano T, Uchino M : The association between dermatomyositis and papillary thyroid cancer: a case report. ***Rheumatol Int***. 32:959-961, 2012

＜和文＞

【和文著書】

1) 木村円, 武田伸一:進行性筋ジストロフィー（ジストロフィノパチー）『今日の神経疾患治療指針 第2版』医学書院, 2013
2) 木村円: Remudyによる患者登録『筋疾患のみかた，考えかた』中外医学社, 2013

H. 知的所有権の出願・登録状況

1. 特許

出願

2. 実用新案登録

なし

3. その他、特記事項

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yokota T, Nakamura A, Nagata T, Saito T, Kobayashi M, Aoki Y, Echigoya Y, Partridge T, Hoffman EP, Takeda S	Extensive and prolonged restoration of dystrophin expression with vivo-morpholino-mediated multiple exon skipping in dystrophic dogs.	***Nucleic Acid Ther.***	22	306-315	2012
Aoki Y, Yokota T, Nagata T, Nakamura A, Tanihata J, Saito T, Duguez SMR, Nagaraju K, Hoffman EP, Partridge T, Takeda S	Bodywide skipping of exons 45-55 in dystrophic mdx52 mice by systemic antisense delivery.	***Proc Natl Acad Sci USA***	109	13763 -13768	2012

NUCLEIC ACID THERAPEUTICS
Volume 22, Number 5, 2012

DOI: 10.1089/nat.2012.0368

Extensive and Prolonged Restoration of Dystrophin Expression with Vivo-Morpholino-Mediated Multiple Exon Skipping in Dystrophic Dogs

Toshifumi Yokota,[1–3] Akinori Nakamura,[3,4] Tetsuya Nagata,[3] Takashi Saito,[3,5] Masanori Kobayashi,[3,6] Yoshitsugu Aoki,[3] Yusuke Echigoya,[1] Terence Partridge,[7,8] Eric P. Hoffman,[7,8] and Shin'ichi Takeda[3]

Duchenne muscular dystrophy (DMD) is a severe and the most prevalent form of muscular dystrophy, characterized by rapid progression of muscle degeneration. Antisense-mediated exon skipping is currently one of the most promising therapeutic options for DMD. However, unmodified antisense oligos such as morpholinos require frequent (weekly or bi-weekly) injections. Recently, new generation morpholinos such as vivo-morpholinos are reported to lead to extensive and prolonged dystrophin expression in the dystrophic *mdx* mouse, an animal model of DMD. The vivo-morpholino contains a cell-penetrating moiety, octa-guanidine dendrimer. Here, we sought to test the efficacy of multiple exon skipping of exons 6–8 with vivo-morpholinos in the canine X-linked muscular dystrophy, which harbors a splice site mutation at the boundary of intron 6 and exon 7. We designed and optimized novel antisense cocktail sequences and combinations for exon 8 skipping and demonstrated effective exon skipping in dystrophic dogs *in vivo*. Intramuscular injections with newly designed cocktail oligos led to high levels of dystrophin expression, with some samples similar to wild-type levels. This is the first report of successful rescue of dystrophin expression with morpholino conjugates in dystrophic dogs. Our results show the potential of phosphorodiamidate morpholino oligomer conjugates as therapeutic agents for DMD.

Introduction

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a lethal and the most common form of muscular dystrophy worldwide, which affects 1 in 3,500 boys (Duchenne, 1867; Zellweger and Antonik, 1975). There is currently no effective cure for DMD. Most patients die in their 20s–30s with respiratory or heart failure. DMD and its milder form, Becker muscular dystrophy, are caused by mutations in the *dystrophin* (*DMD*) gene (Hoffman et al., 1987; Koenig et al., 1987). Antisense oligonucleotide-mediated exon skipping therapy is a most promising approach to curing DMD (Pramono et al., 1996; Dunckley et al., 1998; Goyenvalle et al., 2011; Lu et al., 2011). Antisense oligos such as phosphorodiamidate morpholino oligomers (PMOs, or morpholinos) and 2'O- methyl antisense oligos with phosphorothioate bonds (2'OMePS) against dystrophin mRNA lead to the production of internally deleted in-frame transcripts both *in vitro* and *in vivo* (Pramono et al., 1996; Dunckley et al., 1998; Lu et al., 2005; Yokota et al., 2009a; Yokota et al., 2012). The truncated quasi-dystrophin retains some functions like mild Becker dystrophy or even leads to asymptomatic individuals in some cases (Beroud et al., 2006; Nakamura et al., 2008; Aoki et al., 2010; Goyenvalle et al., 2010). Exon skipping therapies with PMO or 2'OMePS

[1]Department of Medical Genetics, School of Human Development, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada.
[2]The Friends of Garrett Cumming Research & Muscular Dystrophy Canada HM Toupin Neurological Science Research Chair, Edmonton, Alberta, Canada.
[3]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan.
[4]Third Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Japan.
[5]Department of Pediatrics, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan.
[6]Department of Reproduction, Nippon Veterinary and Life Science University, Tokyo, Japan.
[7]Research Center for Genetic Medicine, Children's National Medical Center, Washington, District of Columbia.
[8]Department of Integrative Systems Biology, George Washington University School of Medicine, Washington, District of Columbia.

antisense oligos targeting the exon 51 are currently under phase-2/3 clinical trials (Aartsma-Rus and van Ommen, 2007; van Deutekom et al., 2007; Kinali et al., 2009; Cirak et al., 2011; Goemans et al., 2011).

One of the biggest challenges of exon-skipping therapy is that the single exon skipping is applicable to only approximately 50% of DMD patients (total of each single individual target exon). In contrast, double or multiple exon skipping is potentially applicable to 90% of patients (Aartsma-Rus et al., 2006; Yokota et al., 2007a). The dystrophic dog requires more than one exon skipping (multiple exon skipping targeting exon 6 and exon 8 in the dystrophin mRNA). Previously we reported the first successful multiple (double) exon-skipping treatment in body-wide skeletal muscles in Canine X-linked muscular dystrophy (CXMD) with a cocktail of antisense phosphorodiamidate morpholino oligomers (PMOs, morpholinos) (Yokota et al., 2009a). The dog trial targeting exon 6 and exon 8 of dystrophin mRNA led to 27% normal levels of dystrophin expression in body-wide skeletal muscles detected by western blotting analysis on average. However, unmodified morpholinos exhibit inefficient long-term delivery. The half-life of dystrophin expression was approximately 1–2 months (Wu et al., 2010).

Recently, new generation morpholinos such as cell-penetrating peptide conjugated phosphorodiamidate morpholino oligomers (PPMOs) and vivo-morpholinos (vPMOs) were reported to induce prolonged and extensive rescue of dystrophin expression and ameliorate the function in cardiac muscles in dystrophic *mdx* mice (Wu et al., 2009; Goyenvalle et al., 2010; Jearawiriyapaisarn et al., 2010; Crisp et al., 2011; Widrick et al., 2011; Wu et al., 2011a). vPMOs are morpholino oligomers conjugated with delivery moiety containing eight terminal guanidinium groups on a dendrimer scaffold that enable entry into cells (Fig. 1A) (Morcos et al., 2008). New generation morpholinos are efficiently delivered into various tissues including muscle fibers *in vivo* (Wu et al., 2009). Vivo-morpholino-mediated splice modulation efficiently also rescued Fukuyama congenital muscular dystrophy model mice and primary myotubes from human patients (Taniguchi-Ikeda et al., 2011). Their delivery efficacy is reported to be more than 50 times higher than unmodified morpholinos (Wu et al., 2009).

In this study, we focused on 2 aims. First, we employed a novel backbone (vivo-morpholino) for the antisense therapy in the dog model. Second, we tested novel antisense oligo cocktails designed for multiple exon skipping (exons 6 and 8) in the canine *DMD* gene. We hypothesized that (1) vivo-morpholinos induce extensive and prolonged dystrophin expression in dystrophic dogs, and (2) our novel antisense oligo cocktail can improve the efficacy of exon 6–8 multiple skipping. We tested these hypotheses and the efficacy of multiple (double) exon skipping in dystrophic dogs *in vivo*. Vivo-morpholinos with newly optimized sequences induced near normal level of dystrophin protein and prolonged expression recovery.

Materials and Methods

Ethics Statement

All animal works have been conducted according to relevant national and international guidelines. The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP) Japan approved all experimental protocols in this study. We obtained consent from all of the owners of the dogs involved in this study (All dogs are owned by NCNP).

Animals

The CXMD dog is the beagle dog model of DMD (Shimatsu et al., 2003). They were allowed *ad libitum* access to food and drinking water. Dogs carrying mutations were identified by reverse-transcription polymerase chain reaction (RT-PCR) analysis as previously described (Sharp et al., 1992). Three- to five-month-old dogs were used. Five dystrophic dogs were used for injections. Four dystrophic dogs and three wild-type dogs were used as non-treated controls. Animals were euthanized by exsanguination under general anesthesia.

Antisense oligos

Antisense oligos for targeted skipping of exons 6 and exon 8 in the canine *DMD* gene were used as previously described (Tables 1, 2) (Yokota et al., 2009a; Saito et al., 2010). All PMOs and vPMOs were obtained from Gene-tools, Inc (Morcos et al., 2008). As control oligos, we employed Ex6A only (GTTGATTGTCGGACCCAGCTCAGG) or 3-oligo cocktail containing Ex6A (GTTGATTGTCGGACCCAGCTCAGG), Ex6B (ACCTATGACTGTGGATGAGAGCGTT), and Ex8A (CTTCCTGGATGGCTTCAATGCTCAC) for intramuscular injections as indicated. The dose selection is based on previous mouse studies with PMOs and vPMOs, showing that vPMOs induce more than 10×higher efficacy, and dog studies with PMOs (Wu et al., 2009). The Ex8G dose and ratio were determined based on previous cell experiments (Saito et al., 2010).

Injections

Animals were anesthetized with thiopental sodium induction and maintained by isoflurane (Nacalai Tesque, Inc.) for all intramuscular injections and muscle biopsies. General anesthesia was maintained with isoflurane administered through an endotracheal tube. Skin was excised over the site of injection, muscle exposed, and the injection site marked with a suture in the muscle. Antisense oligonucleotides were delivered by intramuscular injection using 1 mL saline bolus into indicated skeletal muscles using a 27-gauge needle. Antisense oligonucleotides were delivered as a singular or in mixtures as previously described (Yokota et al., 2011). Tibialis anterior, extensor digitorum longus, extensor carpi ulnaris, flexor digitorum superficialis (FDS), flexor carpi ulnaris (FCU), and flexor carpi radialis (FCR) muscles were used for injections. Muscles samples were obtained 2 or 8 weeks after the intramuscular injections. Muscles were obtained immediately, snap-frozen in liquid nitrogen–cooled isopentane, and stored at −80°C for immunohistochemistry and western blotting. Skeletal muscle tissues were cut and collected in microtubes and snap-frozen in liquid nitrogen for RT-PCR analysis.

Immunohistochemical analysis

Antibodies. The following monoclonal antibodies were used for immunofluorescence: anti-dystrophin DYS-1 (Novocastra, Newcastle upon Tyne, UK). Alexa 488, or Alexa 594 conjugated goat anti mouse secondary antibodies (Invitrogen).

Immunofluorescence. Cryosections (7.5 μm) were blocked with 20% goat serum in phosphate buffered saline,

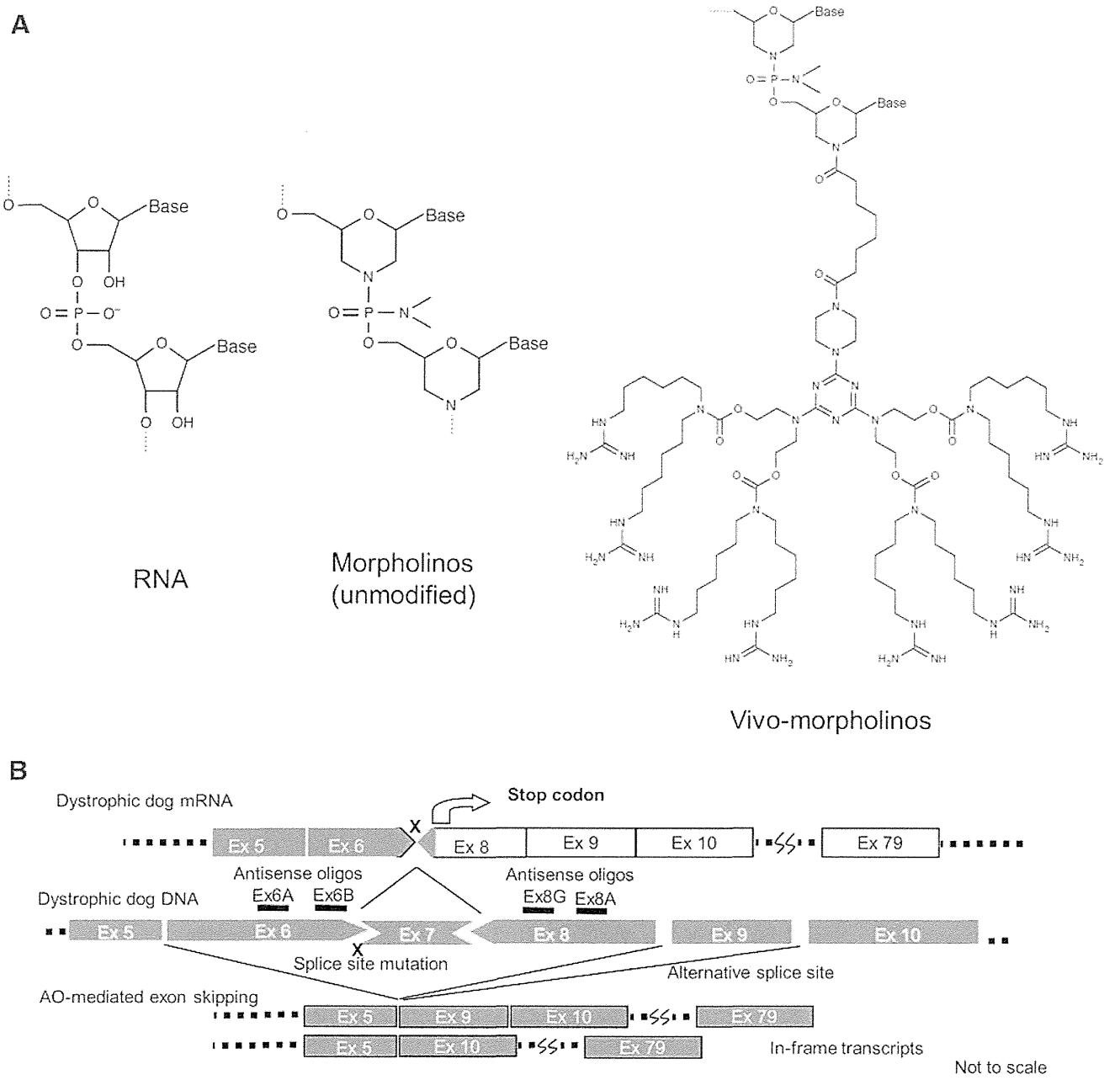

FIG. 1. Antisense chemistry and design of multiple exon skipping for dystrophic dogs. **(A)** Comparison of antisense oligos. **(B)** Schematic design of multi (double) exon skipping therapy for dystrophic dogs. At least two exons (exons 6 and 8) need to be skipped (removed) with antisense oligos to correct their reading frame. Gua, Guanidine.

and then incubated with a primary antibody at 4°C overnight. Alexa 488, or 594-conjugated anti-mouse goat antibody (Invitrogen, Camarillo, CA) was used as the secondary antibody. The sections were viewed and photographed by a laser scanning microscope, FluoView™ (Olympus, Tokyo, Japan) The number of positive fibers for DYS-1 was counted and compared in sections containing the largest number of positive fibers as described previously (Yokota et al., 2006). At least 200 muscle fibers were counted in each section for the analysis.

TABLE 1. ANTISENSE OLIGO SEQUENCES

Oligo name	*Sequence (5′–3′)*
Ex6A	GTTGATTGTCGGACCCAGCTCAGG
Ex6B	ACCTATGACTGTGGATGAGAGCGTT
Ex8A	CTTCCTGGATGGCTTCAATGCTCAC

TABLE 2. ADDITIONAL ANTISENSE OLIGO SEQUENCES FOR EXON 8

Oligo name	*Sequence (5′–3′)*
Ex8G	GGCAAAACTTGGAAGAGTGATGTGA
Ex8I	CCTTGGCAACATTTCCACTTCCTGG
Ex8K	TTTACCTGTTGAGAATAGTGCATTT

Western blotting analysis

Muscle proteins from cryosections were extracted with lysis buffer containing 75 mM Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate, 10 mM EDTA, and 5% 2-mercaptoethanol. Four to 40 μg proteins were loaded onto precase 3%–8% resolving sodium dodecyl sulfate polyacrylamide gel electrophoresis gels following manufacturer's instructions (Bio-Rad, Hercules, CA). The gels were transferred by semidry blotting at 400 mA for 1.5 hours. DYS-1 (Novocastra) antibody against dystrophin and rabbit polyclonal antibody against desmin (Abcam) were used as primary antibodies. Horseradish peroxidase–conjugated anti-mouse or anti-rabbit goat immunoglobulin (Cedarlane Laboratories, Hornby, Ontario,

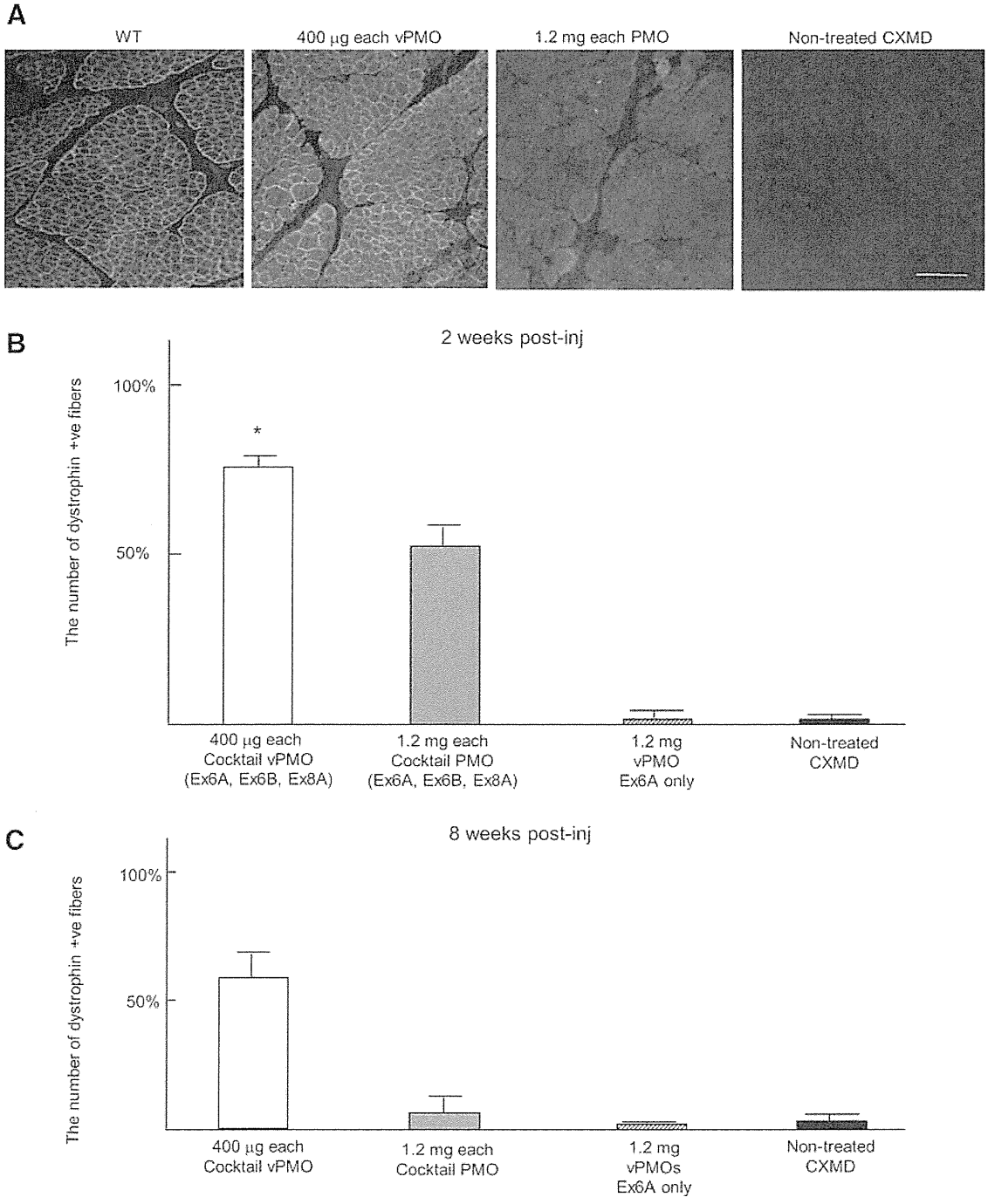

FIG. 2. Vivo phosphorodiamidate morpholino oligomer (vPMO) local injections restore dystrophin expression in TA 8 weeks later. **(A)** Immunohistochemistry with dystrophin (DYS1) antibody 8 weeks after the cocktail vPMO treatment containing Ex6A, Ex6B, and Ex8A in canine X-linked muscular dystrophy (CXMD) (1.2 mg in total as a cocktail, 400 μg of each oligo), and unmodified morpholino treatment containing Ex6A, Ex6B, and Ex8A in CXMD (3.6 mg in total as a cocktail, 1.2 mg of each oligo). **(B)** The number of dystrophin positive fibers 2 weeks after injections. **(C)** The number of dystrophin positive fibers 8 weeks after injections. Scale bar = 200 μm; n = 2–4 in each group; $^*P < 0.05$ compared with non-treated control group.

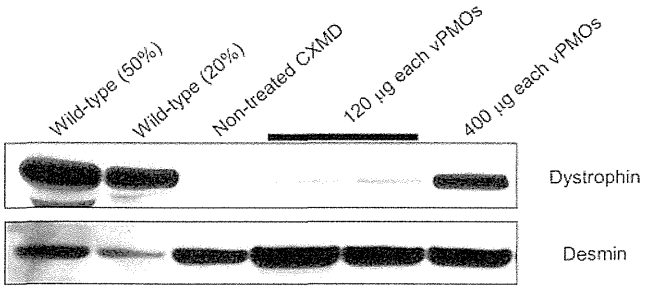

FIG. 3. Prolonged dystrophin expression after vPMO injections. Western blotting analysis on dystrophin expression with DYS-1 antibody 8 weeks after vPMO cocktail injections (120 μg each or 400 μg each of Ex6A, Ex6B, and Ex8A) into TA muscles in dystrophic dogs as indicated.

Canada) was used as a secondary antibody. Enzyme chemiluminescence kit (GE, Fairfield, CT) was used for the detection. Blots were analyzed by ImageJ software (Collins, 2007).

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from frozen tissue sections using TRIzol (Invitrogen). Then RT-PCR was performed on 200 ng of total RNA for 35 cycles of amplification using One-Step RT-PCR kit (Qiagen, Chatsworth, CA) following manufacturer's instructions with 0.6 μM of an exon 5 (CTGACTCTTGGTTTGATTTGGA) forward primer. Reverse primers were exon 10 (TGCTTCGGTCTCTGTCAATG).

Statistical analysis

The data between samples were compared using *F*-test and Student's or Welch's *t*-test. $P<0.05$ was considered statistically significant.

Results

Design of antisense vivo-morpholinos

In this study, we employed a cocktail of antisense vivo-morpholino oligos (Gene-tools) to induce exon skipping of exon 6 and exon 8 in the canine *dystrophin* (*DMD*) gene (Fig. 1A). A vivo-morpholino is comprised of a morpholino oligo with a covalently linked delivery moiety, an octa-guanidine dendrimer. As previously demonstrated, at least two exons (exon 6 and exon 8) need to be removed to restore the reading frame of the splice site mutation in the CXMD (Fig. 1B) (McClorey et al., 2006; Yokota et al., 2009a). Initially, we employed a cocktail oligo with the same sequences and combinations, Ex6A, Ex6B, and Ex8A, as previously used (Yokota et al., 2009a) (Table 1). We compared the efficacy of exon skipping by vPMOs and unmodified morpholinos.

Sustained dystrophin expression after cocktail vPMO injections

Since sustained recovery of dystrophin expression was previously reported after vivo-morpholino injections into dystrophic *mdx* mice, we tested the dystrophin expression levels 2 weeks and 8 weeks after vPMO injections in cranial tibialis (tibialis anterior in humans) muscles in dystrophic dogs (Fig. 2) (Jearawiriyapaisarn et al., 2008; Wu et al., 2009; Widrick et al., 2011; Wu et al., 2011a). In this study, we employed 3- to 5-month-old dystrophic dogs. At this stage the disease progression was relatively mild in these dogs. We employed a cocktail of three antisense oligos named Ex6A, Ex6B, and Ex8A (Table 1). We used anti-rod domain dystrophin antibody because anti-C-terminus dystrophin antibodies cross-react with other dystrophin isoforms (e.g., Dp71).

While unmodified PMO (1.2 mg each, or 3.6 mg in total as a cocktail) injected muscle showed almost no detectable dystrophin expression 8 weeks after injections, extensive dystrophin expression was observed after 400 μg vPMO injected muscles (Fig. 2A). At 2 weeks after the vPMO (400 μg each) injection, approximately 75% of fibers were positive with dystrophin DYS-1 antibody, while 55% were positive after unmodified PMO injection (1.2 mg each) (Fig. 2B). A cocktail of antisense vPMOs was required to induce dystrophin expression (Fig.2B). Injections with single antisense vPMO targeting exon 6 only (Ex6A) did not induce detectable level of dystrophin expression (Fig. 2B). At 8 weeks after vPMO injection, approximately 60% of fibers were still positive with DYS-1 antibody, while only 10% were positive after unmodified PMO injection (Fig. 2C).

The expression level of dystrophin was then examined by western blotting analysis (Fig. 3). Approximately 20% of the level of dystrophin in wild-type was detected in vPMO-injected muscles 8 weeks after the injection with 400 μg each of Ex6A, Exx6B, and Ex8A (or 1.2 mg in total as a cocktail).

Design of novel antisense sequences and combinations for dystrophin exon 8

Next, to further optimize the antisense oligo sequences and combinations, we tested new oligos named Ex8G, Ex8I, and Ex8K (Fig. 4, Table 2). In previous study, these oligos

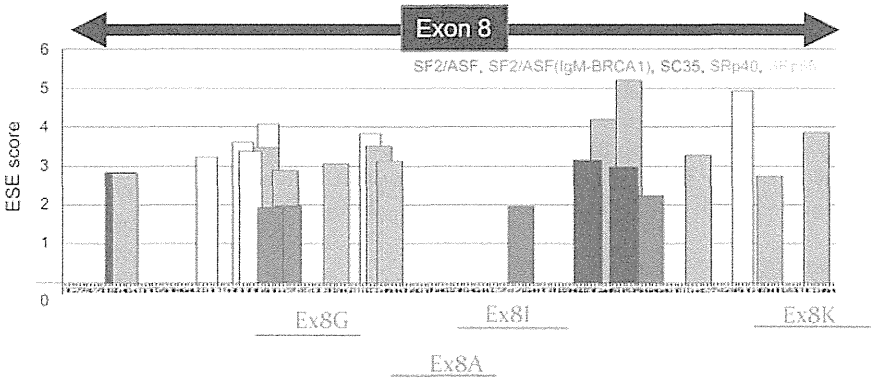

FIG. 4. Schematic outline of the antisense morpholinos targeting exon 8 of dog and human dystrophin mRNA. Antisense oligos against exon 8 of human/dog *DMD* gene used in this study. These 4 oligos were previously reported to be effective for exon 8 skipping in myotubes of dystrophic dogs and human patients *in vitro* (Saito et al., 2010).

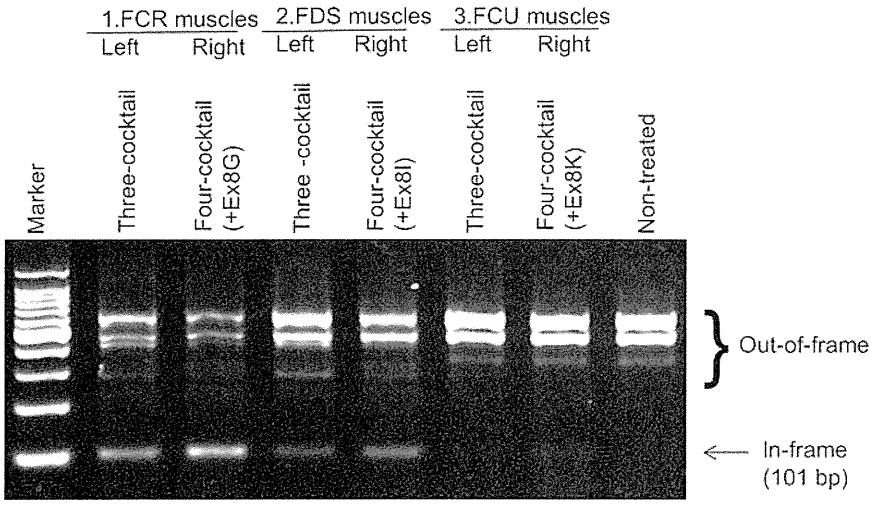

FIG. 5. A 4-oligo cocktail containing Ex8G induces efficient dystrophin expression. Detection of exon 6–9 skipped band with reverse-transcription polymerase chain reaction analysis. Equal amounts (120 μg) of oligos in total were injected into indicated muscles (i.e., 40 μg each in three-oligo cocktails, 30 μg each in four-oligo cocktails). FCR, flexor carpi radialis; FDS, flexor digitorum superficialis; FCU, flexor carpi ulnaris.

efficiently induced exon 8 skipping *in vitro* in dog and human myotubes (Saito et al., 2010). These oligos were designed to target exon/intron borders or exonic splice enhancer (ESE) sites. ESE scores were obtained by using ESE finder software (Cartegni et al., 2003). These oligos target the same conserved sequences in both dog and human dystrophin mRNA.

A novel antisense cocktail induces more efficient exon 8 skipping

We tested the efficacy of newly designed oligos by intramuscular injections into skeletal muscles in dystrophic dogs (Fig. 5). We previously reported a cocktail oligo containing Ex6A, Ex6B, Ex8A, and Ex8G led to the most efficient double exon skipping of exon 6–8 (or triple skipping of exon 6–9) in both human and dog myotubes *in vitro* (Saito et al., 2010). Exon 9 was not targeted by antisense oligos but the exon is known as an alternative splice site which is spontaneously skipped with exon 6–8 skipping induced by antisense oligos in previous studies (Reiss and Rininsland 1994; McClorey et al., 2006). Here, we compared the efficacy of multiple exon skipping induced by the three oligos cocktail which we have previously reported effective for systemic trials in dystrophic dogs (Yokota et al., 2009a), and newly designed 4-oligo cocktails, which we found to be more effective *in vitro* (Saito et al., 2010). Here, 3-oligo cocktail oligos (Ex6A + Ex6B + Ex8A) were injected into right-side muscles of flexor carpi radialis (FCR), Flexor digitorum superficialis (FDS), or flexor carpi ulnaris (FCU) as controls. Different combinations of 4-oligo cocktails were injected into contralateral (left side) muscles (Fig. 5). The same total doses (120 μg) of 3-oligo cocktails or of 4-oligo cocktails were injected (i.e., 40 μg each in 3-oligo cocktails, and 30 μg each in 4-oligo cocktails). The efficacy of multiple exon skipping was initially examined by RT-PCR analysis. While all combinations led to substantial amount of exon 6–9 skipped in-frame mRNA products, the highest efficacy was achieved with the 4-oligo cocktail containing Ex8G (Ex6A + Ex6B + Ex8A + Ex8G), which is consistent with our previous report in myotubes *in vitro* (Saito et al., 2010).

Efficient dystrophin recovery after injections with four-oligo cocktail vPMOs

Next, we examined the recovery of dystrophin expression by immunohistochemistry with DYS-1 anti-dystrophin monoclonal antibody 2 weeks after intramuscular vPMO injections (Fig. 6). Although all tested cocktail oligos induced extensive expression of dystrophin, the highest recovery was obtained with the 4-oligo cocktail containing Ex6A, Ex6B, Ex8A, and Ex8G (Fig. 6). Approximately 70 percent of fibers was positively stained with the four-oligo cocktail (Fig. 6B).

The expression levels of dystrophin after cocktail vPMO injections were also compared with western blotting analysis (Fig. 7). Desmin antibody was used as an internal control. Again, the 4-oligo cocktail injection with Ex6A, Ex6B, Ex8A, and Ex8G led to the highest levels of dystrophin expression.

Discussion

Antisense mediated exon skipping is currently a most promising therapeutic approach to curing DMD (Yokota et al., 2007b; Hoffman et al., 2011; Partridge 2011; Pichavant et al., 2011). Although phase-2/3 clinical trials are currently underway, there are a couple of challenges. One of the most significant challenges is that the effect usually wears off after 3–4 weeks, thus repeated injections are required. Currently, weekly or bi-weekly injections are required for antisense systemic trials (Lu et al., 2005; Alter et al., 2006; Wu et al., 2011b). New generation morpholinos with cell-penetrating moiety, such as PPMOs and vPMOs, were developed to improve the efficacy *in vivo* (Moulton and Jiang 2009; Yokota et al., 2009b). Both PPMOs and vPMOs have the same backbones as conventional unmodified morpholinos (Fig. 1). In vPMOs, cell-penetrating octa-guanidine dendrimers are conjugated, while in PPMOs, arginine rich polypeptides are conjugated (Morcos et al., 2008). Peptide-morpholino conjugates (PPMOs) restored dystrophin to more than 80 percent of wild-type levels in skeletal muscles of *mdx* mice 9 weeks after injections, showing prolonged activity (Moulton et al., 2009). An injection with vPMOs in hDMD mice, a transgenic model carrying the full-length human dystrophin gene, led to more than 70% efficiency of targeted human dystrophin exon skipping *in vivo* systemically (Wu et al., 2011a). Therefore, use of morpholino conjugates such as PPMOs or vPMOs might be able to reduce the frequency of injections.

In this study, we demonstrated the first successful rescue of dystrophin expression with morpholino conjugates in dystrophic dogs. In previous *in vitro* experiments, we used a total

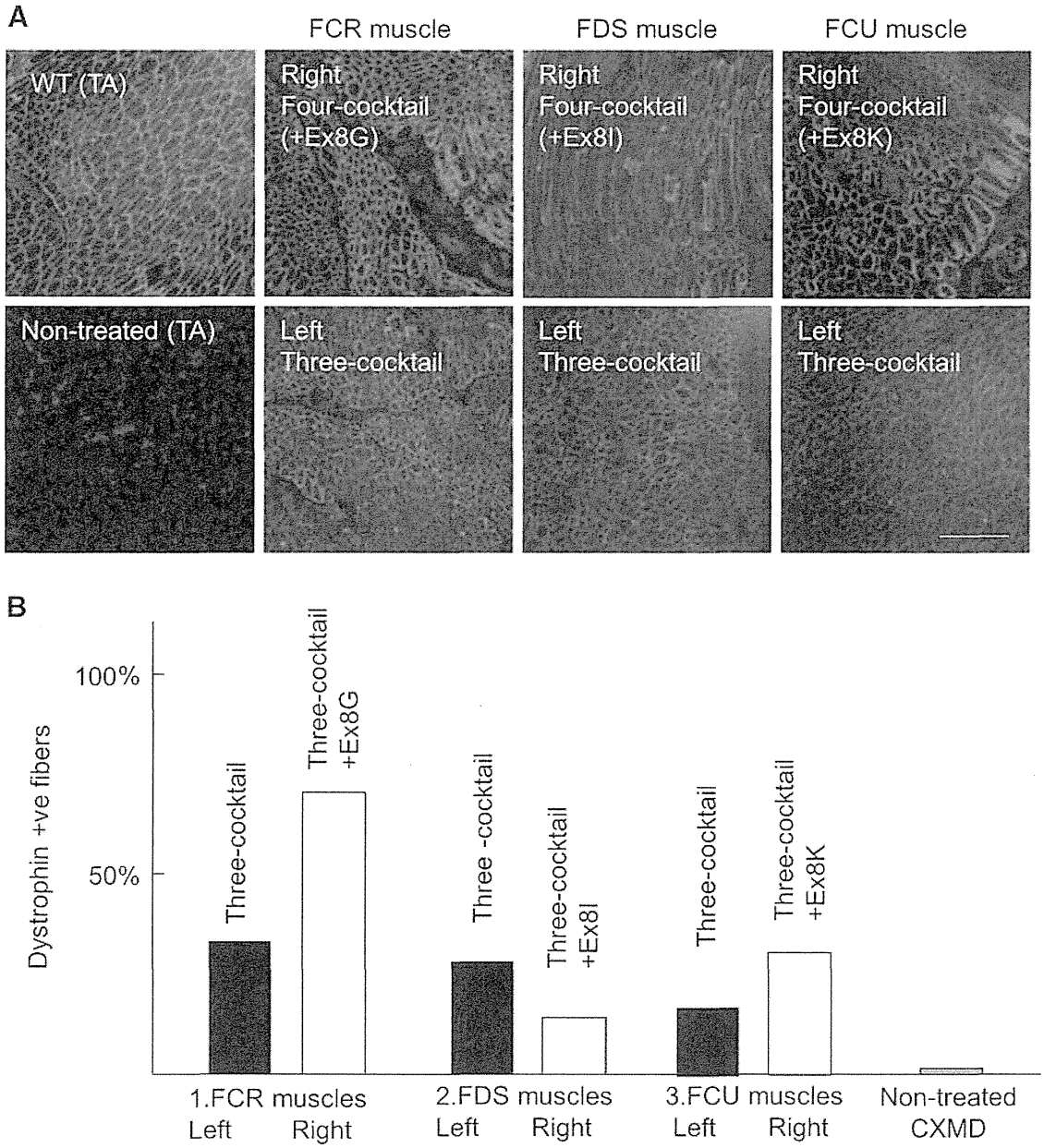

FIG. 6. Immunohistochemistry shows four-oligo cocktails induce efficient dystrophin expression *in vivo*. **(A)** Immunohistochemistry with anti-dystrophin antibody (DYS-1; red) and DAPI nuclei staining (blue). Equal amounts (120 μg) of oligos in total were injected into indicated muscles (i.e., 40 μg each in 3-oligo cocktails, 30 μg each in 4-oligo cocktails). Scale bars = 200 μm. **(B)** The percentage of dystrophin positive fibers after cocktail oligo injections.

of 30 μM for 3 or 4 sequences of PMOs (Saito et al., 2010). In this study, we employed 120μg–1.2 mg of vPMOs for intramuscular injections. The induction of exon 6–9 multiple skipping mediated by cocktail vPMOs was significantly more efficient than that mediated by unconjugated PMOs (Fig. 2). The expression levels were remained very high (60% dystrophin-positive fibers) 2 months after the injection, indicating prolonged persistence (Figs. 2–3). We employed dogs in early stages of the disease, because muscle fibers are replaced by fibrous connective tissue at later stages. This might be generalized to the antisense drug products intended for use in the first-in-human trial. Importantly, a vPMO cocktail efficiently rescued other genetic disorders including the mutation in Fukuyama congenital muscular dystrophy (Taniguchi-Ikeda et al., 2011). These studies clearly indicate that morpholino conjugates are not only useful tools for gene-knockdown study, but also have great potential for treating genetic disorders.

We next compared newly designed antisense oligos against exon 8 of dystrophin mRNA *in vivo* (Figs. 5–7). In accordance with the previous study *in vitro* by Saito et al., the most efficient vPMO cocktail was a 2 oligo cocktail containing Ex8A and Ex8G (Saito et al., 2010). Since exon 6, 7, and 8 are all among the most prevalent targets of exon skipping therapy outside the deletion mutation hotspot (exon 45–55), optimization of antisense oligos against these exons is very

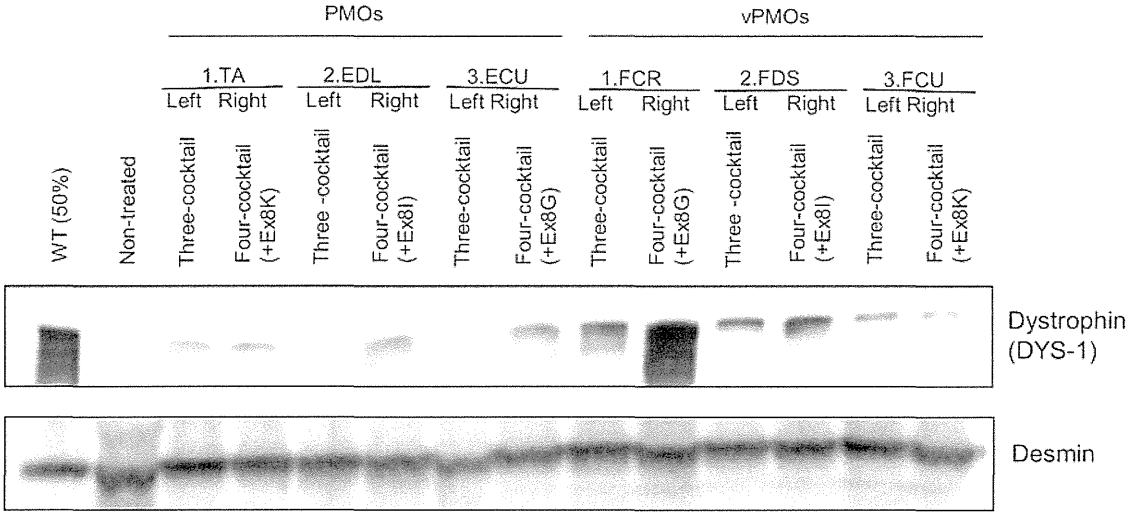

FIG. 7. Restoration of dystrophin expression with f4-oligo cocktail vPMOs. Western blotting analysis with anti-dystrophin (DYS-1) antibody 2 weeks after vPMO injections. Equal amounts (120 μg) of oligos in total were injected into indicated muscles (i.e., 40 μg each in 3-oligo cocktails, 30 μg each in 4-oligo cocktails). The 4-oligo cocktail (Ex6A + Ex6B + Ex8A + Ex8G) leads to the highest level of dystrophin expression. TA, tibialis anterior; EDL, extensor digitorum longus; ECU, extensor carpi ulnaris.

important. In fact, approximately 3.0% of DMD patients can be treated with double skipping of exon 6 and exon 7 (ranked No. 9), and 2.3% can be treated with skipping exon 8 (ranked No. 10) (Aartsma-Rus et al., 2009). Because the exon-skipping approach is fundamentally a mutation-specific personalized medicine, an effective path of drug approval process will be also a key to rescue mutations with a relatively small number of patients.

A major concern of new generation morpholino-mediated antisense therapy is their toxicity. No toxicity of vPMOs has been recorded up to 12 mg/kg of systemic injections in mice (Wu et al., 2009). However, with PPMOs, a high dose (150 mg/kg) of systemic injections led to adverse events such as lethargy, weight loss, elevated blood urea nitrogen, and serum creatinine levels (Amantana et al., 2007). In addition, a test in the cynomolgus monkey revealed mild tubular degeneration in the kidneys after weekly injections with 9 mg/kg PPMOs (Moulton and Moulton, 2010). Although AVI-5038, a PPMO targeting exon 50 of dystrophin mRNA, is in preclinical development, the toxicity of PPMOs might pose a challenge for determination of an effective and safe regimen in man. An immune suppression regimen such as one used for robust adeno-associated virus expression might be effective for systemic trials (Shin et al., 2012; Wang et al., 2007). To test the systemic effect of vPMOs in dogs, precise pharmacokinetics, biodistribution, stability, and toxicity remain to be done. Nevertheless, our results indicate clear potential of the morpholino conjugate as a therapeutic agent to treat DMD and other genetic disorders.

Acknowledgments

We thank Michihiro Imamura, Jing Hong Shin, Takashi Okada, Michiko Wada, Sachiko Ohshima, Jun Tanihata, Satoru Masuda, Kazue Kinoshita, Hideki Kita, Shinichi Ichikawa, Yumiko Yahata, Yuko Kasahara, and Yuko Shimizu (NCNP); and Merryl Rodriguez and Dharminder Panesar (University of Alberta) for useful discussions and technical assistance. This work was supported by Grants-in-Aid for Research on Nervous and Mental Disorders (19A-7), Health and Labor Sciences Research Grants for Translation Research (H19-Translational Research-003 and H21-Clinical Research-015), and Health Sciences Research Grants for Research on Psychiatry and Neurological Disease and Mental Health (H18-kokoro-019) from the Ministry of Health, Labour, and Welfare of Japan, U.S. National Institutes of Health (1P50AR060836; 5T32AR056993), US Department of Defense (W81XWH-09-1-0599) The Friends of Garrett Cumming Research, HM Toupin Neurological Science Research, and Muscular Dystrophy Canada.

TY, TS, AN, and ST conceived and designed study. TY, AN, MK, and TS performed experiments. ST, EH, TN, YA, YO, YE and TP analyzed the data. TY, YA, and TS contributed reagents/materials/analysis tools. TY wrote the manuscript.

Author Disclosure Statement

No competing financial interests exist.

References

AARTSMA-RUS, A., FOKKEMA, I., VERSCHUUREN, J., GINJAAR, I., et al. (2009). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. Hum. Mutat. **30,** 293–299.

AARTSMA-RUS, A., KAMAN, W.E., WEIJ, R., DEN DUNNEN, J.T., et al. (2006). Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. Mol. Ther. **14,** 401–407.

AARTSMA-RUS, A., and VAN OMMEN, G.J. (2007). Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. RNA **13,** 1609–1624.

ALTER, J., LOU, F., RABINOWITZ, A., YIN, H., et al. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat. Med. **12,** 175–177.

AMANTANA, A., MOULTON, H.M., CATE M.L., REDDY M.T., et al. (2007). Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. Bioconjug. Chem. **18,** 1325–1331.

AOKI, Y., NAKAMURA, A., YOKOTA, T., SAITO, T., et al. (2010). In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient mdx mouse. Mol. Ther. **18,** 1995–2005.

BEROUD, C., TUFFERY-GIRAUD, S., MATSUO, M., HAMROUN, D., et al. (2006). Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Hum. Mutat. **28,** 196–202.

CARTEGNI, L., WANG, J., ZHU, Z., ZHANG, M.Q., et al. (2003). ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res. **31,** 3568–3571.

CIRAK, S., ARECHAVALA-GOMEZA, V., GUGLIERI, M., FENG, L., et al. (2011). Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet **378,** 595–605.

COLLINS, T.J. (2007). ImageJ for microscopy. Biotechniques 43, 25–30.

CRISP, A., YIN, H., GOYENVALLE, A., BETTS, C., et al. (2011). Diaphragm rescue alone prevents heart dysfunction in dystrophic mice. Hum. Mol. Genet. **20,** 413–421.

DUCHENNE. (1867). The pathology of paralysis with muscular degeneration (paralysie myosclerotique), or paralysis with apparent hypertrophy. Br. Med. J. **2,** 541–542.

DUNCKLEY, M.G., MANOHARAN, M., VILLIET, P., EPERON, I.C., et al. (1998). Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. Hum. Mol. Genet. **7,** 1083–1090.

GOEMANS, N.M., TULINIUS, M., VAN DEN AKKER, J.T., BURM, B.E., et al. (2011). Systemic administration of PRO051 in Duchenne's muscular dystrophy. N. Engl. J. Med. **364,** 1513–1522.

GOYENVALLE, A., BABBS, A., POWELL, D., KOLE, R., et al. (2010). Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. Mol. Ther. **18,** 198–205.

GOYENVALLE, A., SETO, J.T., DAVIES, K.E., and CHAMBERLAIN, J. (2011). Therapeutic approaches to muscular dystrophy. Hum. Mol. Genet. **20,** R69–78.

HOFFMAN, E.P., BRONSON, A., LEVIN, A.A., TAKEDA, S., et al. (2011). Restoring dystrophin expression in Duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. Am. J. Pathol. **179,** 12–22.

HOFFMAN, E.P., BROWN, R.H., JR., and KUNKEL, L.M. (1987). Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell **51,** 919–928.

JEARAWIRIYAPAISARN, N., MOULTON, H.M., BUCKLEY, B., ROBERTS, J., et al. (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. Mol. Ther. **16,** 1624–1629.

JEARAWIRIYAPAISARN, N., MOULTON, H.M., SAZANI, P., KOLE, R., et al. (2010). Long-term improvement in mdx cardiomyopathy after therapy with peptide-conjugated morpholino oligomers. Cardiovasc. Res. **85,** 444–453.

KINALI, M., ARECHAVALA-GOMEZA, V., FENG, L., CIRAK, S., et al. (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol. **8,** 918–928.

KOENIG, M., HOFFMAN, E.P., BERTELSON, C.J., MONACO, A.P., et al. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell **50,** 509–517.

LU, Q.L., RABINOWITZ, A., CHEN, Y.C., YOKOTA, T., et al. (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc. Natl. Acad. Sci. U. S. A. **102,** 198–203.

LU, Q.L., YOKOTA, T., TAKEDA, S., GARCIA, L., et al. (2011). The status of exon skipping as a therapeutic approach to duchenne muscular dystrophy. Mol. Ther. **19,** 9–15.

MCCLOREY, G., MOULTON, H.M., IVERSEN, P.L., FLETCHER, S., et al. (2006). Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. Gene Ther. **13,** 1373–1381.1373–1381.

MORCOS, P.A., LI, Y., and JIANG, S. (2008). Vivo-Morpholinos: a non-peptide transporter delivers Morpholinos into a wide array of mouse tissues. Biotechniques **45,** 613–614, 616, 618 passim.

MOULTON, H.M., and MOULTON, J.D. (2010). Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy. Biochim. Biophys. Acta **1798,** 2296–2303.

MOULTON, H.M., WU, B., JEARAWIRIYAPAISARN, N., SAZANI, P., et al. (2009). Peptide-morpholino conjugate: a promising therapeutic for Duchenne muscular dystrophy. Ann. N. Y. Acad. Sci. **1175,** 55–60.

MOULTON, J.D., and JIANG, S. (2009). Gene knockdowns in adult animals: PPMOs and vivo-morpholinos. Molecules **14,** 1304–1323.

NAKAMURA, A., YOSHIDA, K., FUKUSHIMA, K., UEDA, H., et al. (2008). Follow-up of three patients with a large in-frame deletion of exons 45–55 in the Duchenne muscular dystrophy (DMD) gene. J. Clin. Neurosci. **15,** 757–763.

PARTRIDGE, T.A. (2011). Impending therapies for Duchenne muscular dystrophy. Curr. Opin. Neurol. **24,** 415–422.

PICHAVANT, C., AARTSMA-RUS, A., CLEMENS, P.R., DAVIES, K.E., et al. (2011). Current status of pharmaceutical and genetic therapeutic approaches to treat DMD. Mol. Ther. **19,** 830–840.

PRAMONO, Z.A., TAKESHIMA, Y., ALIMSARDJONO, H., ISHII, A., et al. (1996). Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. Biochem. Biophys. Res. Commun. **226,** 445–449.

REISS, J., and RININSLAND, F. (1994). An explanation for the constitutive exon 9 cassette splicing of the DMD gene. Hum. Mol. Genet. **3,** 295–298.

SAITO, T., NAKAMURA, A., AOKI, Y., YOKOTA, T., et al. (2010). Antisense PMO found in dystrophic dog model was effective in cells from exon 7-deleted DMD patient. PLoS One **5,** e12239.

SHARP, N.J., KORNEGAY, J.N., VAN CAMP, S.D., HERBSTREITH, M.H., et al. (1992). An error in dystrophin mRNA processing in golden retriever muscular dystrophy, an animal homologue of Duchenne muscular dystrophy. Genomics **13,** 115–121.

SHIMATSU, Y., KATAGIRI, K., FURUTA, T., NAKURA, M., et al. (2003). Canine X-linked muscular dystrophy in Japan (CXMDJ). Exp. Anim. **52,** 93–97.

SHIN, J.H., YUE, Y., SRIVASTAVA, A., SMITH, B., et al. (2012). A Simplified Immune Suppression Scheme Leads to Persistent

Micro-dystrophin Expression in Duchenne Muscular Dystrophy Dogs. Hum. Gene Ther. **23,** 202–209.

TANIGUCHI-IKEDA, M., KOBAYASHI, K., KANAGAWA, M., YU, C.C., et al. (2011). Pathogenic exon-trapping by SVA retrotransposon and rescue in Fukuyama muscular dystrophy. Nature **478,** 127–131.

VAN DEUTEKOM, J.C., JANSON, A.A., GINJAAR, I.B., FRANKHUIZEN, W.S., et al. (2007). Local dystrophin restoration with antisense oligonucleotide PRO051. N. Engl. J. Med. **357,** 2677–286.

WANG, Z., KUHR, C.S., ALLEN, J.M., BLANKINSHIP, M., et al. (2007). Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol. Ther. **15,** 1160–1166.

WIDRICK, J.J., JIANG, S., CHOI, S.J., KNUTH, S.T., et al. (2011). An octaguanidine-morpholino oligo conjugate improves muscle function of mdx mice. Muscle Nerve **44,** 563–570.

WU, B., BENRASHID, E., LU, P., CLOER, C., et al. (2011a). Targeted skipping of human dystrophin exons in transgenic mouse model systemically for antisense drug development. PLoS One **6,** e19906.

WU, B., LI, Y., MORCOS, P.A., DORAN, T.J., et al. (2009). Octa-guanidine morpholino restores dystrophin expression in cardiac and skeletal muscles and ameliorates pathology in dystrophic mdx mice. Mol. Ther. **17,** 864–871.

WU, B., LU, P., BENRASHID, E., MALILK, S., et al. (2010). Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. Gene Ther. **17,** 132–140.

WU, B., XIAO, B., CLOER, C., SHABAN, M., et al. (2011b). One-year treatment of morpholino antisense oligomer improves skeletal and cardiac muscle functions in dystrophic mdx mice. Mol. Ther. **19,** 576–583.

YOKOTA, T., DUDDY, W., ECHIGOYA, Y., and KOLSKI, H. (2012). Exon skipping for nonsense mutations in Duchenne muscular dystrophy: too many mutations, too few patients? Expert Opin. Biol. Ther.

YOKOTA, T., DUDDY, W., and PARTRIDGE, T. (2007a). Optimizing exon skipping therapies for DMD. Acta Myol. **26,** 179–184. Online document at: http://informahealthcare.com/doi/abs/10.1517/14712598.2012.693469 Accessed June 1, 2012.

YOKOTA, T., HOFFMAN, E.P., and TAKEDA, S. (2011). Antisense oligo-mediated multiple exon skipping in a dog model of Duchenne muscular dystrophy. Methods Mol. Biol. **709,** 299–312.

YOKOTA, T., LU, Q.L., MORGAN, J.E., DAVIES, K.E., et al. (2006). Expansion of revertant fibers in dystrophic mdx muscles reflects activity of muscle precursor cells and serves as an index of muscle regeneration. J. Cell Sci. **119,** 2679–2687.

YOKOTA, T., LU, Q.L., PARTRIDGE, T., KOBAYASHI, M., et al. (2009a). Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. Ann. Neurol. **65,** 667–676.

YOKOTA, T., PISTILLI, E., DUDDY, W., and NAGARAJU, K. (2007b). Potential of oligonucleotide-mediated exon-skipping therapy for Duchenne muscular dystrophy. Expert Opin. Biol. Ther. **7,** 831–842.

YOKOTA, T., TAKEDA, S., LU, Q.L., PARTRIDGE, T.A., et al. (2009b). A renaissance for antisense oligonucleotide drugs in neurology: exon skipping breaks new ground. Arch. Neurol. **66,** 32–38.

ZELLWEGER, H., and ANTONIK, A. (1975). Newborn screening for Duchenne muscular dystrophy. Pediatrics **55,** 30–34.

Address correspondence to:
Dr. Shin'ichi Takeda, MD, PhD
Department of Molecular Therapy
National Institute of Neuroscience
National Center of Neurology and Psychiatry
Ogawa-higashi 4-1-1
Kodaira, Tokyo 187-8502
Japan

E-mail: takeda@ncnp.go.jp

or

Dr. Toshifumi Yokota, PhD
Department of Medical Genetics
University of Alberta
Faculty of Medicine and Dentistry
8812-112 Street
Edmonton, AB, T6G 2H7
Canada

E-mail: toshifum@ualberta.ca

Received for publication May 17, 2012; accepted after revision July 3, 2012.

Bodywide skipping of exons 45–55 in dystrophic *mdx52* mice by systemic antisense delivery

Yoshitsugu Aoki[a], Toshifumi Yokota[a,b,c,d,1], Tetsuya Nagata[a], Akinori Nakamura[a,e], Jun Tanihata[a], Takashi Saito[a,f], Stephanie M. R. Duguez[b,g], Kanneboyina Nagaraju[b,h], Eric P. Hoffman[b,h], Terence Partridge[b,h], and Shin'ichi Takeda[a,1]

[a]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan; [b]Research Center for Genetic Medicine, Children's National Medical Center, Washington DC 20010; [c]Department of Medical Genetics, School of Human Development, Faculty of Medicine and Dentistry and [d]The Friends of Garrett Cumming Research and Muscular Dystrophy Canada HM Toupin Neurological Science Research Chair, University of Alberta, Edmonton, AB, T6G 2H7 Canada; [e]Department of Medicine (Neurology and Rheumatology), Shinshu University School of Medicine, Matsumoto 390-8621, Japan; [f]Department of Pediatrics, School of Medicine, Tokyo Women's Medical University, Shinjuku, Tokyo 162-8666, Japan; [g]Thérapie des Maladies du Muscle Strié, Institut de Myologie Unité Mixte de Recherche en Santé 974, Université Pierre et Marie Curie Paris 6, Unité 974, Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 7215, Centre National de la Recherche Scientifique, Association Institut de Myologie 47, 75651 Paris cedex 13, France; and [h]Department of Integrative Systems Biology, George Washington University School of Medicine, Washington DC 20010

Edited by Louis M. Kunkel, Children's Hospital Boston, Harvard Medical School, Boston, MA, and approved June 28, 2012 (received for review March 20, 2012)

Duchenne muscular dystrophy (DMD), the commonest form of muscular dystrophy, is caused by lack of dystrophin. One of the most promising therapeutic approaches is antisense-mediated elimination of frame-disrupting mutations by exon skipping. However, this approach faces two major hurdles: limited applicability of each individual target exon and uncertain function and stability of each resulting truncated dystrophin. Skipping of exons 45–55 at the mutation hotspot of the *DMD* gene would address both issues. Theoretically it could rescue more than 60% of patients with deletion mutations. Moreover, spontaneous deletions of this specific region are associated with asymptomatic or exceptionally mild phenotypes. However, such multiple exon skipping of exons 45–55 has proved technically challenging. We have therefore designed antisense oligo (AO) morpholino mixtures to minimize self- or heteroduplex formation. These were tested as conjugates with cell-penetrating moieties (vivo-morpholinos). We have tested the feasibility of skipping exons 45–55 in *H2K-mdx52* myotubes and in *mdx52* mice, which lack exon 52. Encouragingly, with mixtures of 10 AOs, we demonstrated skipping of all 10 exons in vitro, in *H2K-mdx52* myotubes and on intramuscular injection into *mdx52* mice. Moreover, in *mdx52* mice in vivo, systemic injections of 10 AOs induced extensive dystrophin expression at the subsarcolemma in skeletal muscles throughout the body, producing up to 15% of wild-type dystrophin protein levels, accompanied by improved muscle strength and histopathology without any detectable toxicity. This is a unique successful demonstration of effective rescue by exon 45–55 skipping in a dystrophin-deficient animal model.

personalized medicine | nucleic acid therapy | molecular therapy | oligonucleotides | gene therapy

Duchenne muscular dystrophy (DMD), the commonest form of muscular dystrophy, is characterized by progressive deterioration of muscle function (1). DMD is caused mainly by frame-shifting deletion or nonsense mutations in the *DMD* gene, which encodes the protein dystrophin (2). At the milder end of the disease spectrum, Becker muscular dystrophy (BMD) is a form of dystrophin deficiency that presents with a large spectrum of severities, from borderline DMD to almost asymptomatic cases. BMD typically results from in-frame deletions in the *DMD* gene that allow the expression of limited amounts of an internally truncated but partly functional protein (3).

Skipping of exons in DMD muscle so as to restore an in-frame and asymptomatic or very mild Becker-like transcript is among the more promising therapeutic approaches to treatment of DMD (4). To this end, systemic administration of antisense oligonucleotides (AOs) targeting specific exon(s) in the *DMD* gene has been shown to restore the reading frame and induce body-wide production of partially functional BMD-like dystrophin in mouse and dog models of DMD (5–7). Recently, phase I/II human clinical trials with AOs targeting exon 51 have been completed (8, 9).

Although promising, future development of exon skipping to encompass a wider range of mutations faces two major hurdles. First, each exon must be targeted by a specific bespoke antisense sequence. This strategy requires the design and testing of many different antisense reagents to treat all of the different mutation patterns, entailing substantial investment in time and money to perform toxicology and safety assessments (10). Second, although in-frame mutations are associated with milder BMD forms in 80% of cases, the function and stability of each resulting truncated dystrophin are still unknown (11).

A potential solution for these two issues arises from the observation that exon 45–55 deletions are overwhelmingly associated with remarkably mild clinical phenotypes, sometimes almost asymptomatic, with elevated serum creatine kinase (CK) levels as the main symptom (12) (Fig. S1*A*). It has also been noted that exons 45–55 cover the main mutation "hotspot" of the *DMD* gene so that, theoretically, up to 63% of DMD patients with dystrophin deletion mutations could be treated if we were able to skip the entire exon 45–55 region, to generate an asymptomatic or remarkably mild BMD-like protein that appears to retain most of the function of the intact protein (13) (Fig. 1 *A* and *B* and Fig. S1*B*). Although several investigations have demonstrated successful skipping of two or three exons both in vitro and/or in vivo (5, 14, 15), there is no report of 10 exons being efficiently skipped by administration of antisense sequences; where attempted, the levels of multiple exon-skipped products have been very low (16). We therefore redesigned mixtures of phosphorodiamidate morpholino oligomer (PMO) sequences targeting exons 45–55, paying attention to the minimization of self- and cross-annealing. Additionally we maximized the efficiency of delivery of these improved sequences by combining them with a cell-penetrating moiety (vivo-morpholino or vPMOs) (Fig. S2).

We demonstrate here that intramuscular or systemic injections of a vPMO mixture of these improved reagents generated extensive dystrophin expression in dystrophic skeletal muscles of mice harboring a deletion mutation of exon 52 (*mdx52*), unaccompanied by any detectable toxicity.

Author contributions: Y.A., T.Y., A.N., and S.T. designed research; Y.A., T.Y., T.N., J.T., and T.S. performed research; Y.A., T.Y., S.M.R.D., K.N., and T.P. contributed new reagents/analytic tools; Y.A., T.Y., T.N., A.N., T.S., E.P.H., T.P., and S.T. analyzed data; and Y.A., T.Y., and T.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]To whom correspondence may be addressed. E-mail: toshifumi.yokota@ualberta.ca or takeda@ncnp.go.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1204638109/-/DCSupplemental.

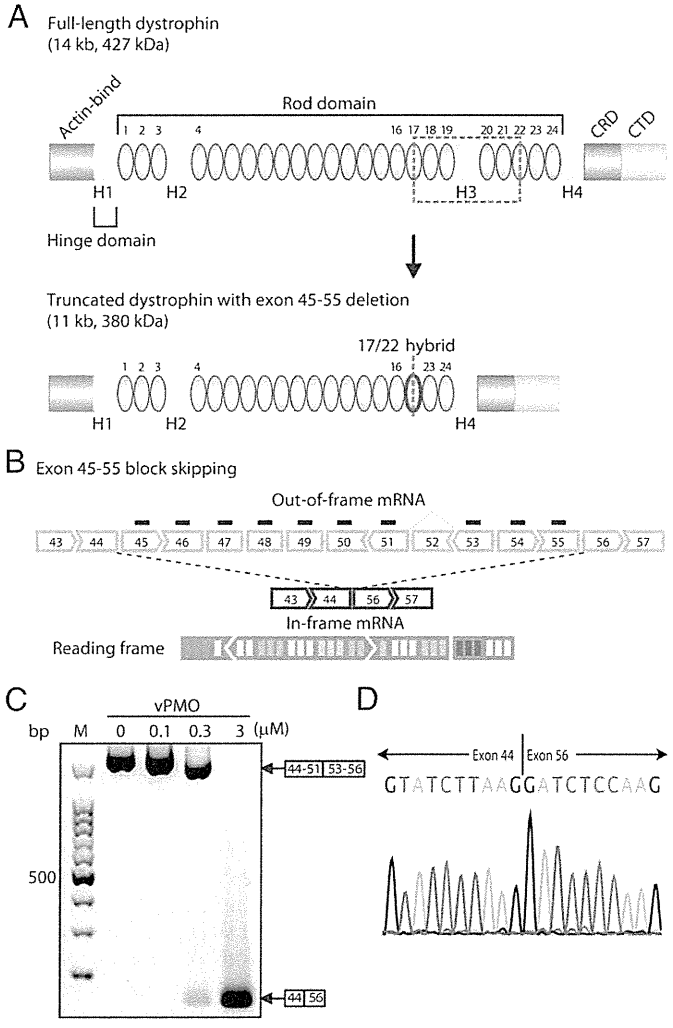

Fig. 1. Efficacy of exon 45–55 multiskipping in *H2K-mdx52* cells in vitro. (*A*) Structure of full-length and quasidystrophin. The quasidystrophin produced by exon 45–55 deletion (skipping) has a hybrid rod repeat of rods 17 and 22. Actin-bind, actin-binding domain; CRD, cysteine-rich domain; CTD, C-terminal domain. (*B*) *Mdx52* mouse lacks exon 52 in the mRNA of the murine *Dmd* gene, leading to out-of-frame products (yellow broken line). Exon 45–55 skipping with mixture vPMOs (blue broken line) restores the reading frame of dystrophin mRNA. (*C*) RT-PCR results after 0.1, 0.3, or 3 μM in total of mixture vPMO transfected into *H2K-mdx52* myotubes as indicated. M, molecular marker; 0, no vPMO transfection. (*D*) Confirmation of correct exon 45–55 block skipping by direct sequencing of the PCR products. Sequencing of the most intense band shows exon 45–55 skipped dystrophin mRNA sequence.

Results

In Vitro Evaluation of the vPMO Sequences in *H2K-mdx52* Myotubes. To examine the feasibility of skipping exons 45–55 in *mdx52* mice, we designed AO sequences against the 10 exons between exons 45 and 55, which target the exonic splice enhancers (ESEs) or the exon/intron boundaries of each exon except exon 52 (Table S1 and Fig. S3). We initially transduced *H2K-mdx52* myotubes with specific AO sequence to each separate targeted exon between exons 45 and 55. Skipping efficiency varied between exons, and although good levels of skipping were obtained for exons 45, 46, 47, 48, 49, 50, 51, 54, and 55, only a faint band was detected in the case of exon 53 after 0.1 or 1 μM in total of single vPMO (45A, 46A, 47A, 48A, 49A, 50A, 51A, 53A, 54A, or 55A) transfection (Fig. S4*A*).

As a next step, we sought to skip the entire exon 45–55 region with 10 vPMO mixtures in vitro. Efficient exon 45–55 skipping of the entire exon 45–55 region was detected by RT-PCR with a forward primer 44F at exon 44 and a reverse primer 56R at exon 56 after transfections of the mixture-ESE2 (Table S2 and Fig. 1*C*). The targeted splicing products with skipped exons 45–55 were confirmed by directly sequencing the RT-PCR band using both primer pairs (Fig. 1*D*). RT-PCR results after transfections with other mixture combinations are also shown in Fig. S4*B*.

Local Injections for Exon 45–55 Block Skipping. To restore dystrophin expression with exon 45–55 skipping in vivo, we injected the mixtures of 10 vPMOs, at 1.5 μg in total, into tibialis anterior (TA) muscle of 5-wk-old *mdx52* mice and took muscle samples 2 wk after the injection. We detected the PCR product that was equivalent to the mRNA lacking the exon 45–55 region most efficiently after injection with mixture-ESE2 (Fig. 2*A* and Table S2 and Fig. S4*C*). Taking these results together, we concluded that the mixture-ESE2 can skip exons 45–55 of the murine *Dmd* gene efficiently both in vitro and in vivo. We also observed extensive dystrophin expression by immunohistochemistry after the mixture-ESE2 injection (Fig. 2 *B* and *C*). Dystrophin expression following exon 45–55 skipping was detected by P7 against exon 57, but not by MANEX46B against exon 46 or MANEX50 against exon 50 (Fig. 2*D*). Western blotting revealed a 380-kDa band that conformed to the size of the product estimated to be derived from deletion of exons 45–55; a truncated quasidystrophin found in the very mild Becker muscular dystrophy associated with deletion of these same exons as well as dystrophin-associated proteins (Fig. 2*E* and Fig. S5*A*). These results suggest that exon 45–55 block skipping is feasible in vivo.

Recovery of Dystrophin-Associated Proteins with Exon 45–55 Skipping. We examined the expression of components included in the dystrophin–glycoprotein complex in the TA muscle by immunohistochemistry and Western blotting. In all dystrophin-positive fibers, the expression of α1-syntrophin and β-dystroglycan at the subsarcolemma was evident; however, the expression of neuronal nitric oxide synthase (nNOS) at the subsarcolemma was minimal compared with that of WT (Fig. 2*D*). Western blotting reveals that the expression levels of α1-syntrophin, β-dystroglycan, and α-sarcoglycan in the TA muscle were at 80–100% of normal levels, whereas the expression level of nNOS was 20% of normal levels (Fig. 2*E* and Fig. S5*B*).

Repeated Systemic Delivery of the Mixture-ESE2 Induces Efficient Exon 45–55 Skipping in Whole Body Skeletal Muscles. Next, we performed serial i.v. injections of the mixture-ESE2 into 5-wk-old *mdx52* mice. After five biweekly (every 2 wk) i.v. injections of 12 mg/kg of the mixture per injection, we detected dystrophin-positive fibers in all skeletal muscles by immunohistochemistry (Fig. 3*A*). Dystrophin expression was evaluated by semi-quantitative Western blotting (Fig. 3*B*). The dystrophin expression levels in the quadriceps, TA, gastrocnemius, triceps brachii, abdominal, paraspinal, and diaphragm muscles were ~8–15% of normal levels, whereas the dystrophin expression level in the heart muscle was 2% of normal levels (Fig. 3*C*).

Exon 45–55 Skipped Quasidystrophin Ameliorates Skeletal Muscle Pathology. We then evaluated the detailed histological changes in quadriceps, TA, gastrocnemius, and diaphragm muscles of treated mice compared with the changes in those of untreated mice. After five i.v. mixture-ESE2 injections, we observed less muscle degeneration and fewer cellular infiltrates in the treated quadriceps, TA, gastrocnemius, and diaphragm muscles compared with the untreated muscles by H&E staining (Fig. 4*A*). We found that the percentage of centrally nucleated fibers (CNFs) were significantly lower in the quadriceps, TA, and gastrocnemius muscles compared with the untreated mice (Fig. 4*B*). These changes reflect the amelioration of dystrophic changes in the treated *mdx52* mice.

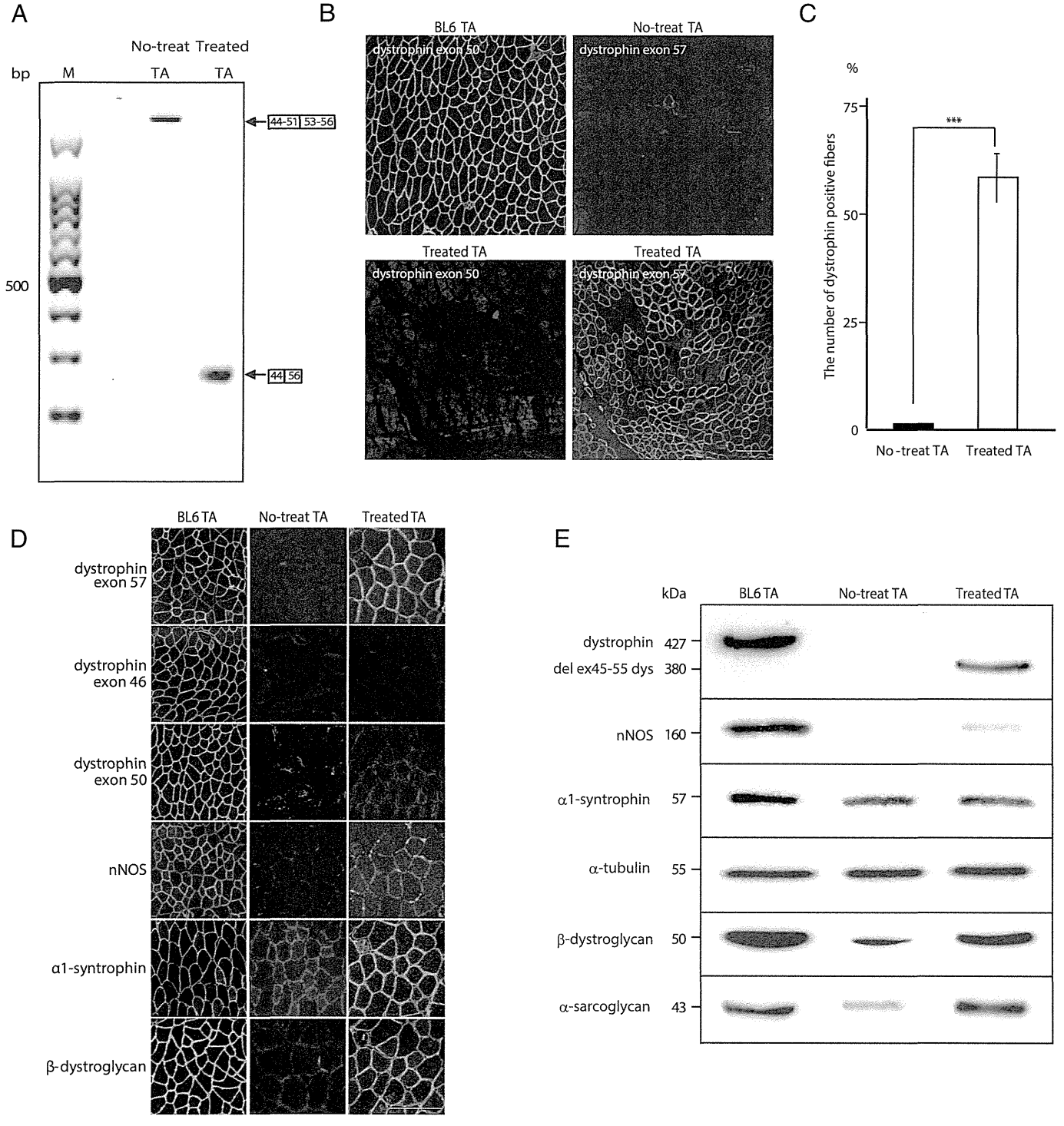

Fig. 2. Exon 45–55 multiskipping and rescue of dystrophin expression in *mdx52* mice by local mixture-ESE2 injections. (*A*) Detection of exon 45–55 skipped dystrophin mRNA by RT-PCR with primers flanking exons 44 (44F1) and 56 (56R1) at 2 wk after injection of mixture-ESE2, targeting exons 45–55 except exon 52 into tibialis anterior (TA) muscles. Representative data are shown. M, molecular marker; no-treat TA, untreated TA muscles from *mdx52* mice; treated TA, treated TA muscles from *mdx52* mice. (*B*) Immunohistochemical staining of dystrophin exon 50 in the TA muscle of WT and treated *mdx52* mice (*Left*) and dystrophin exon 57 in the TA muscle of untreated and treated *mdx52* mice (*Right*). Representative data are shown. BL6 TA, TA muscle from a wild-type C57/BL6. (Scale bar, 100 μm.) (*C*) Percentage of dystrophin-positive fibers after local injections with the 10 vPMO cocktail. Data ($n = 6$) are presented as mean ± SD ***$P < 0.001$. (*D*) Recovery of dystrophin-associated proteins with exon 45–55 skipping. Immunohistochemical staining of dystrophin exons 57, 46, and 50, neuronal nitric oxide synthase (nNOS), α1-syntrophin, and β-dystroglycan in the TA muscle of WT, untreated, and treated *mdx52* mice. Representative data are shown. BL6TA, TA muscle from a wild-type C57/BL6; no-treat TA, untreated TA muscles from *mdx52* mice. (Scale bar, 100 μm.) (*E*) Western blotting after the mixture 10 vPMOs local injections to detect the expression of full-length dystrophin, 380-kDa quasidystrophin, nNOS, α1-syntrophin, α-tubulin, β-dystroglycan, and α-sarcoglycan in TA muscles of WT, untreated, and treated *mdx52* mice. Representative data are shown.

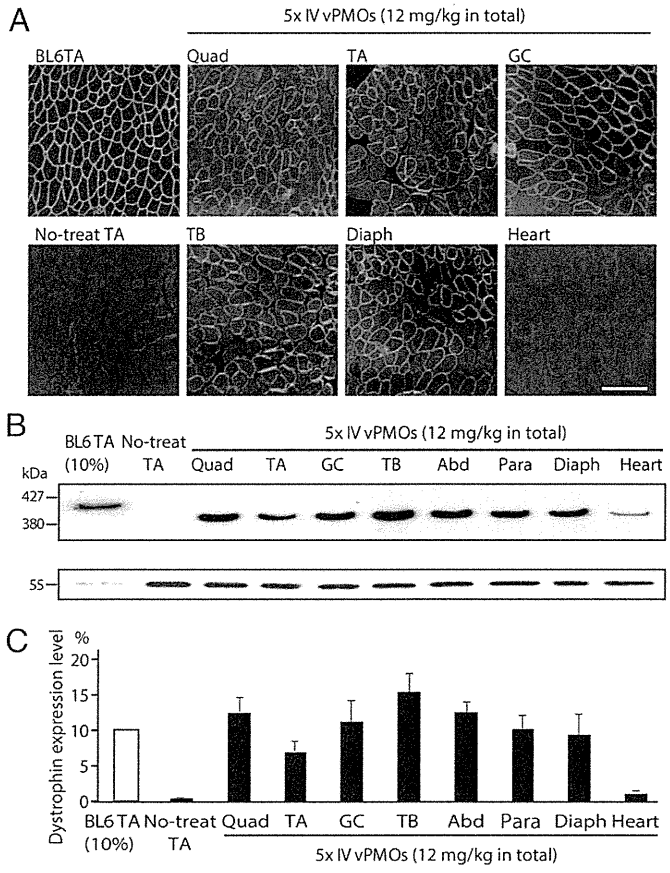

Fig. 3. Systemic i.v. injections of the mixture-ESE2 in *mdx52* mice restore dystrophin expression in body-wide skeletal muscles. (*A*) Immunohistochemical staining of dystrophin exon 57 in quadriceps (Quad), TA, gastrocnemius (GC), triceps brachii (TB), diaphragm (Diaph), and heart muscles in *mdx52* mice after five consecutive biweekly systemic injections of 12 mg/kg of the mixture-ESE2. Representative data are shown. BL6TA, TA muscle from wild-type C57/BL6; no-treat TA, untreated TA muscle from *mdx52* mice. (Scale bar, 100 μm.) (*B*) Western blotting analysis with mouse monoclonal antibody DYS2 after the repeated vPMOs systemic injections into *mdx52* mice. Representative data are shown. vPMO-injected muscles show 380-kDa quasidystrophin bands (*Upper*) and α-tubulin (*Lower*) in Quad, TA, GC, TB, abdominal (Abd), paraspinal (Para), Diaph, and heart muscles of treated *mdx52* mice. BL6TA (10% wt/wt), TA muscle from a 10% (wt/wt) extract of wild-type C57/BL6 mice. (*C*) Semiquantitative analysis of dystrophin expression after AO injection. Data (n = 4) are presented as mean ± SD $*P < 0.05$; $**P < 0.01$.

Exon 45–55 Skipped Quasidystrophin Ameliorates Skeletal Muscle Function. To examine the function of the exon 45–55 skipped quasidystrophin, we performed a battery of physiological and blood tests after five biweekly i.v. injections with the mixture-ESE2. Serum CK levels were significantly reduced in the treated mice, suggesting the protection of muscle fibers against degeneration (Fig. S6*A*). In addition, significant improvement in maximum forelimb grip force and improvement tendency in treadmill endurance and time latency to fall in rotarod test were observed in treated *mdx52* mice compared with untreated *mdx52* mice (Fig. S6 *B–D*).

No Detectable Toxicity After Repeated Delivery of AOs into *mdx52* Mice. To further monitor any potential toxicities in the major organs induced by treatment with AOs, we compared a series of standard serum markers as indicators of liver and kidney dysfunction in WT, untreated, and treated *mdx52* mice after five biweekly 12 mg/kg injections with the mixture-ESE2. Serum aspartate amino transferase was reduced in the treated mice (Fig. S7). No significant differences were detected between untreated and treated *mdx52* mice groups in the levels of aspartate transaminase, alanine aminotransferase, total bilirubin, alkaline phosphatase, blood urea nitrogen, creatinine, sodium ion, chloride ion, and potassium ion (Fig. S7). These data confirm that this AO combination was nontoxic in vivo.

Discussion

In this paper, we demonstrated that rescue of dystrophin expression by skipping exons 45–55 is feasible both in *H2K-mdx52* myotubes in vitro and in dystrophic *mdx52* mice in vivo. As far as

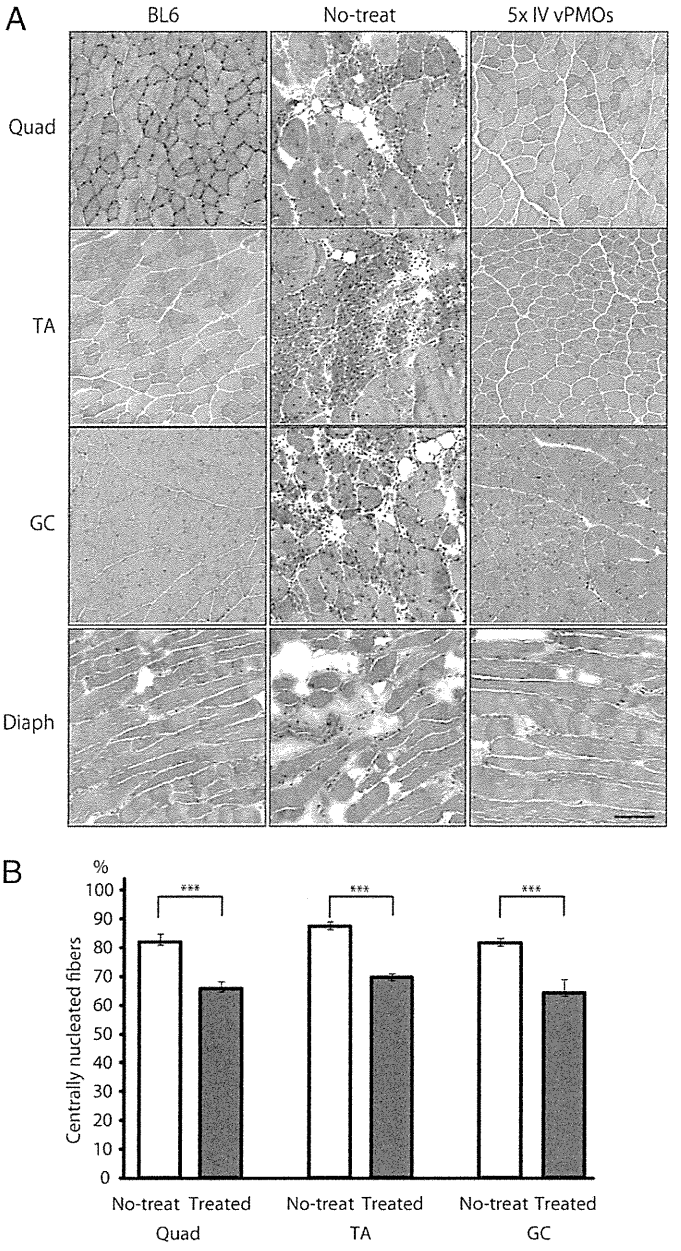

Fig. 4. Exon 45–55 skipped quasidystrophin ameliorates skeletal muscle pathology in *mdx52* mice. (*A*) H&E staining in quadriceps (Quad), TA, gastrocnemius (GC), and diaphragm (Diaph) muscles of WT (BL6), untreated (no-treat), and treated *mdx52* mice (five times i.v. vPMOs). Representative data are shown. (*B*) Measurement of centrally nucleated fibers (CNFs) after systemic exon 45–55 skipping (treated) and nontreated *mdx52* muscles (no-treat) in Quad, TA, and GC muscles. Data (n = 4) are presented as mean ± SD $***P < 0.001$.

we know, this is a unique report of a successful rescue of dystrophin using this technique to skip a large block of 10 exons. Currently, trials of exon 51 skipping using systemic delivery of PMO and 2′O-methyl phosphorothioate antisense oligonucleotides (2′O-MePS) AOs are underway (8, 9). Although exon 51 is the single target exon in the *DMD* gene whose skipping would restore ORF to the largest proportion of DMD mutations, it would still be applicable to only some 10% of DMD patients (or 17% of DMD patients with deletion mutations) (Fig. S1*B*) (17). The goal of treating a broader range of DMD patients with a single treatment has fostered interest in skipping multiple exons. In particular block skipping of exons 45–55 commends itself as a most promising approach (13, 18). Exon 45–55 skipping is known to have two major advantages. First, it would be applicable to ~63% of DMD patients with dystrophin deletion mutations (13). Second, the majority of individuals with a deletion of exons 45–55 of the *DMD* gene fall into the category of BMD patients with exceptionally mild, sometimes almost asymptomatic skeletal muscle involvement (12, 13). Nakamura et al. reported two Japanese patients with exon 45–55 deletions (28 and 42 y old) showing no symptoms except for high blood CK level (12). Similarly, Ferreiro et al. reported a 69-y-old individual with exon 45–55 deletion showing only high CK level without showing any symptoms (19). Such observations raise the hope that antisense-mediated exon 45–55 skipping will have the potential not only to convert DMD patients to a mild BMD clinical phenotype but also to ameliorate the condition of some of the more severe BMD patients with mutations within the exon 45–55 hotspot region.

It is not known why deletion of exons 45–55 leads to very mild phenotypes. The Leiden database used in this study consists of reports from many different countries and sites, raising the possibility of inconsistencies arising from incorrect mapping of some patients or differences in the criteria of diagnosis for Duchenne, Becker, and intermediate cases. However, we have no reasons to suspect any systematic bias with this large dataset. The resulting products of exon 45–55 skipping lead to a truncation at the middle of two rod spectrin repeats (rod repeats 17 and 22) as we have previously pointed out (Fig. 1*A*) (11). Interestingly, the number of rod repeats in this quasidystrophin between the remaining adjacent hinge domains H2 and H4 (16 spectrin repeats) is exactly the same as that between H2 and H3 in full-length dystrophin, which might indicate a requirement of such a spacing for protein function or stability (Fig. 1*A*).

Our results indicate that the truncated dystrophin induced by skipping exons 45–55 restored all of the dystrophin-associated proteins except nNOS. It was reported that nNOS is anchored at the subsarcolemma through its binding to the rod domain of dystrophin at the 16 and 17 rod repeats encoded by exons 42–45 and by interaction with α1-syntrophin (20, 21). The truncated dystrophin induced by skipping exons 45–55 would lack half of the 17 rod repeat, thus disturbing the site-responsible anchoring of nNOS and possibly rendering its subsarcolemmal localization unstable. Although nNOS was not restored after the mixture oligo injections, it is encouraging to note that mild BMD patients with deletions of exons 45–55 also lack nNOS at the subsarcolemma (22).

A previous in vitro study by van Vliet et al. showed that the exon 45–55 skipping frequencies with 2′O-MePS AOs were minimal and comparable to those observed in untreated myocytes of DMD patients with exon 45–47 deletions (16). Here, we redesigned the mixture of vPMOs using online software called Human Splicing Finder and ESEfinder to detect ESEs (23, 24), but paying attention to the avoidance of formation of self- or heteroduplex of the AOs, which, we reasoned, could diminish the efficacy of multiple exon skipping. With this aim, we used OligoAnalyzer 3.1 (25) to design the mixture in which most of combinations of ΔG forces of different AOs were above −5 kcal/mole.

After i.v. injections of our mixture vPMOs into *mdx52* mice, we observed extensive dystrophin-positive fibers and an average of some 8–15% of wild-type levels of dystrophin protein, as determined by Western blotting analysis in a range of skeletal muscles (Fig. 3). The pathology of skeletal muscles was ameliorated; but the skeletal muscle function was only marginally recovered, probably due to the incomplete restitution of quasi-dystrophin (Fig. 4 and Fig. S6).

Whereas conventional PMOs are relatively safe, high-dose administration is required to induce exon skipping systemically (5, 7). Here, we observed high efficacy for systemic rescue with vPMOs that contain a cell-penetrating moiety of octaguanidine dendrimer (Fig. S2*B*). Although the sequences we have used are specific to the mouse, our data validate the principle that carefully designed AOs may be used to realize block skipping of exons 45–55 and, by this means, generate effective amounts of quasidystrophin of near-optimal structure in some 60% of DMD deletion patients. This multiple exon skipping therapy was achieved by use of vPMOs, which produced no evidence of toxicity. The most significant barrier to translating the "mixture approach" to a therapeutic for DMD is the lack of adequate delivery, especially to the heart. Nevertheless, we suggest that a similar approach might pave the way for use of a single-mixture antisense drug that could be applied to treatment of 40–45% of DMD patients.

Materials and Methods

Animals. Exon 52-deficient X chromosome-linked muscular dystrophy mice (*mdx52* mice) were produced by a gene-targeting strategy and are maintained in our facility (26); they have been backcrossed to the C57BL/6J (WT) strain for more than eight generations. Five-week-old male *mdx52* and WT mice were used in this study. The mice were allowed ad libitum access to food and drinking water. The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Japan, approved all experimental protocols in this study.

Antisense Oligos. AOs for targeted skipping of exons 45–55 in the mouse *Dmd* gene were designed using ESEfinder and Human Splicing Finder software to anneal to the ESEs of each exons (Table S1) (23, 24). All antisense mixtures consist of equal amounts of each antisense oligo. Unmodified morpholinos (PMOs) or octaguanidine dendrimer-conjugated morpholinos (vivo-morpholinos or vPMOs) were used in this study (Gene-Tools) (27, 28).

***H2K-mdx52* Myoblasts.** *H-2Kb*-tsA58 × *mdx52*/*mdx52* F_1 male mice yielded dystrophin-deficient *H2K-mdx52* myoblasts (29). *H2K-mdx52* myoblasts were grown at 33 °C in medium containing γ-IFN at a concentration of 20 units/mL and 20% (vol/vol) FBS. After the treatment, the cells were grown in differentiation medium containing 5% (vol/vol) horse serum at 37 °C for 1 d.

AO Transfection. Myotubes were differentiated from *H2K-mdx52* cells and were transfected with the vPMO or the PMOs as previously reported (30). In the differentiation medium, the final concentration of the vPMO was a total of 0.1–3 μM and that of the PMO was a total of 10 μM for 10 sequences. After 48 h incubation with the vPMO or the PMO, total RNA was extracted from myotubes using TRIzol (Invitrogen).

AO Injections. Animals were anesthetized by inhalation of sevoflurane (Wako Pure Chemical Industries) for injections. A total of 1.5 μg of vPMOs or 10 μg of PMOs targeting exons 45–55 in a total volume of 36 μL of saline were used for each TA muscle in the *mdx52* mice. Muscle samples were obtained 2 wk after the intramuscular injection.

A total of 12 mg/kg dose of vPMOs in 100 μL of saline was injected into the tail vein of *mdx52* mice, five times at biweekly (every 2 wk) intervals. The mice were examined 2 wk after the final injection. Muscles were obtained immediately, snap frozen in liquid nitrogen-cooled isopentane, and stored at −80 °C for immunohistochemistry and Western blotting. Skeletal muscle tissues were cut and collected in microtubes and snap frozen in liquid nitrogen for reverse transcription PCR (RT-PCR).

RT-PCR and Sequencing of cDNA. Total RNA from the muscles of WT, untreated, or *mdx52* mice were extracted as previously described (7). Two hundred nanograms of RNA template was used for a 20-μL RT-PCR using