was observed in the DEX group, but not in the DEX plus BCAA group. In contrast, there was no significant difference in the size of slow type I fibers among the three treatment groups. Moreover, the therapeutic effects of BCAA were inhibited by rapamycin (Figures 7B–7E). Therefore, we conclude that the administration of BCAA elicits mTOR activation and intervenes in GR-dependent catabolic transcriptional regulation to ameliorate DEX-induced muscle atrophy.

DISCUSSION

In skeletal muscle, we suggested that GR activates a secondary transcription network driven by KLF15; that the promoter regions of atrogin-1 and MuRF1 contain KLF15 binding sites as well as those of FoxOs; and that KLF15 induces the expression of these atrogenes. Although the molecular mechanism remains elusive, the functional cooperativity of GR, FoxOs, and KLF15 in the expression of the atrogenes may represent the molecular basis for the involvement of GR in muscle atrophy associated with a number of pathological conditions including diabetes and sepsis. From the metabolic viewpoint, these GR-driven transcriptional cascades appear to be relevant for providing rapid and integrated cues toward muscle breakdown and nutrient supply from muscle to other organs, i.e., to the liver, under stressful conditions associated with excess levels of glucocorticoids.

BCAT2 catalyzes the initial step for BCAA degradation, and BCAT2 activity is a critical determinant of cellular BCAA content in skeletal muscle; mice with systemic inactivation of BCAT2 gene are reported to have approximately ten times or higher concentrations of plasma BCAA (She et al., 2007). We demonstrated that BCAA content was decreased with a reciprocal increase in alanine levels in L6 myotubes after the exogenous expression of KLF15 (Figure 4F). Although it is generally known that BCAA is supplied via protein breakdown during skeletal muscle atrophy (Wagenmakers, 1998; Yu et al., 2010), it was reported that net increase in muscle BCAA concentrations after glucocorticoid treatment (~150% increase compared to control) were strikingly lower than those of diabetic rats (~400% increase compared to control) (Aftring et al., 1988; Hundal et al., 1991). This difference in BCAA concentrations is most likely to be due to increased BCAT2 activity in glucocorticoid-treated rats. The glucocorticoid-driven GR-KLF15-BCAT2 axis may negatively modulate the intracellular availability of BCAA and result in a negative impact on mTOR function in skeletal muscle. Indeed, exogenous KLF15 increased mRNA expression of the atrogenes and BCAT2 and decreased mTOR activity and BCAA concentrations in cultured myotubes (Figures 4E–4G). Moreover, the introduction of KLF15 decreased myofiber size in cultured myotubes and caused atrophy in the tibialis anterior muscle, even in the absence of glucocorticoids (Figures 4G and 4H). Therefore, we may conclude that KLF15 is a crucial GR target gene acting as a catabolic modulator of skeletal muscle.

In addition to the KLF15-BCAT2 axis, it should be noted that a number of glucocorticoid-induced products can repress mTOR activity in skeletal muscle cells. Among others, myostatin (Ma et al., 2001; Gilson et al., 2007) and REDD1 (Figure S1) (DeYoung et al., 2008) are direct targets of GR. Moreover, atrogin-1 was recently reported to inhibit S6K1 activity via eIF3f (Csibi et al., 2010). Therefore, it is likely that the mTOR system is negatively regulated by a variety of factors in the presence of excess glucocorticoids in a distinct fashion. Given that the glucocorticoid-GR axis is a major catabolic regulator for homeostatic control (Munck et al., 1984), this multimodal repression of mTOR via the GR axis appears to be rational. In any case, this type of negative mTOR modulation is not reported in other types of muscle atrophy, and may be a striking feature in glucocorticoid-induced muscle atrophy. Interestingly, muscle-specific inactivation of mTOR was reported to exacerbate the myopathic features of type I and type II fiber-rich muscles in a distinct fashion; type I fiber-rich muscles showed prominent dystrophic features with less impact on muscle mass and CSA compared to type II fiber-rich muscles, and a decrease in muscle mass and CSA are characteristic of type II fiber-rich muscles with less dystrophic appearance (Bentzinger et al., 2008; Risson et al., 2009). Therefore, we speculate that type II fiber-rich glycolytic muscles have an evolutionally preserved role for the storage of nutrients under the control of the glucocorticoid-GR axis and that the GR-triggered gene expression program is a purposeful and efficient compensatory mechanism for nutrient supply from those muscles.

An important question is how the GR-driven proteolytic cascades can be shut down when necessary in skeletal muscle. We clearly demonstrated that mTOR activation negatively modulated GR-mediated transcription. Given that the effect of mTOR is rapamycin sensitive, the involvement of mTORC1 is strongly indicated in this interaction. The role of the mTOR pathway in the determination of glucocorticoid sensitivity has not yet been highlighted, except in certain hematologic malignancies (Beesley et al., 2009; Gu et al., 2008; Yan et al., 2006a). It was postulated that the treatment of cultured cells with FK506 or rapamycin enhances glucocorticoid-inducible reporter gene expression, most possibly via their interaction with heat shock proteins and the promotion of the ligand-dependent nuclear entry of GR (Ning and Sanchez, 1993). In contrast, we documented that rapamycin, without any alteration in the cytoplasmic-nuclear distribution of GR, increased GR recruitment onto the promoter (Figures 5E and 5F), and these effects were not reproduced by FK506 (data not shown).

and quantified band densities of S6K1 and p-S6K1(T389) relative to GAPDH (n = 5). Right, representative fluorescent microscopic images of the myotubes and quantified diameters of the myotubes (500 < n < 510).

(H) Effects of ectopic KLF15 expression on mTOR activity and myofiber cross-sectional area (CSA) in rat tibialis anterior. Left, representative immunoblots and quantified band densities (n = 5). Right, immunostaining for type IIB myosin heavy chain (MHC IIB, red in left photographs), type I myosin heavy chain (MHC I, red in right photographs), and type IV collagen (green) of transverse cryosections. CSA distribution of MHC IIB fibers (left) and MHC I fibers (right) are presented as frequency histograms (500 < n < 510).

(A–H) Error bars show SD. *$p < 0.05$, †$p < 0.05$ versus vehicle-treated rats. ‡$p < 0.05$ versus mock-transfected cells.

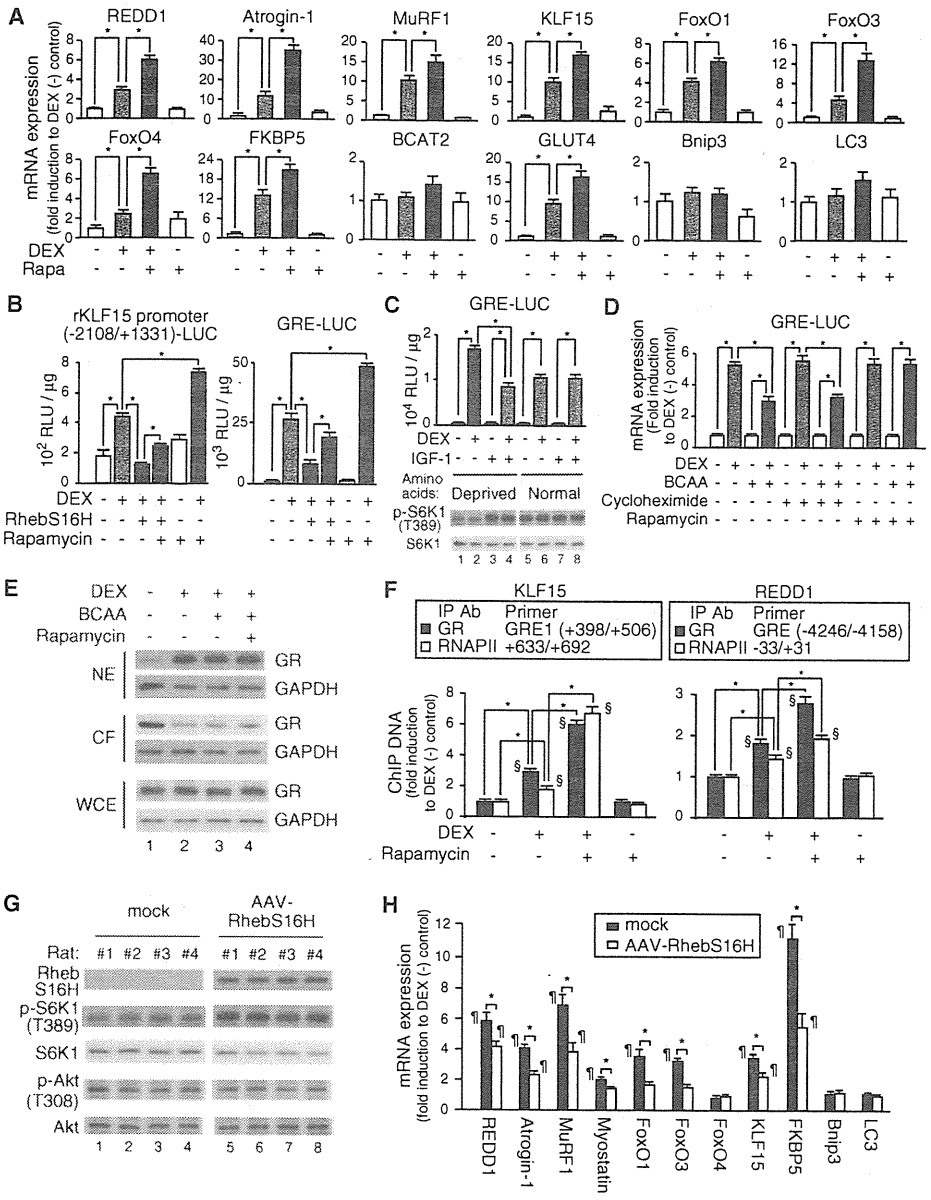

Figure 5. Negative Regulation of GR-Mediated Transcription by mTOR

(A) qRT-PCR analysis of L6 myotubes treated with DEX and rapamycin (Rapa) for 24 hr.

(B) Attenuation of GR-dependent reporter gene expression by mTOR. L6 myoblasts were transfected with rKLF15 promoter-LUC or GRE-LUC, with or without the expression plasmid for a constitutive active Rheb (RhebS16H), and treated with DEX and rapamycin for 18 hr.

(C) Effects of IGF-1 on mTOR activity and GR-dependent reporter gene expression. L6 myoblasts were transfected with GRE-LUC and cultured in amino acid-deprived DMEM (lanes 1–4) or normal DMEM (lanes 5–8) in the presence or absence of IGF-1 and/or DEX for 9 hr. Top, luciferase activities. Bottom, representative immunoblots.

(D) Effects of DEX, BCAA, cycloheximide, and rapamycin on GR-dependent reporter gene expression. L6 myoblasts were transfected with GRE-LUC and cultured in amino acid-deprived DMEM in the presence or absence of 10 mM BCAA cocktail, cycloheximide, rapamycin, and DEX for 6 hr.

(E) Effects of DEX, BCAA, and rapamycin on protein levels and subcellular localization of GR. L6 myotubes were cultured in amino acid-deprived DMEM in the presence or absence of DEX, 10 mM BCAA cocktail, and rapamycin for 30 min. Representative immunoblots of the nuclear extracts (NE), cytoplasmic fractions (CF), and whole-cell extracts (WCE) are shown (n = 3).

(F) Effects of rapamycin on DEX-dependent recruitment of GR onto target gene promoters. L6 myotubes were treated with 1 μM DEX and rapamycin for 2 hr (for KLF15) or 20 min (for REDD1) and processed for ChIP assays.

(G and H) Effects of ectopic expression of RhebS16H on mTOR activity and DEX-mediated mRNA expression. AAV-RhebS16H was infected to rat tibialis anterior for 7 days. (G) Representative immunoblots (n = 7). (H) qRT-PCR analysis of the muscles from the rats 6 hr after intraperitoneal injection with DEX.

(A–D, F, and H) Error bars show SD (n = 5). *$p < 0.05$, §$p < 0.05$ versus ChIP with normal IgG, ¶$p < 0.05$ versus vehicle-treated rats.

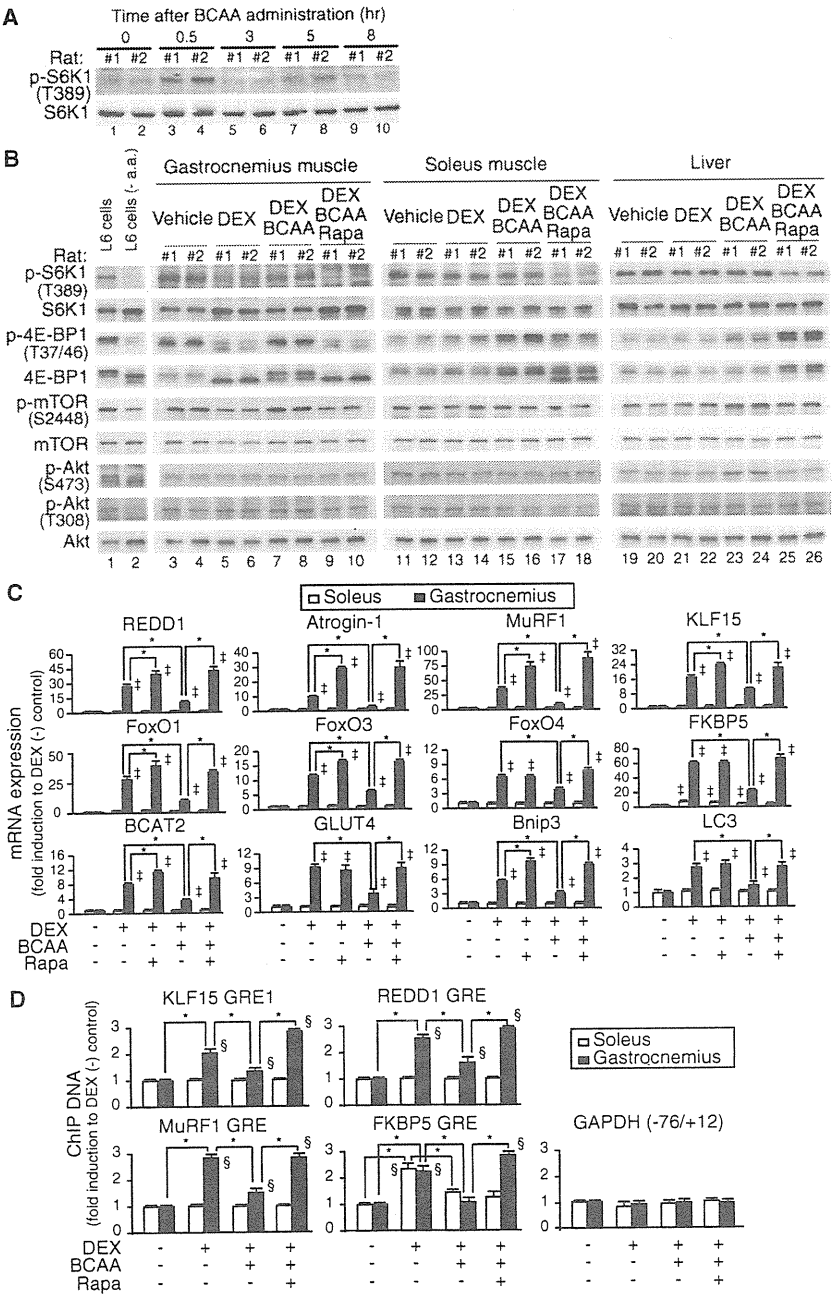

Figure 6. In Vivo Activation of mTOR and Attenuation of GR-Mediated Transcription after Programmed Administration of BCAA

(A) Time course of mTOR activity in rat gastrocnemius after BCAA administration. Representative immunoblots are shown (n = 5).

(B–D) Effects of DEX, BCAA, and rapamycin on mTOR activity; mRNA expression of atrophy-related genes; and GR recruitment onto the target gene promoters. Rats were treated with DEX, BCAA cocktail, and rapamycin for 5 days as described in the Supplemental Information. (B) Representative immunoblots (n = 17). L6 myotubes cultured in normal DMEM and in amino acid-deprived DMEM (–a.a.) for 1 hr were served as controls. (C) mRNA expression of atrophy-related genes. (D) Recruitment of GR onto its target genes. ChIP was performed using anti-GR antibody.

(C and D) Error bars show SD (n = 17). $^{*}p < 0.05$, $^{\ddagger}p < 0.05$ versus vehicle-treated rats, $^{\S}p < 0.05$ versus ChIP with normal IgG.

In skeletal muscle, this nutrition sensor-driven inhibition of GR function may be one of the mechanisms by which nutrients modulate the internal cellular milieu. Intriguingly, GR-mediated transcription was not repressed by insulin/IGF-1 under normal culture conditions, but did so when amino acids were deprived from the culture media (Figure 5C). This indicates that mTOR may be constitutively activated to a certain extent by nutrients and growth factors to protect cells from GR-driven catabolism in skeletal muscle. Under fasting conditions, however, blood concentrations of insulin/IGF-1 are low, and glucocorticoids may be allowed to efficiently drive the catabolic atrophy program for nutrient supply. Thus, our hypothesis may provide an insight into how muscle cells critically determine their volume after sensing endocrine hormones and the nutritional conditions for homeostatic regulation. In this context, GR-mTOR crosstalk might be a key for creating an interdisciplinary research area that bridges nutrition and medicine.

Therefore, the mTOR-mediated inhibition of GR in skeletal muscle is likely to be due not to the modulation of its chaperone activity but to its intervention in the access of GR to target DNA. It is becoming apparent that mTOR is intimately involved with the transcriptional apparatus in concert with a variety of transcription factors and cofactors (Cunningham et al., 2007). Since mTOR is reported to dock in the nucleus in association with, for example, PML (Bernardi et al., 2006), it would be of particular interest to identify such a factor that tethers GR and mTOR in the nucleus.

The biochemical rationale for the usage of BCAA as a therapeutic tool in glucocorticoid-induced muscle atrophy is that BCAA increases the association between Rheb and mTOR and, at least in part, mimics the effect of Rheb overexpression (Sancak et al., 2010). In our model, BCAA administration repressed mRNA expression of almost all GR-regulated genes (Figure 6C). ChIP analysis strongly supported the notion that BCAA administration inhibited GR recruitment onto the promoters of its target genes (Figure 6D). Moreover, these effects of BCAA were efficiently counteracted by rapamycin.

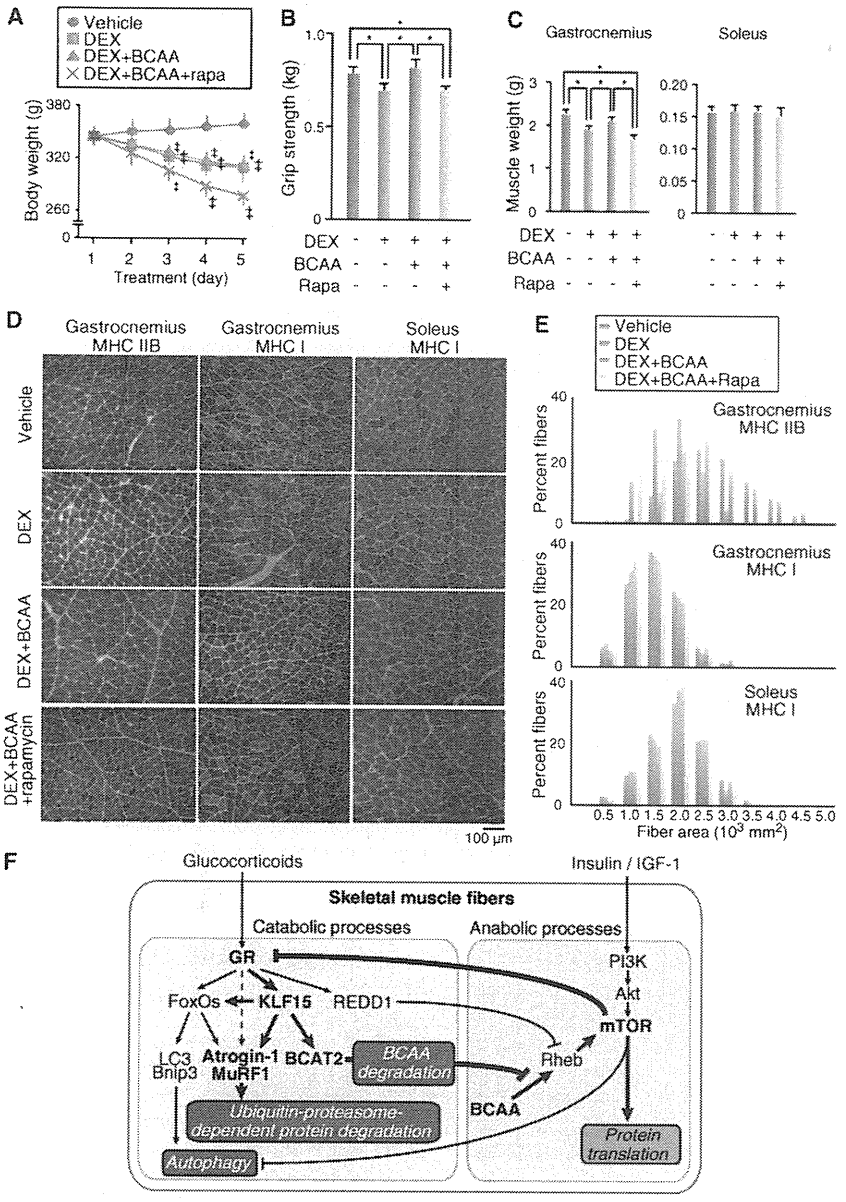

Figure 7. Restoration of Muscle Fiber Mass and Strength by mTOR Activation in DEX-Induced Skeletal Muscle Atrophy Model

(A–E) Effects of DEX, BCAA, and rapamycin on body weight (A), grip strength of forearms (B), muscle weight (C), muscle pathology (D), and CSA of skeletal muscle fiber (E). Rats were treated with DEX, BCAA, and rapamycin for 5 days as described in the Supplemental Information. (A) Time course of body weight (n = 15). (B) Grip strength of forearms at 5 hr after DEX injection on the day 5 ($47 < n < 51$). (C) Weight of gastrocnemius and soleus at 6 hr after DEX injection on the day 5 (n = 15). (D) Immunostaining for MHC IIB (red in left photographs), MHC I (red in middle and right photographs), and type IV collagen (green) of transverse cryosections. (E) CSA distribution of MHC IIB fibers (gastrocnemius) and MHC I fibers (gastrocnemius and soleus) presented as frequency histograms ($500 < n < 510$).

(F) Schematic model of mutual crosstalk between catabolic processes and anabolic processes in skeletal muscle.

(A–C) Error bars show SD (A and C) or SEM (B). $^{*}p < 0.05$, $^{‡}p < 0.05$ versus vehicle-treated rats.

Therefore, we are convinced that the therapeutic effects of BCAA could, at least in part, be ascribed to GR inhibition by the BCAA-mediated activation of mTOR. BCAA administration also resulted in the decreased mRNA expression of autophagy-related genes (Figure 6C), indicating that this therapeutic regimen repressed the vicious circuit connecting the initial induction of GR-triggered gene expression to degradation and atrophy. Of course, we cannot rule out other mechanisms for the effects of BCAA, including the non-GR-mediated repression of atrophy and/or autophagy, and further studies are clearly needed to clarify this issue.

There are conflicting results concerning the biological effects of BCAA, e.g., the overactivation of amino acid-dependent mTOR-mediated signaling can lead to the inhibition of the proximal part of the insulin signaling pathway (Um et al., 2006). Moreover, in obese humans, BCAA in association with a high-fat diet is linked to the elevation of insulin resistance (Newgard et al., 2009). On the other hand, it is suggested that an increase in type II fibers in obese mice may reduce fat mass and improve metabolic parameters (Izumiya et al., 2008). Therefore, it is necessary, for the validation of BCAA therapy, to evaluate the influence of long-term BCAA administration on various metabolic parameters.

In conclusion, we revealed that GR and mTOR act as catabolic and anabolic liaisons for skeletal muscle metabolism, respectively, and these molecules interact with each other at multiple levels. This issue would be of particular importance to understand the molecular mechanism underlying the regulation of the volume and metabolism of muscle and for the development of treatments for glucocorticoid-induced and wasting disorder-related skeletal muscle atrophy.

EXPERIMENTAL PROCEDURES

Rats

All animal experiments were approved by the institutional committee and conducted according to the institutional ethical guidelines for animal experiments. Rapamycin, RU486, the BCAA cocktail, and DEX administration were performed as described in the Supplemental Information. Excised tissues were snap frozen in isopentane cooled by liquid nitrogen, and crushed using Cryo-Press (Microtec, Funabashi, Japan) prefrozen in liquid nitrogen, or processed to serial 10 μm transverse cryostat sections.

Cell Culture

L6 rat myoblasts, C2C12 mouse myoblasts, and COS-7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Culture conditions for myotube formation, drug treatment, and amino acids deprivation are described in the Supplemental Information.

In Silico Promoter Analysis

Putative FoxO1- and FoxO3-binding sequences, as well as putative GREs which are conserved between rat and human genomes, were searched for in the genomic regions (−5000 to +2000) of KLF15, REDD1, atrogin-1, and MuRF1 using rVISTA 2.0 as described in the Supplemental Information. KLF15-binding sequences (see the Supplemental Information) were searched for in the promoters of rat atrogin-1 (−4141 to +1191) and MuRF1 (−3223 to +1547) genes.

Chromatin Immunoprecipitation Assay

Cells or crushed tissues were treated with 1% formaldehyde in PBS for 10 min at 37°C, incubated in 125 mM glycine for 5 min, resuspended in buffer S (50 mM Tris [pH 8.0], 1% SDS, 10 mM EDTA) supplemented with 1 mM DTT, 100 nM MG132, and protease and phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and incubated at 10°C for 10 min. Samples were sheared to an average size of 500 bp by sonication. Lysates corresponding to 2×10^6 cells or 200 mg of crushed tissues were diluted 10-fold in buffer D (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8.1], 167 mM NaCl) supplemented with 100 nM MG132, and protease and phosphatase inhibitor cocktail, and incubated with 5 μg of antibodies listed in the Supplemental Information at 4°C for 18 hr. Protein A or G agarose/salmon sperm DNA (Millipore, Billerica, MA) was added and further incubated at 4°C for 1 hr. Precipitated DNA were quantified as described in the Supplemental Information.

Indirect Immunofluorescent Staining and Fluorescence Imaging

Muscle cryosections were treated with 0.1% Triton X-100, blocked with 5% goat serum/1% BSA in PBS, and incubated with antibodies listed in the Supplemental Information. After washing with PBS, specimens were incubated with secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen, 1:1000) and analyzed as described in the Supplemental Information. For imaging cultured myotubes, GFP was expressed in myotubes by infecting 10 multiplicity of infection of Ax1CAgfp (RIKEN DNA Bank, Tsukuba, Japan).

Statistical Analysis

Data were analyzed with Student's t test for unpaired data. P values below 0.05 were considered statistically significant. Graphs represent means ± SD or means ± SEM as specified in each figure legend.

SUPPLEMENTAL INFORMATION

Supplemental Information include one figure, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2011.01.001.

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The Status of Exon Skipping as a Therapeutic Approach to Duchenne Muscular Dystrophy

Qi-Long Lu[1], Toshifumi Yokota[2], Shin'ichi Takeda[3], Luis Garcia[4], Francesco Muntoni[5] and Terence Partridge[2]

[1]McColl-Lockwood Laboratory for Muscular Dystrophy Research, Neuromuscular/ALS Center, Carolinas Medical Center, Charlotte, North Carolina, USA; [2]Research Center for Genetic Medicine, Children's National Medical Center, Washington, District of Columbia, USA; [3]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan; [4]INSERM U974, UMR 7215 CNRS, Institut de Myologie, UM 76 Université Pierre et Marie Curie, Paris, France; [5]Institute of Child Health, London, UK

Duchenne muscular dystrophy (DMD) is associated with mutations in the dystrophin gene that disrupt the open reading frame whereas the milder Becker's form is associated with mutations which leave an in-frame mRNA transcript that can be translated into a protein that includes the N- and C- terminal functional domains. It has been shown that by excluding specific exons at, or adjacent to, frame-shifting mutations, open reading frame can be restored to an out-of-frame mRNA, leading to the production of a partially functional Becker-like dystrophin protein. Such targeted exclusion can be achieved by administration of oligonucleotides that are complementary to sequences that are crucial to normal splicing of the exon into the transcript. This principle has been validated in mouse and canine models of DMD with a number of variants of oligonucleotide analogue chemistries and by transduction with adeno-associated virus (AAV)-small nuclear RNA (snRNA) reagents encoding the antisense sequence. Two different oligonucleotide agents are now being investigated in human trials for splicing out of exon 51 with some early indications of success at the biochemical level.

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INTRODUCTION

From the moment of its identification, the Duchenne muscular dystrophy (*DMD*) gene, was clearly going to test the ingenuity of would-be gene therapists. The need to achieve body-wide distribution of the largest known gene is compounded by its structural role as the keystone of a transmembrane cell–surface protein complex; removing the possibility, even with a fully functional protein, of the amplifying effect of an enzyme and implying the need for near-normal molar concentrations to approach normal function. Strange then, that one of the more promising strategies for treating DMD, the skipping of mutated sites, is actually facilitated by the large size and modular structure of dystrophin: its major functional binding sites being separated by a long stretch of rod-like "spacer" that carries no essential function and is the site of the more common DMD mutations.

Use of antisense oligonucleotides to modulate splicing of the dystrophin gene so as to restore a translatable mRNA transcript was mooted some years ago on the basis of *in vitro* data[1,2] but firm evidence for practical utility of this approach awaited studies in the *mdx* mouse model of DMD.[3–6] These, in turn, set in train a concerted effort to advance the technology toward human trials, as summarized in the following accounts of work presented and discussed at a meeting held in the Banbury Center at Cold Spring Harbor from the 14th to the 17th of October 2008.

CHEMISTRY AND MODIFICATIONS: CRUCIAL FOR REALIZING THERAPEUTIC POTENTIAL

Progressive advances in exon skipping for DMD have been related to the application of new antisense oligomer chemistries and their modification for improved delivery (**Figure 1**). The most widely used chemistry is the 2′O-methylphosphorothioate-modified (2′OMePS) antisense oligoribonucleotide (AON). This modification provides resistance to nuclease degradation while retaining negative charge to facilitate effective delivery in cell culture systems by most delivery reagents.[7] The potential of this chemistry for treating DMD was initially demonstrated in dystrophic *mdx* mice[5,6] and more recently by intramuscular injection in DMD patients.[8] However, for systemic delivery, 2′OMePS showed limited efficiency in the *mdx* mouse. Three intravenous (i.v.) injections of 2 mg 2′OMePS/mouse (~60–80 mg/kg) at weekly intervals did induce detectable dystrophin expression in all skeletal muscles, but only in sparse focal patches of muscle fibers within each muscle and never at >5% of normal levels. Disappointingly too, little or no dystrophin expression was seen in cardiac muscle. No toxicity to liver or kidney was observed. Thus, assuming that the preclinical model recapitulates precisely the efficiency and pharmacokinetics of administration to DMD boys, 2′OMePS appear safe but it is uncertain whether their systemic use would induce sufficient dystrophin expression to have a therapeutic impact in DMD boys.[4]

Correspondence: Terence Partridge, Research Center for Genetic Medicine, Children's National Medical Center, 111 Michigan Ave NW, Washington, District of Columbia, USA. E-mail: tpartridge@cnmcresearch.org

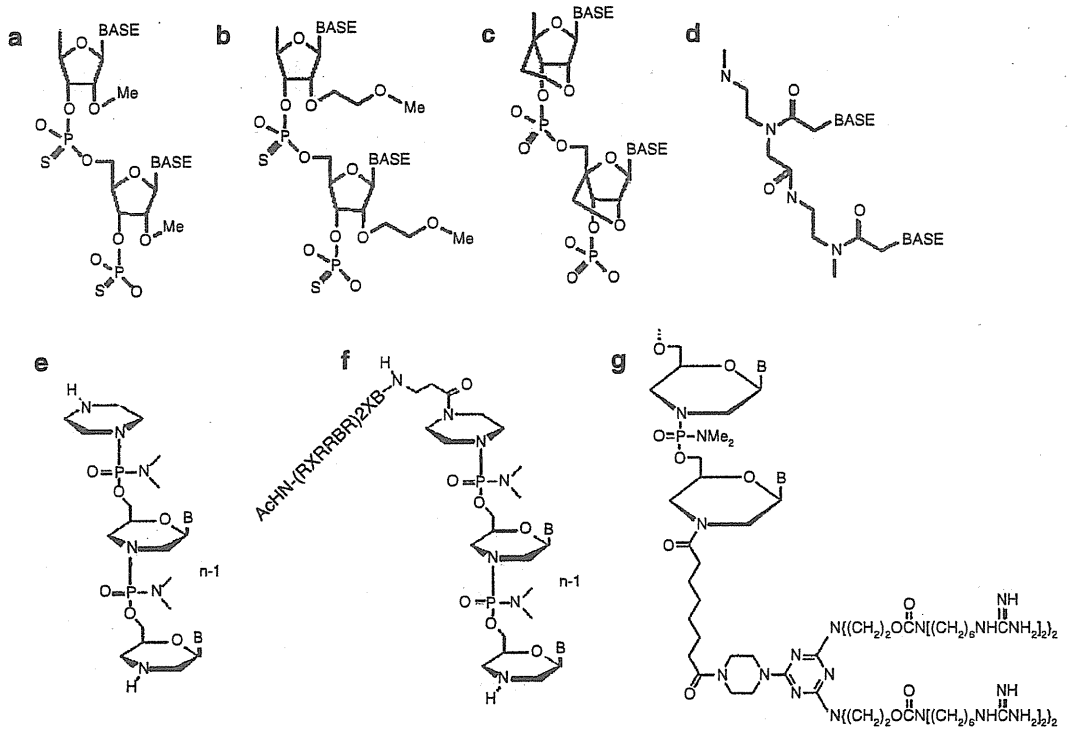

Figure 1 Chemistries of antisense oligomers. (**a**) 2′-O-Methylphosphorothioate (2′OMePS AON); (**b**) 2′-O-methoxyethyl phosphorothioate; (**c**) locked nucleic acid (LNA); (**d**) peptide nucleic acid (PNA); (**e**) phosphorodiamidate morpholino oligomers (PMO); (**f**) AcHN-(RXRRBR)2XB peptide-tagged PMO (R, arginine, X, 6-aminohexanoic acid and B, ®- alanine) (PPMO); G, octa-guanidine PMO.

More recently, phosphorodiamidate morpholino oligomers (PMO) have been explored for exon skipping in the dystrophin gene. In PMO, the phosphodiester bond is replaced by phosphorodiamidate linkage and the ribose replaced by a morpholino moiety (**Figure 1**). PMOs are charge-neutral and refractory to biological degradation. This chemistry has long been used for translational blockade in zebrafish; penetrating the cells of the developing fishes relatively easily.[9] It has also been applied to cultured mammalian cells[10] where its delivery appears to be impeded by its nonionic nature. In response to this problem, "scrape-loading" (creating pores in the membrane) and "leashing" (complexing PMO with negatively charged complementary DNA sequences) were then developed to enhance delivery by use of commercially available delivery reagents, such as polyethyleneimine and lipofectin.[11] However, on direct injection into muscles the leash adjunct proved toxic and was therefore not tested by i.v. administration. Despite the poor entry of unmodified PMO into cells in tissue culture, it was later found to enter muscle fibers better than 2′OMePS *in vivo* in the dystrophic *mdx* mouse. A single intramuscular injection of 10 μg PMO induced significantly higher levels of dystrophin expression than the same PMO complexed with leash and lipofectin.[3] Furthermore, regular weekly i.v. injections of PMO targeting mouse dystrophin exon 23 induced up to 50% of normal levels of dystrophin in body-wide skeletal muscles in the *mdx* mice, with improved muscle pathology, decreased serum levels of muscle creatine kinase and partial restoration of normalized muscle strength. Even after systemic administration for 1 year, no toxicity has been detected in muscles or other organs. A more recent investigation at higher dosages[12] confirmed that PMO produced higher levels of exon 23 skipping than 2′OMePS and thus appears to be a promising antisense oligomer chemistry for the treatment of DMD patients.[3]

Although both 2′OMePS and PMO induce exon skipping systemically, it was disappointing to find that dystrophin expression was highly variable within and between muscles, even after repeated i.v. injections.[3,4,12] Why this is so, is not clearly understood, but may be due to the reliance on passive diffusion for entry into muscle fibers. For PMO, the lack of charge may present less of an impediment to cell surface contact thus allowing more efficient entry than 2′OMePS into muscle fibers, particularly those with leaky membranes as seen in dystrophic muscles. Such dependence on muscle damage for effective delivery of AONs, would have the advantage of limiting the amount of AON entering untargeted and undamaged nonmuscle cells, thus diminishing possible side effects. However, for long-term effective treatment of DMD, it would carry the disadvantage that muscle fibers rescued by PMO-induced exon skipping would have to re-enter a myopathic state to permit further PMO entry. Such a requirement for recurring cycles of rescue and degeneration in treated muscles could severely limit the value of antisense therapy for DMD patients.

The requirement of muscle damage for effective delivery and AON induced dystrophin expression is further demonstrated by the relative lack of dystophin expression in cardiac muscle of *mdx* mice after systemic injection of either 2′OMePS AON or PMO.[3,4,12] Cardiac muscles in the mice are less affected than skeletal muscle by the dystrophic process and neither conspicuous pathological change nor functional impairment are seen until late stages. Consistently, only trace amounts of dystrophin are detected in

cardiac muscle even after repeated injections into *mdx* mice of either 20′ MePS or PMO AON[3,4,12] even with doses of PMO that induce high levels of dystrophin in skeletal muscles. However, direct injection of AON or adeno-associated virus (AAV)-mediated AON delivery induced effective dystrophin expression in cardiac muscles, suggesting that efficiency of delivery rather than of exon-skipping is the critical factor in this organ.[13]

One way of enhancing intracellular delivery is to employ cell-penetrating peptides or polymers to provide active transport of AON into muscle fibers. Earlier studies showed that conjugation to an arginine-rich peptide significantly improved PMO-mediated antiviral activity[14] as well as delivery of PMO for dystrophin exon skipping in cell cultures and on intramuscular injection into muscles.[15] More recently, Jearawiriyapaisarn *et al.*[16] used a transgenic mouse that expresses enhanced green fluorescent protein as a positive readout for the efficiency of exon exclusion to evaluate the potency, functional biodistribution, and toxicity of PMOs conjugated to various arginine-rich cell-penetrating peptides containing 6-aminohexanoic acid (X) and/or β-alanine. The greatest restoration of enhanced green fluorescent protein expression in both skeletal and cardiac muscles was observed with PMO tagged with a peptide of (RXRRBR)2XB (PPMO). When applied to the dystrophic *mdx* mice model of DMD, a single i.v. injection of 30 mg/kg of PPMO restored dystrophin in all skeletal muscles to almost normal levels[17] that were maintained by regular biweekly administration over 12 weeks and accompanied by improvement in muscle strength and pathology, with significant lowering of serum creatine kinase levels. Most importantly, i.v. injections of PPMO elicited near-normal levels of dystrophin in cardiac muscle (**Figure 2**) and prevented dobutamine-induced cardiac failure. Efficient exon skipping was also achieved in smooth muscles in other organs such as the esophagus. Treatment with the PPMO did not cause detectable toxicity. Recently, this PPMO has been shown to considerably ameliorate the severe pathology in the dystrophin–utrophin double null mouse.[18] Together, these findings illustrate the theoretical feasibility of using PPMO to rescue dystrophin expression in both skeletal and cardiac muscles of DMD patients.

However, use of peptides to enhance delivery raises the possibility of an immune response that may prevent repeated administration or cause rejection of targeted tissues or both, especially because DMD patients would require regular life-long administration. Although no immune response was observed in the above study[17] or in previous reports with similar peptides in animal models,[14,19] immunogenicity varies considerably between species, arguing for longer-term studies in a range of animal models. But final verification can come only from clinical trials. It is, therefore, important to develop nonpeptide alternatives to enhance delivery of oligomers. The known sequence and structure of the peptide used in the PPMO provides a basis for modeling such nonpeptide polymers as delivery vehicles with similar or improved function. With this in mind, Wu *et al.* exploited a nonlinear, nonpeptidic dendrimer as a transporter for delivery of PMO. This consists of eight guanidinium head groups bonded to a trifunctional triazine as a core scaffold, which is then conjugated to PMO targeting exon 23 (ref. 6) (termed Vivo-PMO).[20] The study demonstrated that the Vivo-PMO targeting mouse dystrophin exon 23 (Vivo-PMOE23) is highly effective for exon skipping and dystrophin induction in *mdx* mice. A single i.v. injection of 6 mg/kg Vivo-PMOE23 generated dystrophin expression in skeletal muscles at levels equivalent to the injection of 300 mg/kg unmodified PMOE23. Repeated injections of 6 mg/kg Vivo-PMOE23 achieved ~50% and 10% wild-type levels of dystrophin expression in body-wide skeletal muscles and in cardiac muscle respectively, without eliciting a detectable immune response. Vivo-PMOs showed no signs of toxicity at the effective dosage regime that reduced the serum levels of creatine kinase significantly.[20] These results thus offer prospects for the development of new nonpeptide delivery moieties with improved function and low toxicity.

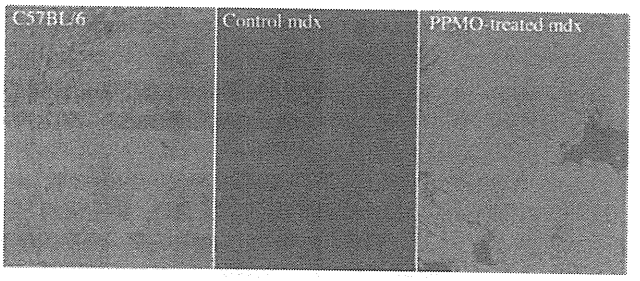

Figure 2 Restoration of dystrophin in cardiac muscles of mdx mice after six intravenous injections (at biweekly intervals) of 30 mg/kg of the PPMOE23 targeting mouse dystrophin exon 23. Muscles were examined 2 weeks after the last injection. Left panel, muscles from heart of normal *C57BL/6*. Middle panel, muscle from heart of control *mdx* mouse. Right panel, PPMO-treated *mdx*. Dystrophin was detected by immunochemistry with the polyclonal rabbit antidystrophin antibody, P7, and visualized with Alexa 594 tagged goat-anti-rabbit Igs. Blue nuclear staining with DAPI.

MULTIEXON SKIPPING IN DYSTROPHIC DOGS

Although antisense-mediated exon skipping clinical trials currently conducted in United Kingdom and Netherlands targeting exon 51 show promising results,[8,21] such single exon skipping covers only a proportion of DMD patients. Even if antisense oligos against most exons in the *DMD* gene become available, approximately half of DMD patients will require multiexon skipping by targeting of more than one exon, depending, not on the size but on the type of mutation (*e.g.*, deletion, duplication, point mutation, etc.) and the "phase" of the mutated exon and its neighboring exons. For example, to treat a patient with deletion of exon 7, one needs to target at least two exons (*e.g.*, both exon 6 and exon 8) to put the mutation back in frame (**Figure 1**). In fact, canine X-linked muscular dystrophy harbors such mutation[22] (*i.e.*, a splice site mutation in intron 6 that excludes exon 7 from the mRNA transcript (**Figure 3**)) and is therefore, a good model for testing the efficacy and efficiency of double-exon skipping.[23] The dystrophic dog has several further advantages over the *mdx* mouse. First, it provides the prospect of more detailed analyses of clinical condition, such as clinical grading, magnetic resonance imaging, three-dimensional-echocardiography, and electrocardiogram.[23] Second, the canine X-linked muscular dystrophy model, is closer in clinical phenotype than the *mdx* mouse model to human DMD. Indeed, it shows, if anything, a more severe progression than DMD; this, in combination with its similarity in body weight, makes it especially useful for physiological and

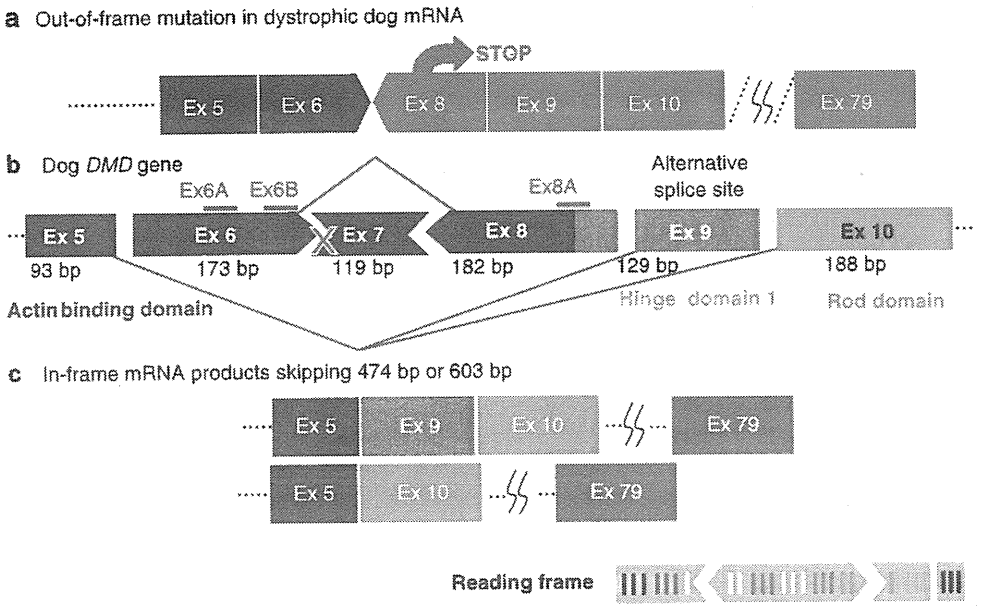

Figure 3 Diagram to illustrate the exon-skipping strategy to restore open reading frame at the mutation site in the CXMD dystrophic dog. A point mutation in the acceptor splice site in intron 6 preceeding exon 7 (X) leads to exclusion of exon 7 from the transcript and loss of open reading frame when exon 6 is spliced to exon 8. To restore open reading frame, requires the loss of at least two further exons: 6 and 8. In the event, exon 9 is also excluded from the transcript but, because it contains a whole number of codon triplets, this does not disrupt the translation of the resultant mRNA. CXMD, canine X-linked muscular dystrophy.

toxicological studies.[23] Finally, there may be some advantage in the fact that many target sites for exon skipping show identity of DNA (mRNA) sequences between dog and human. Drug regulation authorities such as US Food and Drug Administration are inclined to regard antisense oligonucleotides (AOs) of different sequences as different drugs; thus, to target the range of mutations encountered in DMD patients, many AO sequences will need to be designed, tested, and approved. Between man and mouse targeting homologous sites has little predictive value, perhaps due to minor sequence differences.[24] Dogs and humans however, share considerable sequence identity; for most exons in the *DMD* gene one can design a single 20–25mer antisense sequence that is applicable to both and comparison of targeting efficiencies between these species should be explored further.

Overall, the dog experiments provide a promising message for DMD patients. McClorey and colleagues transfected cultured myotubes from dystrophic dogs *in vitro* with a cocktail of antisense oligos targeting exons 6 and 8, noting restoration of reading frame in mRNA.[25] Recently, we sought to test efficacy and toxicity of i.v. PMO induced exon skipping *in vivo* in the DMD dog model.[26] We identified a cocktail that, by either intramuscular injection or systemic i.v. delivery, resulted in extensive dystrophin expression to therapeutic levels. Weekly or biweekly systemic i.v. injections, over the course of 5–22 weeks, with a three-morpholino cocktail (120–200 mg/kg in total of three oligos/injection) targeting exon 6 and exon 8, induced therapeutic levels of dystrophin expression throughout the body, to an average of 26% of normal levels. Expression of dystrophin was associated with significant functional and clinical stabilization, being accompanied by reduced inflammation as observed histologically and by magnetic resonance imaging, improved or stabilized clinical symptoms and timed running tests. Histology and blood tests indicated no evidence of toxicity. Dystrophin expression was also detected in cardiac muscles by immunohistochemistry but, as in the *mdx* mouse,[3,4] less than in skeletal muscles and concentrated in small patches. Recently, we have found that an i.v. injection of peptide-conjugated morpholinos (PPMOs) at 12 mg/kg elicited increased dystrophin expression in the canine heart, as detected by western blotting (Yokota *et al.*, data not shown).

An unexpected observation in the dog study was that, in tissue culture, either of the two antisense oligonucleotide components of the cocktail directed against exon 6 were able, alone, to efficiently induce the desired exon 5–10 splicing in the absence of the sequence against exon 8. By contrast, they did not do this *in vivo*. In addition, excision of exon 8 by the exon 6-specific sequences alone occurred only in the context of the mutant exon 7 splice site (*i.e.*, it did not occur in wild-type dog cells). Similarly, AO administration to human cells produced some disparities in skipping between patients carrying small mutations in the *DMD* gene and wild-type cells.[27] The differences between patterns of skipping *in vivo* versus *in vitro* and between wild-type versus mutant genotypes indicate that the pattern of exon skipping is greatly influenced by variables other than the local presence of target sequence. Thus, it is prudent to consider testing of selected target sequences in multiple systems before committing to a specific sequence for subsequent clinic trials.

SIGNIFICANCE OF MULTIEXON SKIPPING

Theoretically, multiple exon skipping could restore open reading frame in >80% both of deletion and nonsense mutations in the *DMD* gene.[28–31] Moreover, since some in-frame deletions are

associated with milder phenotypes than others, selective skipping of more exons than are required for simple restoration of reading frame offers the prospect of selecting options that optimize the functionality of the resultant dystrophin protein. Thus, it has been proposed that a cocktail of AOs targeting exons 45–55, a deletion associated with a high percentage of asymptomatic or mild BMD clinical phenotypes[32] would potentially be applicable to 63% of patients with dystrophin deletions. Currently, techniques for skipping 11 exons simultaneously are not available but might be achieved in future by improved efficacy of AO chemistry or more efficient delivery methods.

AAV U7 GENERATION OF ANTISENSE OLIGONUCLEOTIDES

Perhaps the most efficient way to achieve long lasting exon skipping, without recurrent infusions of antisense oligonucleotides, would be to generate the antisense agent within the target cells. Current studies have used gene vectors expressing modified U7 or U1 small-nuclear RNAs as antisense shuttles (AS-snRNAs).[33–35] Because these expression cassettes are very small (AS-U7 is about 400 nucleotides) there is sufficient room within gene vectors to combine several copies of different AS-snRNAs designed to target multiple exons within a gene or even different genes simultaneously.

Although a number of viral vectors could be used for the delivery of such AS-snRNA chimeras in tissue culture as well as *in vivo*, AAV have come to the fore, offering the advantage of stable long-term expression. Current AAV8-, AAV1- and AAV6-capsids effect efficient and widespread transduction of muscles in mice after tail vein administration,[36] with promising new serotypes pending.[37,38] Systemic delivery of AAV vectors harboring AS-U1 in the *mdx* mouse resulted in effective body-wide dissemination of the therapeutic construct and significant improvement of muscle function suggestive of overall maintenance of muscle mass and strength.[39] Similar results have been obtained with the AS-U7 system:[35] sustained dystrophin rescue to near wild-type levels and restoration of normal levels of muscle resistance to mechanical stress. In addition, no immune response has been reported, against the rescued dystrophin, due perhaps to fact that the rescued truncated dystrophin is represented in the repertoire of pre-existing revertant fibers, which naturally occur in dystrophic mice. However, while the long-term stability of corrected fibers was clearly demonstrated in the *mdx* mouse,[13,40] the AAV(AS-snRNA) approach still faces problems arising from immune sensitization against AAV, that would prevent the application of repeated treatment unless an effective regime of immunomodulation can be developed.[36]

For most myopathic disorders, to be of practical clinical therapeutic value, a genetic therapy would, ideally, provide treatment of the whole skeletal and cardiac musculature. As has been demonstrated by initial experimental trials in murine models, this cannot be achieved by intramuscular injections; only a systemic injection can approach this objective. Such a systemic delivery procedure is not without risk and entails long and expensive development, in particular to overcome the immune problems.[41,42] First, production of the large quantity of vector required to treat even a single patient is a daunting task that is the objective of a number of methods for large scale AAV production currently being developed.[43] Second, practicability of the AAV(AS-snRNA) technology requires development of a safe and effective protocol for systemic administration. This needs to be tested in a large animal, such as the canine X-linked muscular dystrophy dog, to permit evaluation of the dose range and the protocols of administration of the vectors required to achieve therapeutic effectiveness while remaining safe. In order to anticipate, on a rational basis, the adaptation of such a protocol to trials in man, it is important to conduct such studies in conditions that mimic clinical practice as closely as possible.

ONGOING THERAPEUTIC TRIALS USING ANTISENSE OLIGONUCLEOTIDES

Two European consortia are involved in clinical trials using two different antisense oligonucleotide chemistries. One group is based in Holland, closely associated with the Leiden University Medical School (Prof Gert van Ommen and Dr Jan Verschuuren) and works in close collaboration with the company Prosensa, which also sponsored these studies. The second group is based in United Kingdom, where a consortium of four Universities (MDEX consortium) is led by F.M., and works in close collaboration with AVI Biopharma, which is sponsoring the present study.

Both groups are targeting exon 51, although using two different primary sequences, and different backbones. The Dutch study utilizes a 21-mer 2′OMePS,[7] whereas the MDEX Consortium is employing a 30 PMO.[44] Both groups elected to study patients with deletions who would benefit from exon 51 skipping (50, 52, 52–63, 45–50, 48–50, and 49–50), both because cumulatively these account for 13% of all DMD deletions,[7,28] and more especially because the resulting protein has been clearly demonstrated to be extremely functional, as suggested by several multigenerational families deleted for the same domains with no symptoms whatsoever.[45–47]

The Dutch consortium have completed and published in 2007 the result of a proof of concept study in which four DMD boys have received a single injection of the 2′OMePS into the tibialis anterior. This was well tolerated and accompanied by specific skipping of exon 51 as well as detection of sarcolemmal dystrophin in 64–97% of myofibers of the biopsied muscle; the amount of dystrophin ranged from 3 to 12% of that found in the normal control muscle and with intensities in individual fibers ranging from 17 to 35%.[8]

The MDEX consortium performed a similar study using the PMO AO, but with a different design: a dose escalation study in seven DMD boys, who received either 0.09 or 0.9 mg in one of the two extensor digitorum brevis muscles, whereas the contralateral muscle received saline. The results, recently published,[21] demonstrated clearly detectable dystrophin expression in 44–79% of myofibers, with intensity of dystrophin staining averaging 17% greater than the levels in the contralateral muscle and, in the most positive fibers, up to 42% of that in healthy muscle fibers.[21]

Both studies have been followed by repeated systemic administration studies. The Dutch consortium recently completed a study in which four group of DMD boys received escalating doses of the 2′OMePS antisense to skip exon 51, subcutaneously, at doses of 0.5, 2.0, 4.0, and 6.0 mg/kg, weekly for 5 weeks. All 12 children (3/group) had a muscle biopsy at the beginning and the end of the study. While the results of this study have not yet been published, Dr Goemans reported at the World Muscle Society meeting in

2009 (Geneva)[48] that the study drug was well tolerated and that a dose–response in exon skipping and dystrophin production was observed. All boys who received the 2′OMePS AO have been enrolled in an extension study that is currently underway.

Encouraging results have also been announced by the analysis of the first four cohorts of the boys recruited into the MDEX systemic study using the PMO. In this study, seven groups of DMD boys received escalating doses of PMO (0.5, 1.0, 2.0, 4.0, 10, and 20 mg/kg) for a period of 12 weeks. All patients had a pretreatment and post-treatment muscle biopsy. At the time of writing only the first four cohorts have completed the study, and the preliminary analysis indicates that in the three patients in the 2.0 and 4.0 mg/kg cohorts there was accurate skipping of exon 51. In one of the patients at the 2.0 mg/kg dose, the appearance of skipped mRNA was accompanied by a several fold increase in expression of dystrophin protein in the post-treatment samples using both western blotting and immunofluorescent analysis (fivefold on western blot and approximately sevenfold on immunocytochemistry). While the results of the patients recruited into the last two cohorts will not be available until the 2nd quarter of 2010, both these results, and those from the Dutch consortium are very encouraging. Two pivotal multicentric phase III studies are currently being planned, one by Prosensa/GSK, using the 2′OMePS AO, and one by AVI Biopharma, using the PMO AO, and are both likely to start in 2010. The design will be a randomized placebo controlled study which is likely to last for ~1 year. Additional studies are also being planned by Prosensa (a multicentre phase I/II study targeting exon 44 with a 2′OMePS, whereas target optimization for exon 43, 45, 46, and 52 are being pursued, possibly followed by further clinical studies in 2011–2012). In addition AVI Biopharma has initiated a preclinical program which is anticipated to lead to an IND/IMPD filing in early 2010 for it's lead peptide-conjugated PMO (PPMO) to skip exon 50 and thus into a clinical study which is currently being planned.

Prospects

As attested by the above accounts, the potential for use of exon skipping as a therapeutic strategy for DMD has developed from a plausible notion in the mid-1990's[1,2] to the point where early clinical trials show that it holds realistic prospects of providing genuine therapeutic benefit. There remain, however, substantial barriers: some scientific, some regulatory, with occasional interaction between the two.

The major scientific issues concern the choice of sequence for any given exon and the enhancement of delivery and effectiveness of that sequence to the majority, ideally all, of the muscle fibers in the body.

Although, effective sequences that promote skipping of a number of exons have been identified, we have no reliable method for determining whether any given sequence is optimal. A thorough screen for optimal sequences alone and in combination requires the ease of use of a tissue culture system and although a broad correspondence has shown between the *in vitro* and *in vivo* activities of different chemistries and adjuncts[49,50] it is evident from the canine studies[26] that myogenic cultures cannot be relied upon to inform us accurately as to the *in vivo* activity of various sequences. A recent study of equivalent sequences that target human and mouse exons confirms the view that the efficacy of targeting is highly context dependent[24] and that we should be wary of generalizing the applicability of specific results from one test system.

As to delivery, most work in the *mdx* mouse favors PMO over 2′OMePS backbone chemistry, but neither shows great promise for entering cardiac muscle in useful amounts and even in skeletal muscle, effectiveness is patchy. We are therefore in need of developments such as the addition of cell-penetrating moieties which, in turn, will entail extensive animal studies to establish dosage regimes that provide efficacy with minimal toxicity.

For the AAV(AS-snRNA) approach, the ideal would be a single body-wide delivery to 100% of cardiac and skeletal muscle cells, with the reasonable expectation that this would need to be repeated rarely, perhaps never if we are lucky. At present, such efficient delivery does not seem to be possible with a single infusion and the potential immune complications; generation of neutralizing antibodies and of cell-mediated response to residual viral antigens mandates a thorough appraisal of multiple delivery protocols.

For regulatory bodies, antisense induced exon skipping represents an extreme example of agents that are highly targeted to the individual patient, and poses a potentially educative challenge to the appropriateness of standard procedures. The combination of a need for at least one different oligonucleotide sequence for each target exon and the large number of different exons, together with the small numbers of patients who might benefit from skipping of some specific exons, raises considerable obstacles to the conduct of standard safety and efficacy regimes. The problem is further compounded by the fact that sequence-specific side effects are likely to be species-specific and therefore not reliably assessable on animal models. A requirement for a full toxicological workup of each individual sequence would be a major disincentive for manufacturers to extend their interests beyond a small number of the more widely applicable target exons or even to seek to optimize sequences for the commoner exon targets. Moreover, many target exons would be relevant to too few patients to permit conduct of any form of conventionally designed trial. Thus, imposition of the normal regulatory processes would constitute a major impediment to the application of exon-skipping therapy across the range of patients who might benefit from it. A positive exploration of these issues would act as a trailblazer to the benefit of the progress of personalized medicine in general.

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Review

Gene therapy for muscle disease

*Yuko Miyagoe-Suzuki, Shin'ichi Takeda**

Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan

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ABSTRACT

The molecular mechanisms of Duchenne muscular dystrophy (DMD) have been extensively investigated since the discovery of the dystrophin gene in 1986. Nonetheless, there is currently no effective treatment for DMD. Recent reports, however, indicate that adenoassociated viral (AAV) vector-mediated transfer of a functional dystrophin cDNA into the affected muscle is a promising strategy. In addition, antisense-mediated exon skipping technology has been emerging as another promising approach to restore dystrophin expression in DMD muscle. Ongoing clinical trials show restoration of dystrophin in DMD patients without serious side effects. Here, we summarize the recent progress in gene therapy, with an emphasis on exon skipping for DMD.

Contents

* *Corresponding author.* Fax: +81 42 346 1750.
E-mail address: takeda@ncnp.go.jp (S. Takeda).

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Introduction

Muscular dystrophies are heterogeneous genetic disorders, characterized by progressive degeneration and weakness of the skeletal and cardiac muscles. DMD is severe and the most common type of muscular dystrophy; worldwide, approximately one in every 3500 boys born is afflicted with DMD.

The *DMD* gene is the largest known gene in humans, comprising over 79 exons, with a coding sequence of 11 kb and spans no less than 2.3 Mb of genomic DNA. DMD is caused by deletion (65%), duplication (15%), or nonsense and other small mutations (20%) in the *DMD* gene, all of which disrupt the open reading frame [1].

The *DMD* gene encodes dystrophin, which is located beneath the sarcolemma, assembles the dystrophin–glycoprotein complex at the sarcolemma, and links the internal cytoplasmic actin filament network and extracellular matrix, providing physical strength to muscle fibers [2]. At present, there is no effective therapy to stop the lethal progression of the disease, but several therapeutic approaches hold great potential. Here we focus on gene therapy for DMD and summarize AO-mediated exon skipping technology as a most promising therapy.

Adenoassociated virus -mediated gene therapy

Updates on rAAVs

The adenoassociated virus (AAV) is a tiny single-stranded, nonpathogenic, nonreplicative DNA virus belonging to the Parvovirus family. So far, more than 12 serotypes have been identified in primates [3]. Recombinant AAV (rAAV) is a powerful tool to deliver therapeutic genes to skeletal muscle [4–6]. Even in immunologically competent mice, the expression of the exogenous gene was shown to continue for years without evoking immune responses.

Importantly, rAAV has several serotypes that show tropisms to skeletal muscle. rAAV1 and rAAV2 are commonly used for direct delivery to skeletal muscle and mainly used in local treatment. rAAV-6 [7] plus the more recently developed rAAV-8 [8,9], and rAAV-9 [10–12] are powerful in systemic delivery of the therapeutic genes via the circulation to the musculature bodywide, including the diaphragm and heart.

Limited packaging size of rAAV

rAAV has a limitation in the length of the transgene it can accommodate (less than 5.0 kb). Full-length dystrophin, which is nearly 11 kb, cannot be incorporated into an AAV vector. To overcome this limitation, truncated but functional microdystrophins with a large deletion in the central rod domain have been constructed because studies of the genotype–phenotype relationships in DMD and Becker muscular dystrophy (BMD), a milder form of muscular dystrophy with near-normal life expectancy, have suggested that the rod domain has limited function and is largely dispensable [4]. Several types of microdystrophin were administrated to *mdx* mice locally [13] or systemically [7,14–16] and ameliorated pathology and improved muscle function. To expand the packaging capacity of the AAV vector, trans-splicing (ts) of two vectors and recombination of two overlapping (ov) rAAV vectors have been tested (reviewed in Trollet et al. [4]). A hybrid dual-vector system, which combines the features of the ts and ov vectors into a single system, has been reported to work well in skeletal muscle [17].

Immunity against rAAV in dog models

Based on the improvement of pathology and muscle function due to successful AAV-mediated gene transfer into dystrophic mice, preclinical studies using dystrophic dogs [18,19] and nonhuman primates [20,21] were performed. In dogs, considerable cellular immune response was often observed [18,19,22], and transient immune suppression was needed [23]. However, there is no clear explanation of why rAAVs evoke much stronger immune responses in dogs than mice.

Clinical trials

Immunity to AAVs is also a big concern in rAAV-mediated gene therapy for DMD. First, natural AAV infection is quite common in human populations, and preexisting antibodies could block AAV vector-mediated therapy. Second, after the first injection of rAAV vectors, the second injection is known to be much less effective due to a neutralizing antibody. Indeed, clinical trials using AAV vectors suggest that immune response to the vector and/or transgene product is the most important limitation of the rAAV-mediated gene therapy. To diminish a host immune response against the transgene product, utilization of a muscle-specific promoter active in both skeletal and cardiac muscles [24,25] is desirable. Codon optimization has also been demonstrated to be effective to reduce the virus titer [26]. A phase I/II clinical trial of intramuscular delivery of microdystrophin by AAV2.5-CMV-Mini-Dystrophin was initiated in 2006 (PI: JR Mendell; Trial ID: US-679; clinicaltrials.gov identifier: NCT00428935). More information can be obtained at http://www.wiley.co.uk/genetherapy/clinical/, http://www.clinicaltrials.gov, or http://www.mda.org.

Lentiviral vector-mediated gene transfer into muscle stem cells

Lentiviral vectors have a relatively large transgene carrying capacity (7.5–9 kb), integrate into the genomes of both dividing and nondividing cells, and achieve long-term transgene expression in a wide variety of tissues including skeletal muscle. Previously, lentiviral vectors have been used to introduce a mini-dystrophin gene into mouse skeletal muscle [27]. Because the expression levels of mini-dystrophin were low after direct injection of lentiviral

vectors into diseased muscle, this system seemed to be useful in modifying genetically autologous cells *ex vivo* rather than in direct injection *in vivo*. In fact, lentiviral vectors expressing mini-dystrophin transduced mouse satellite cells efficiently, and the transduced cells regenerated muscle fibers after transplantation [28]. Quenneville et al. [29] showed that lentiviral vectors are useful in transducing monkey muscle stem cells. The lentiviral vector has been recently used to modify muscle stem cells to deliver an antisense sequence linked to a modified U7 [30] or U1 [31] small nuclear RNA for restoration of the reading frame.

Antisense oligonucleotide (AO)-mediated exon skipping for *DMD* gene

Skipping of targeted exons

DMD is caused by mutations in the *DMD* gene that disrupt the open reading frame. BMD is also caused by mutations in the *DMD* gene, but in the case of BMD, the open reading frame is maintained. If we can skip (splice out) targeted exons by modification of splicing patterns and restore the reading frame, a shorter dystrophin protein can be restored in the DMD muscle, converting the DMD phenotype to a BMD phenotype. To this end, a number of antisense oligonucleotides (AOs) have been designed and tested *in vitro* [32–34] and *in vivo* [35–37]. Fig. 1 illustrates the skipping of exon 51 using one AO. Whether the resultant shortened dystrophin is functional or not depends largely on the function of the deleted part. In general, truncation of the rod domain is thought to be relatively harmless.

Single exon 51 skipping is expected to be suitable for approximately 13% of DMD patients. Multiple exon skipping is estimated to be applicable to more than 80% of DMD patients. Theoretically, the AO-mediated exon skipping strategy cannot treat patients with mutations in the promoter region, deletion of the first or last (79th) exon, deletion of the domain bound by dystroglycan: exons 62–69 [38] or large deletions (>35 exons) [39]. However, these mutations are rare, and the majority of patients have a mutation in the hotspot located between exons 43 and 55.

Design of AOs

AOs are designed to hybridize specific sequences, such as exon–intron boundaries, and exon splicing enhancer (ESE) sequences in transcripts. AOs interfere sterically with the splicing machinery [40,41]. There are several software programs, such as ESEfinder (http://rulai.cshl.edu/tools/ESE), to design antisense oligonucleotides, but extensive empirical analysis is still required for each exon.

AO chemistry, delivery in vivo, and toxicity

Among the AOs tested so far, AOs having a 2′-*O*-methyl phosphorothioate backbone (2′-O-MeAO) and phosphorodiamidate morpholino oligomers (PMOs) (Fig. 2) are commonly used in animal models and in clinical trials [42,43]. 2′-O-MeAOs have a chemically modified RNA structure (Fig. 2). The modifications increase the half-life and distribution to tissues. 2′-O-MeAOs have been well tolerated in clinical trials. PMOs have a morpholino backbone, are uncharged, are not recognized by cellular proteins, and, therefore, are rapidly cleared from plasma and excreted in urine. Very high doses of PMOs are reported to be well tolerated by animal models. This would be partly because PMOs hardly evoke innate immune responses.

In vivo delivery of AOs

One limitation of PMO-mediated exon-skipping therapy is that PMOs do not easily enter cardiac muscle. Recently, to improve the uptake of PMOs by cardiocytes, peptide-tagged PMOs (PPMOs) [44] and Octa-guanidine PMOs [45] were developed. These modified

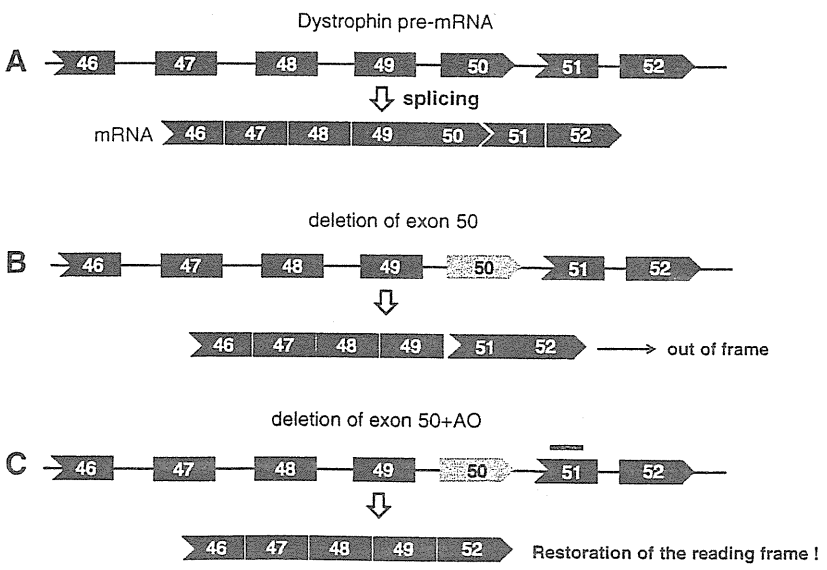

Fig. 1 – Exon skipping therapy for DMD patients with deletion of exon 51. (A) Normal dystrophin transcript and mRNA. (B) Deletion of exon 50 disrupts the open reading frame, leading to a premature stop codon, unstable mRNA, and a truncated protein. (C) Targeted skipping of exon 51 using AO restores the reading frame and produces a shorter but functional dystrophin that lacks exons 50 and 51. Blue bar indicates AO targeting the sequence in exon 51.

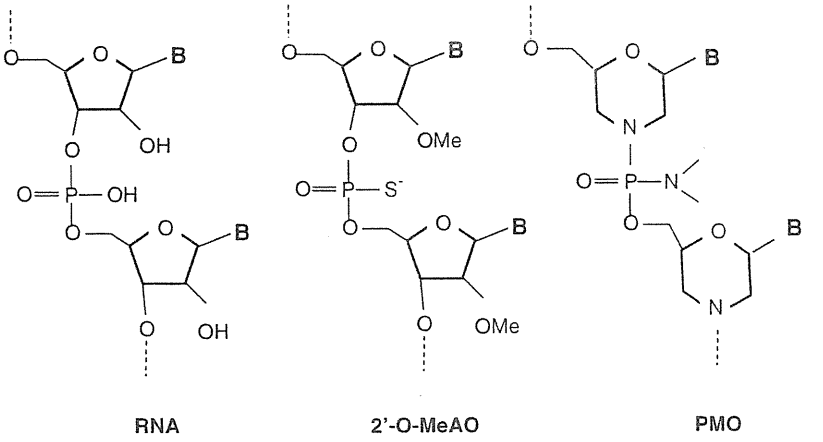

Fig. 2 – Structure of RNA, 2′-O-MeAO, and PMO. B: bases (adenine, cytosine, guanine, and thymine).

morpholinos are reported to be more effective than native PMOs in inducing exon skipping in cardiac muscle after intravascular injection. But there are potential concerns that PPMOs might elicit an immune response or have toxicity compared with PMOs due to the protein moiety.

Skipping multiple exons

If fully approved, AVI-4658 and PRO051, both of which target dystrophin exon 51, will be able to treat 13% of DMD patients. To treat more patients, elimination of two or more exons from the final mRNA is required. Theoretically, multiexon skipping using a cocktail of AOs can restore the reading frame of the *DMD* gene in more than 83% of the all DMD patients. Double-exon skipping using AOs has been shown to be feasible in patient-derived cells [46], mouse models, and dystrophic dogs [37]. On the other hand, the efficiency of multiexon skipping is much lower than expected [47]. This is presumably because partial exon skipping results in out-of-frame transcripts. It will be some time before multiple-exon skipping is applied to DMD patients.

Ongoing clinical trials of exon skipping

Clinical trials using intramuscular administration of 51 AOs, PRO051 (2′-O-Me AO), and AVI-4658 (PMO) have been performed in Europe by Prosensa and AVI BioPharma respectively. PRO051 and AVI-4658 were both designed to induce exon 51 skipping in the *DMD* gene and, therefore, can treat DMD patients with deletions such as 45–50, 47–50, 48–50, 49–50, 50, or 52. AVI BioPharma reported the initial data of systemic treatment with AVI-4658 (a phase 1b/2 clinical study) in the United Kingdom, which resulted in the successful restoration of dystrophin in the 2-mg/kg dose cohort (http://www.avibio.com/). AVI-4658 is well tolerated and so far has caused no serious side effects in treated patients. A phase 1/2 dose-ranging safety study using PRO051 was performed on 12 patients at two European clinical centers. The study demonstrated that PRO051 was also well tolerated up to 6 mg/kg and that novel dystrophin expression was detected in the patients in response to injections above 0.5 mg/kg [48] (also refer to http://prosensa.eu/technology-and-products/Pipeline/PRO-051.php or http://www.parentproject.org.au/html/s02_article/article_view.asp?art_id=679&nav_catid=214&nav_top_id=78).

However, the consequences of long-term administration of both AOs should be carefully examined because AOs have a transient effect and must be readministered to sustain the effect.

Conclusions

Development of gene therapy for DMD has long been a challenge, but recent strategies, such as AAV-8 or AAV-9-mediated systemic delivery of microdystrophin and exon skipping, hold great potential. AO-induced exon skipping is a mutation-specific approach. Both the mutation and splicing patterns of dystrophin mRNA must be examined individually, and the AO sequences used would differ from patient to patient. One concern is that the efficacy and safety of each variation must be tested on the same backbone, requiring more time to get approval from the regulatory authorities.

Although AO-mediated exon skipping has shown promising results, the authors predict that a combination of exon skipping and other therapeutic approaches, such as viral vector-mediated gene transfer, stem cell-based therapy, or additional strategies of enhancing muscle regeneration, will become the standard approach for future DMD therapy.

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In-frame Dystrophin Following Exon 51-Skipping Improves Muscle Pathology and Function in the Exon 52–Deficient *mdx* Mouse

Yoshitsugu Aoki[1,2], Akinori Nakamura[1], Toshifumi Yokota[1,3], Takashi Saito[1,4], Hitoshi Okazawa[5], Tetsuya Nagata[1] and Shin'ichi Takeda[1]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan;* [2]*Department of System Neuroscience, Medical Research Institute, Tokyo Medical and Dental School University Graduate School, Tokyo, Japan;* [3]*Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC, USA;* [4]*Department of Pediatrics, Tokyo Women's Medical University, Tokyo, Japan;* [5]*Department of Neuropathology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan*

A promising therapeutic approach for Duchenne muscular dystrophy (DMD) is exon skipping using antisense oligonucleotides (AOs). In-frame deletions of the hinge 3 region of the dystrophin protein, which is encoded by exons 50 and 51, are predicted to cause a variety of phenotypes. Here, we performed functional analyses of muscle in the exon 52–deleted *mdx* (*mdx*52) mouse, to predict the function of in-frame dystrophin following exon 51-skipping, which leads to a protein lacking most of hinge 3. A series of AOs based on phosphorodiamidate morpholino oligomers was screened by intramuscular injection into *mdx*52 mice. The highest splicing efficiency was generated by a two-oligonucleotide cocktail targeting both the 5′ and 3′ splice sites of exon 51. After a dose-escalation study, we systemically delivered this cocktail into *mdx*52 mice seven times at weekly intervals. This induced 20–30% of wild-type (WT) dystrophin expression levels in all muscles, and was accompanied by amelioration of the dystrophic pathology and improvement of skeletal muscle function. Because the structure of the restored in-frame dystrophin resembles human dystrophin following exon 51-skipping, our results are encouraging for the ongoing clinical trials for DMD. Moreover, the therapeutic dose required can provide a suggestion of the theoretical equivalent dose for humans.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe muscle disorder characterized by mutations in the *DMD* gene that mainly disrupt the reading frame leading to the absence of functional protein.[1] A related allelic disorder, Becker muscular dystrophy (BMD), which shows a much milder phenotype, typically results from shortened but in-frame transcripts of the *DMD* gene that allow expression of limited amounts of an internally truncated but partially functional protein.[2] Antisense oligonucleotide (AO)-mediated exon-skipping therapy for DMD, which is a splice modification of out-of-frame dystrophin transcripts, has been demonstrated to exclude specific exons, thereby correcting the translational reading frame, resulting in the production of "Becker-like," shortened but partially functional protein.[3–7] As a result of exon skipping, DMD could be converted to the milder BMD.[4]

The principle underlying exon-skipping therapy for DMD has been demonstrated in cultured mouse or human cells *in vitro*.[7–13] In addition, *in vivo* studies in murine or canine animal models have provided preclinical evidence for the therapeutic potential of AO-mediated exon-skipping strategies for DMD.[11,14–19] However, the number of patients who have the same mutation as the mice or dogs is estimated to be quite low.[20,21] On the other hand, a hot spot for deletion mutations between exons 45 and 55 accounts for >60% of DMD patients with deletion mutations.[20,22] In particular, exon skipping that targets exon 51 is theoretically applicable to the highest percentage (13%) of DMD patients with an out-of-frame deletion mutation.[20,23–25] Recently, efficient in-frame dystrophin expression following an exon 51-skipping approach has been successfully demonstrated in human subjects using local intramuscular AO injection.[23,24]

The functionality of the dystrophin protein produced by exon 51-skipping has been inferred by the identification of patients harboring the corresponding in-frame deletions (*e.g.*, in BMD patients).[6,25] In-frame deletions near hinge 3 (refs. 12,26,27), which is encoded by exons 50 and 51, are predicted to lead to BMD; however, the severity of this disease can vary considerably.[25,28–32] Consequently, it is desirable to use an animal model to investigate the molecular functionality of in-frame dystrophin lacking hinge 3 following exon 51-skipping. In the exon 52–deficient *mdx* mouse (*mdx*52), exon 52 of the *Dmd* gene has been deleted by gene-targeting, resulting in the production of a premature termination codon in exon 53 (refs. 7,33). This mouse lacks dystrophin and shows dystrophic features as well as muscle hypertrophy.[33] It would be meaningful in predicting whether exon 51-skipping led to an accumulation of BMD-like dystrophin that was able to

Correspondence: Shin'ichi Takeda, Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawa-higashi, Tokyo, Japan. E-mail: takeda@ncnp.go.jp