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Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology

Julia Alter[1], Fang Lou[2], Adam Rabinowitz[1], HaiFang Yin[1], Jeffrey Rosenfeld[3], Steve D Wilton[4], Terence A Partridge[1] & Qi Long Lu[3]

For the majority of Duchenne muscular dystrophy (DMD) mutations, antisense oligonucleotide (AON)-mediated exon skipping has the potential to restore a functional protein. Here we show that weekly intravenous injections of morpholino phosphorodiamidate (morpholino) AONs induce expression of functional levels of dystrophin in body-wide skeletal muscles of the dystrophic *mdx* mouse, with resulting improvement in muscle function. Although the level of dystrophin expression achieved varies considerably between muscles, antisense therapy may provide a realistic hope for the treatment of a majority of individuals with DMD.

Mutations in the dystrophin gene underlie two allelic forms of muscular dystrophy. Severe DMD is characterized by mutations that create premature termination of translation, thus preventing production of functional protein[1]. Becker muscular dystrophy, ranging from almost asymptomatic to milder forms of DMD, is typified by mutations that create shortened but in-frame transcripts, permitting production of partially functional protein. The musclar form of dystrophin protein can be divided into amino terminal, rod, cysteine-rich and carboxy terminal domains, with most of the known functions being assigned to the two terminal domains and the cysteine-rich domain[2]. By good fortune, the rod domain, spanning about half the length of the protein, seems to be of limited functional importance and seems to be the region in which the majority of DMD mutations occur[3,4]. Thus, DMD provides an unparalleled prospect for gene correction by AON-mediated skipping of exons that bear mutations or disrupt the reading frame.

AON-mediated exon skipping has been shown to remove specific exons effectively from the dystrophin transcript, creating shortened but functional proteins[5–7]. In *mdx* mice, intramuscular delivery of 2′O methyl phosphorothioate AONs (2OMeAONs) induced skipping of exon 23 bearing a nonsense mutation and local dystrophin expression with functional improvement of the targeted muscles[8]. But effective treatment relies on restoration and maintenance of dystrophin expression in most, if not all, of the affected muscles throughout the body, and although 2OMeAONs delivered systemically have been shown to induce body-wide expression of dystrophin, the levels were far too low to be of therapeutic value[9].

Among the alternative chemistries to substitute for classic oligonucleotides[10], morpholino phosphorodiamidate oligonucleotides (morpholinos), in which the phosphodiester bond is replaced by phosphoroamidate linkage and the ribose replaced by a morpholino moiety seem most promising, with higher affinity for their target nucleic acid sequences and greater resistance to degradation than conventional nucleic acids[11]. Their nonionic nature, however, seems to impede their delivery into cells[12], prompting use of 'scrape-loading' or complexing morpholinos with negatively charged complementary DNA sequences (called leashes) and lipids to enhance delivery[13,14]. We therefore first compared the antisense effect of the morpholino alone or complexed with a previously reported leash and Lipofectin[14]. We used the AON sequence M23D+07–18, specific for the removal of exon 23 of the mouse dystrophin gene (*Dmd*)[14] (**Supplementary Fig. 1** online). In both cases, we injected 2 μg of morpholino intramuscularly into tibialis anterior muscles of 6-week-old dystrophic *mdx* mice. We detected similar numbers of dystrophin-positive fibers (up to 250) and amounts of dystrophin protein, about 10% of normal levels, with both formulations (**Supplementary Fig. 2** online). But larger amounts of morpholino complex induced noticeable tissue damage without significant increase in dystrophin-positive fibers, whereas morpholino alone elicited a dose-dependent increase in the number of dystrophin-positive fibers. Ten micrograms of morpholino induced strong dystrophin expression in $>70\%$ of fibers in injected tibialis anterior muscles with up to 60% of normal dystrophin levels (**Supplementary Fig. 2** online). Equivalent efficiencies of dystrophin induction were also achieved in young (3-week-old) and older (6-month-old) *mdx* mice (**Supplementary Fig. 2** online). We therefore used uncomplexed morpholinos in all subsequent experiments.

To examine the systemic route of delivery, we injected 2 mg morpholino intravenously into 6-week-old *mdx* mice. Two weeks after single injection, *Dmd* mRNA of a size expected for deletion of exon 23 was detected by nested RT-PCR in all skeletal muscles, and immunostaining showed dystrophin in skeletal muscles body-wide, with the strongest signals and largest numbers of positive fibers in diaphragm, intercostal and abdominal muscles (**Fig. 1a**). Up to 5% of normal levels of dystrophin protein was detected in intercostal and quadriceps muscles (**Fig. 1b**), but none in cardiac muscles.

Three consecutive weekly intravenous injections produced considerably more dystrophin-positive fibers in all skeletal muscles together with transcripts of a size expected for deletion of exon 23, reaching 30% of the normal transcript product in the quadriceps, abdominal and intercostal muscles, and lower levels in tibialis anterior and

[1]Muscle Cell Biology, MRC Clinical Science Centre, Hammersmith Hospital, Du Cane Road, London, W12 0NN, UK. [2]School of Life Science, University of Hertfordshire, College Lane, Hatfield, Herts, AL10 9AB, UK. [3]Muscular Dystrophy Laboratory, Neuromuscular/ALS Center, Carolinas Medical Center, 1000 Blythe Boulevard, Charlotte, North Carolina 28231, USA. [4]Australian Neuromuscular Research Institute, Centre for Neuromuscular and Neurological Disorders, QEII Medical Centre University of Western Australia, Nedlands 6009, Western Australia. Correspondence should be addressed to Q.L.L.(qi.lu@carolinashealthcare.org).

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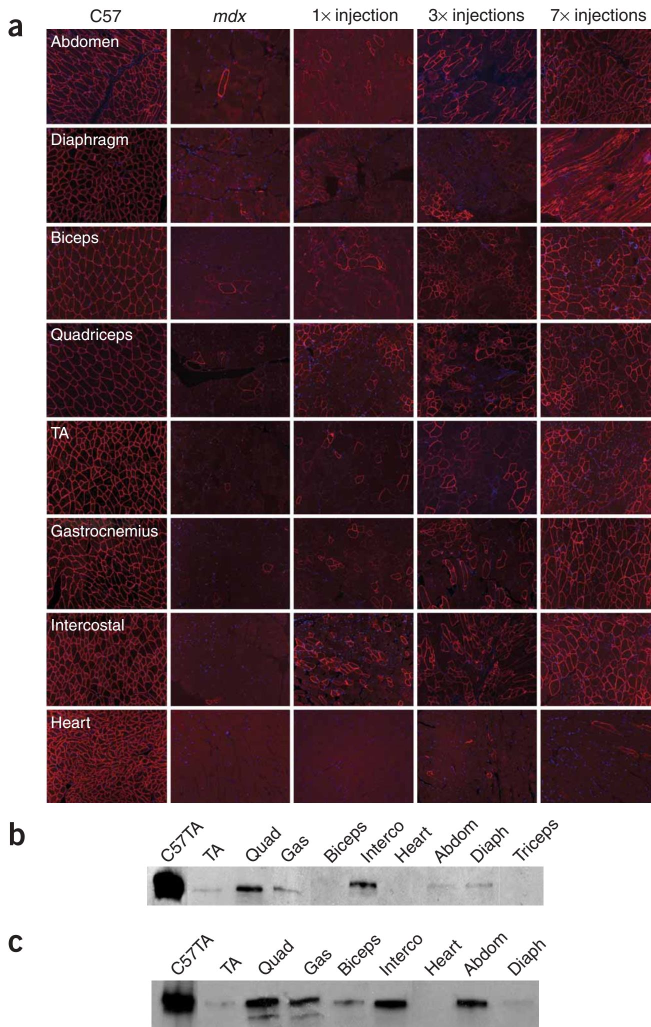

Figure 1 Induction of dystrophin expression in muscles from *mdx* mice with intravenous injection of morpholino AONs. (**a**) Immunohistochemistry. C57 (left column), muscles from *C57Bl/10* normal controls; *mdx*, muscles from control *mdx* mice; muscles from *mdx* mouse after one (1× injection), three (3× injections) and seven intravenous injections (7× injections) of the morpholinos at weekly intervals. Muscles were examined 2 weeks after the last injection. Sections were stained with rabbit polyclonal antibody P7 to dystrophin[9] (original magnification, ×200). (**b**,**c**) Western blots for dystrophin expression in muscles 2 weeks after single (**b**) or three weekly (**c**) intravenous injections of the morpholino AONs. Abdomen, abdominal muscles; TA, tibialis anterior; Quad, quadriceps; Gas, gastrocnemius; Interco, intercostal muscle; Diaph, diaphragm. C57TA, tibialis anterior muscle from *C57Bl/10* mouse as a positive control. Equal loading of 5 μg protein from each sample. Mice were injected initially at 6 weeks of age. We used M23D(+07-18) (5′-GGCCAAACCTCGGCTTACCTGAAAT-3′) against the boundary sequences of exon and intron 23 of the dystrophin gene and the sense oligonucleotide (5′-AGGTAAGCCGAGGTTTGGCC-3′) as a control. We dissolved 2 mg morpholino in 200 μl saline and injected it into the tail vein for each injection. Muscles were removed and snap-frozen in liquid nitrogen–cooled isopentane and stored at −80 °C. Immunohistochemistry and western blots were performed as described previously[9]. The positive bands with size of full-length dystrophin were scanned and intensities of bands in each lane were measured with US National Institutes of Health imaging software (Gel blotting macros). The intensity of the band from *C57Bl/10* muscle samples was taken as a reference of 100% for samples from *mdx* muscles[9]. $n = 4$–6 per group.

diaphragm, but remaining undetectable in the heart. We detected dystrophin by immunostaining in 25% of fibers in most muscles examined, including intercostal and abdominal muscles (**Fig. 1a**), and by western blots, at >20% of normal levels in quadriceps, intercostal and abdominal muscles (**Fig. 1c**), but at only about 5% in the diaphragm. Levels of dystrophin expression varied considerably both between and within muscles (**Fig. 1a**). A large proportion of dystrophin-positive fibers in some muscles, including biceps and quadriceps, were of small caliber, suggesting that they were newly regenerated.

Because previous results of AON-induced dystrophin expression put its half-life at less than 2 months[8], we extended our regime to seven weekly intravenous injections of morpholino to achieve the maximum cumulative antisense effect. As expected, numbers of dystrophin-positive fibers in most skeletal muscles, 2 weeks after the seventh injection, were conspicuously larger than in the equivalent muscles after three injections (**Fig. 1a**), most notably in the quadriceps and gastrocnemii, where >70% fibers were dystrophin positive (**Figs. 1a** and **2a**,**b**). Moreover, both the spatial distribution and the range of signal intensities were more uniform than after three injections, although some area-to-area variation remained, most conspicuously in the diaphragm. Western blots also showed, in nearly all skeletal muscles, higher levels of dystrophin protein than after three injections: up to 50% of normal levels in gastrocnemii and quadriceps (**Fig. 2b**), and up to 10–20% of normal levels in tibialis anterior, intercostal, abdominal and triceps muscles (data not shown). RT-PCR further confirmed the presence of high levels of transcript lacking exon 23, with intensities equal to or higher than those of the normal transcripts in gastrocnemii, quadriceps and intercostal muscles (**Supplementary Fig. 3** online) accompanied by a minor PCR product consistent with loss of both exons 22 and 23, as confirmed by sequencing (**Supplementary Fig. 3** online). As we found previously with 2OMeAONs[9], no dystrophin protein or truncated *Dmd* mRNA was detected in the heart. It may be that antisense delivery to cardiac muscle is less effective than delivery to dystrophic skeletal muscles where degeneration and regeneration, which are associated with vascular leakiness, are much more conspicuous than in cardiac muscle of the dystrophic mouse.

Morpholino-induced expression of dystrophin produced several functional benefits. First, almost all fibers expressing dystrophin showed membrane localization of α-sarcoglycan, β-dystroglycan and neuronal nitric oxide synthase, with staining intensities correlated with that for dystrophin. Second, physiological examination of tibialis anterior muscles 2 weeks after three weekly intravenous injections of the AONs showed a significantly higher normalized maximum isometric tetanic force in all tibialis anterior muscles from the AON-treated mice than that of tibialis anterior muscles from control *mdx* mice, although still less than that of muscles from normal *C57Bl/10* mice (**Fig. 2c**,**d**). This is particularly heartening because the morpholinos induced <20% normal dystrophin levels in tibialis anterior muscles, suggesting that low levels of dystrophin can have a crucial physiological impact in some muscles. Histologically, dystrophin-positive fibers in all muscles except diaphragm showed more uniform and larger diameters after seven intravenous injections than after three intravenous injections of morpholino, suggesting some protection against degeneration in this population (**Fig. 1a**). This result was strengthened by the distinctly lower percentage of centrally nucleated fibers in gastrocnemii, quadriceps, tibialis anterior and abdominal muscles of the morpholino-treated *mdx* mice than in corresponding muscles of age-matched control *mdx* mice (**Supplementary Fig. 4** online). Strong evidence of a widespread protective effect of dystrophin on myofiber integrity was the significantly lower serum creatine phosphokinase levels observed in morpholino-treated *mdx* mice than

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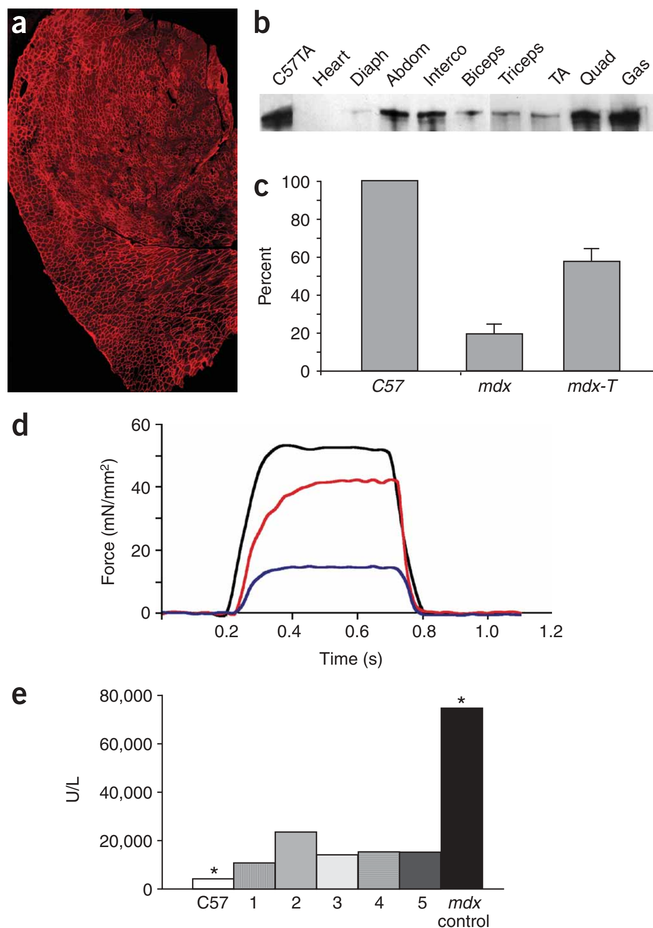

Figure 2 Immunohistochemistry and western blot analysis showing induction of dystrophin expression. Muscles were examined 2 weeks after seven intravenous injections of the morpholinos at weekly intervals. (**a**) Cross-section of the quadriceps showing expression of dystrophin in >80% of fibers in the entire cross-sectional area of the muscle (original magnification, ×50). Antibody P7 to dystrophin was used. (**b**) Levels of dystrophin expression in the treated *mdx* mouse reached >50% of normal levels in gastocnemius and quadriceps. Monoclonal antibody DYS1 to dystrophin (NovoCastra) was used. C57TA, tibialis anterior from *C57Bl/10* mice; Diaph, diaphragm; Abdom, abdominal; TA, tibialis anterior; Quad, quadriceps; Gas, gastrocnemius. (**c**,**d**) Physiological analysis of tibialis anterior muscles from *C57Bl/10,* control *mdx* mice (*mdx*) and *mdx* mice treated with the morpholino AONs (three weekly intravenous injections, *mdx–T*). (**c**) Normalized maximum tetanic force expressed as a percentage of *C57Bl/10* control. Means ± s.e.m. Student *t*-test; $n = 3$ mice. (*$P = 0.03$). (**d**) Graphic representation of normalized tetanic force in tibialis anterior muscles. Morpholino-treated *mdx* muscle (red line) and saline-injected control (blue line) compared with age-matched *C57Bl/10* (black line). The distal tendon of the tibialis anterior muscle was dissected from the bone and the force was measured when the proximal tendon remained attached to the bone[8] (**Supplementary Methods** online). (**e**) Significant drop in serum creatine kinase activity in *mdx* mice treated with the morpholino AONs (seven intravenous injections, mouse numbers 1–5) when compared to control *mdx* mice. *Means of 3 mice for both *C57Bl/10* (C57) and control *mdx* mice. (ANOVA; $P = 0.01$). Creatine phosphokinase activity in serum was assayed with the Randox CK 110 Kit (Randox Laboratories Ltd) according to the manufacturers' instructions[9] (**Supplementary Methods** online). $n = 4–6$ for each treated group.

in control *mdx* mice, although still distinctly higher than those in normal *C57Bl/10* mice (**Fig. 2e**).

We did not observe any overt histological signs of tissue damage in the lung, liver and kidney from the morpholino-treated *mdx* mice. No general sign of ill health and no deaths were noted during the period of the experiment.

Our results show that morpholino-mediated AON therapy can achieve and maintain therapeutic levels of dystrophin throughout the body musculature and may provide a realistic option for the treatment of the majority of DMD. It possesses a number of immediate advantages over the main alternative approach using recombinant U7 plasmids in adeno-associated virus–based vectors for exon skipping in the dystrophin gene[15]. The viral strategy provides highly effective and long-term exon skipping, but this robustness, together with the need for high-pressure intravascular delivery and the immune problems associated with viral proteins, raises a number of practical hurdles. With morpholino AONs, the limited duration of effect, generally regarded as problematic, is mitigated by the simple and safe delivery protocol and the lack of immunological problems. The impermanence itself greatly facilitates the conduct of clinical trials because it provides the flexibility to terminate, to change dose or to switch to new regimes using more potent chemistries or target sequences. Moreover, AONs are more easily manufactured on a large scale to high quality for clinical trials. Our studies also show some remaining obstacles to optimum effectiveness of AON treatment, principally the achievement of homogeneous restoration of dystrophin expression in muscles and the failure to induce dystrophin in the heart.

Experiments on mice were done under animal licence 70/5177, Great Britain, and approved by Institutional Animal Care and Use Committee, protocol No. 01-05-02A, Carolinas Medical Center, Charlotte, North Carolina, USA.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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