Journal Pre-proof

Optimized lentiviral vector for restoration of full-length dystrophin via a cell-mediated approach in a mouse model of Duchenne muscular dystrophy

Jinhong Meng, Marc Moore, John Counsell, Francesco Muntoni, Linda Popplewell, Jennifer Morgan

PII: S2329-0501(22)00063-8

DOI: https://doi.org/10.1016/j.omtm.2022.04.015

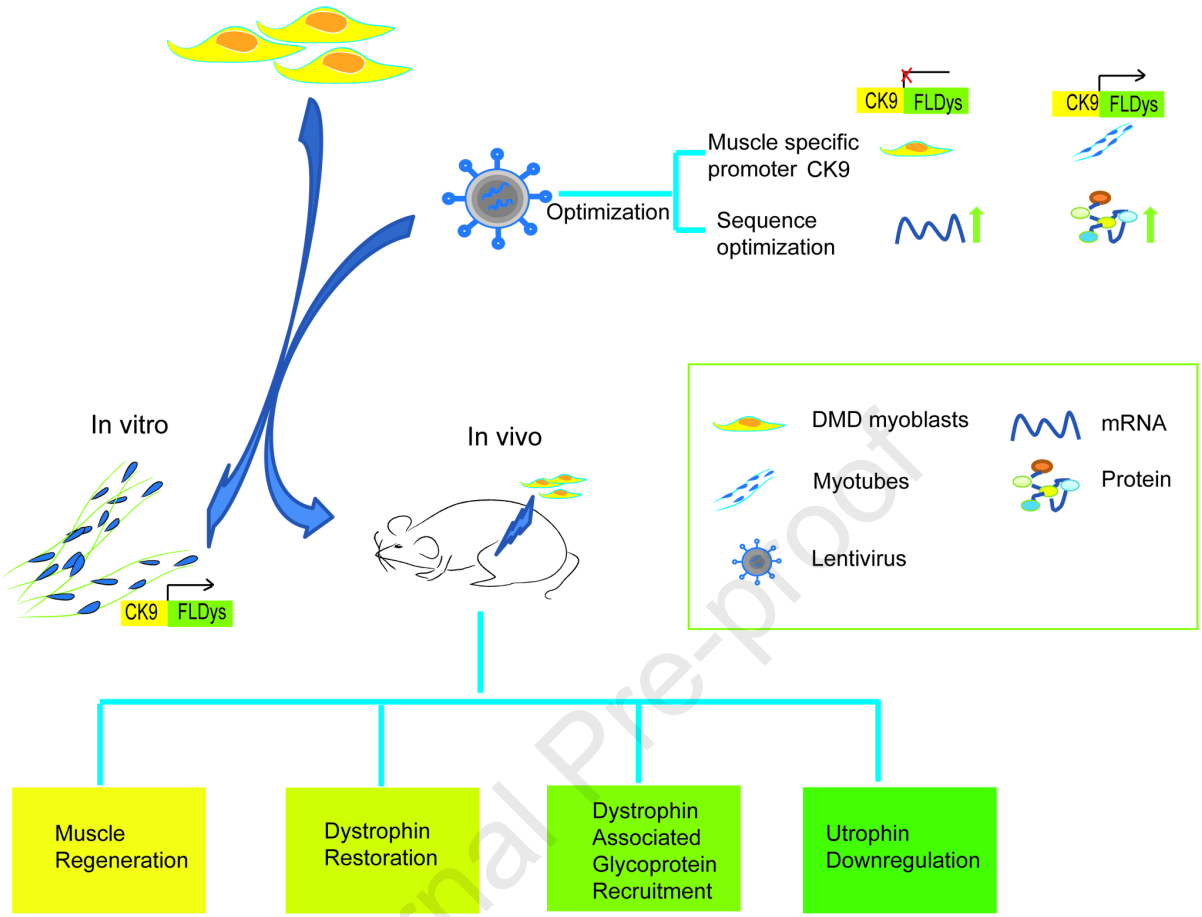
Reference: OMTM 871

To appear in: *Molecular Therapy: Methods & Clinical Development*

Received Date: 12 July 2021

Accepted Date: 28 April 2022

Please cite this article as: Meng J, Moore M, Counsell J, Muntoni F, Popplewell L, Morgan J, Optimized lentiviral vector for restoration of full-length dystrophin via a cell-mediated approach in a mouse model of Duchenne muscular dystrophy, *Molecular Therapy: Methods & Clinical Development* (2022), doi: https://doi.org/10.1016/j.omtm.2022.04.015.



Optimized lentiviral vector for restoration of full-length dystrophin via a cell-mediated approach in a mouse model of Duchenne muscular dystrophy

Jinhong Meng[1,4], Marc Moore[1,2,4], John Counsell[1,3,4], Francesco Muntoni[1,4], Linda Popplewell[2], Jennifer Morgan[1,4]*.

1. Dubowitz Neuromuscular Centre, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London WC1N 1EH
2. Department of Biological Sciences, School of Life Sciences and the Environment, Royal Holloway University of London, Egham Hill, Egham, TW20 0EX
3. UCL Division of Surgery and Interventional Science, Charles Bell House, 43-45 Foley Street, London, W1W 7TY
4. National Institute for Health Research, Great Ormond Street Institute of Child Health Biomedical Research Centre, University College London, London WC1N 1EH

*Correspondence should be addressed to J.E.M (jennifer.morgan@ucl.ac.uk)

Dubowitz Neuromuscular Centre, Molecular Neurosciences Section, Developmental Neuroscience Programme, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London WC1N 1EH

Email: jennifer.morgan@ucl.ac.uk

Telephone: 00442079052874

Fax: 00442079052832

Short title

Restoration of full-length dystrophin *in vivo*

Abstract

Duchenne muscular dystrophy (DMD) is a muscle wasting disorder caused by mutations in the *DMD* gene. Restoration of full-length dystrophin protein in skeletal muscle would have therapeutic benefit, but lentivirally-mediated delivery of such a large gene *in vivo* has been hindered by lack of tissue-specificity, limited transduction and insufficient transgene expression. To address these problems, we developed a lentiviral vector, which contained a muscle-specific promoter and sequence optimized full-length dystrophin, to constrain the dystrophin expression to differentiated myotubes/myofibres and enhance the transgene expression. We further explored the efficiency of restoration of full-length dystrophin *in vivo*, by grafting DMD myoblasts that had been corrected by this optimized lentiviral vector intramuscularly into an immunodeficient DMD mouse model. We showed that these lentivirally-corrected DMD myoblasts effectively reconstituted full-length dystrophin expression in 93.58±2.17% of the myotubes *in vitro.* Moreover, dystrophin was restored in 64.4±2.87% of the donor-derived regenerated muscle fibres *in vivo*, which was able to recruit members of the dystrophin glycoprotein complex at the sarcolemma. This study represents a significant advance over existing cell-mediated gene therapy strategies for DMD that aim to restore full-length dystrophin expression in skeletal muscle.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder caused by mutations within the *DMD* gene, leading to progressive muscle fibre necrosis and muscle wasting and weakness.[1, 2] Restoration of dystrophin protein in the affected muscles is the main therapeutic strategy for DMD. Adeno-associated viral (AAV) vectors coding mini- or micro- dystrophins are showing promising therapeutic effects in DMD clinical trials [3, 4]. However, these vectors are unable to deliver the full-length *DMD* cDNA whose length of 11Kb is far beyond the 5Kb packaging capacity of AAVs; only a tri-AAV vector system can deliver full length dystrophin, albeit at low efficiency.[5, 6] Although viral vectors with high packaging capacity, such as adenovirus,[7-9] herpes simplex virus[10], foamy virus[11, 12] or lentivirus[13] can accommodate the full-length *DMD* cDNA, the direct delivery of such vectors to skeletal muscles is challenging, as their bio-production scalability and myotropism remain suboptimal. Alternatively, a cell-mediated strategy can be explored to deliver the full-length dystrophin in DMD animal models.

Stem cell therapy is a potential treatment for DMD, as transplanted cells contribute to muscle regeneration and functionally reconstitute the muscle stem cell pool,[14, 15] following their intra-muscular injection in mouse models. But systemic delivery of stem cells to skeletal muscle remains challenging, due to the large number of cells required and inefficient targeting of skeletal muscle following intra-arterial or intravenous delivery. It has been suggested by patient groups[16] that preserving or improving the function of hand muscles of older DMD patients would be immensely beneficial for their quality of life. The thenar muscles of the hand control the fine movements of the thumb, including gripping and would be key muscles that would benefit from dystrophin restoration. Although satellite cell-derived myoblasts are not systemically deliverable[17] and have limited diffusion after local delivery,[18-21] they can still be considered to treat key muscles such as thenar muscles of DMD patients

via intramuscular injection. Autologous stem cells genetically-modified to express full-length dystrophin[12, 13, 22] are preferable to allogeneic cells, as they are less likely to be rejected.[23] We have previously shown that the full-length *DMD* cDNA can be packaged into a lentiviral vector[13] and produce full-length dystrophin in myotubes differentiated from transduced myoblasts. However, the strategy requires further optimization and preclinical validation before progressing to clinical application.

In normal skeletal muscle, dystrophin is expressed in activated satellite cells[24] and differentiated myofibres, but not in proliferating myoblasts.[25] We have previously reported that expression of mini-dystrophin in DMD muscle stem cells can adversely affect their proliferation and myogenic differentiation *in vitro.*[26] Therefore, it would be advantageous to use a muscle specific promoter that drives transgene expression only in differentiated myotubes/myofibres and is small enough to fit into the lentiviral vector together with the large full-length *DMD* human cDNA.

The level of transgene expression is a key issue; many factors affect the transcription and translation of transgenes and these can play an important role in delivering an effective therapy. In order to elicit functional benefit[27-29] within the treated muscle, restored dystrophin protein level has to reach between 5-30% of normal dystrophin levels; it is better to have a lower level of dystrophin in the majority of fibres than a high level of dystrophin in a few fibres.[29-33] In an effort to enhance expression, the full length *DMD* cDNA was subject to multi-parametric sequence optimisation, in which native sequence was modified with focus upon GC content, codon optimisation,[34, 35] mRNA transcription and stability and protein translation. Sequence optimisation of this nature has been used in the engineering of micro-[36] and mini- dystrophin transgenes and has been successfully exploited in both a large animal model[37] and clinical trials (NCT03375164 and GNT0004).[38]

A lentiviral vector, containing a muscle specific promoter and sequence optimized full-length *DMD* transgene, could constitute an effective cell-mediated gene therapy to treat all DMD patients, regardless of their *DMD* mutation.

Results

The CK9 promoter drives transgene expression predominantly in differentiated myotubes

We transduced DMD myoblasts carrying an out-of-frame deletion of exon 52 (del Ex52) with lentiviruses expressing EGFP driven by a panel of promoters, to identify the optimal candidate for use in a lentiviral gene therapy context. In cells transduced with viral vectors containing enhanced synthesized promoter (ESyn) (654bp)[39] or creatine kinase promoter 9 (CK9) (429bp),[40] there was little, if any, EGFP expression prior to myogenic differentiation and EGFP was strongly expressed in differentiated myotubes (Figure 1A and B). In contrast, the majority of cells that were transduced with vectors driven by phosphoglycerate kinase (PGK) or spleen focus forming virus (SFFV) promoter had strong EGFP expression both pre- and post- myogenic differentiation (Figure 1A, a-e). These findings were confirmed by western blot (Figure 1B, Supplementary Figure S1, A-C). Thus we chose the CK9 promoter, the smaller of the two promoters which have increased expression after myogenic differentiation, for use in our modified lentiviral vector.

Modification of the lentiviral vector by utilizing a muscle specific CK9 promoter

In order to make the lentiviral vector coding for full-length dystrophin more clinically compatible and to limit transgene expression to muscle, we modified the original full-length dystrophin vector[13] by removing the EGFP cassette from the reading frame and replacing the SFFV with the CK9 promoter (Figure 2A), to produce the lentivirus, LV-CK9-native full-length dystrophin (nFLDys; lentivirus in which expression of full length dystrophin is driven by the CK9 promoter). *In vitro* assays of DMD myoblasts which were transduced with different MOIs of this lentivirus showed that increasing the amount of lentivirus (MOIs>20) may have had an adverse effect on cell proliferation (supplementary Figure S2), thus cells transduced with lower MOIs (0, 0.5, 5 or 10) were subsequently expanded and induced to

undergo myogenic differentiation, to evaluate the transgene expression. We found that dystrophin was present in myotubes in all the transduced groups. While there were only a few dystrophin positive myotubes in MOI 0.5 transduced cells, the majority of the myotubes in MOI 5 or 10 transduced groups contained dystrophin (Figure 2B). These results demonstrate that the titre of the lentivirus was high enough to produce dystrophin in the majority of the myotubes derived from the transduced cells (at MOI >5), without having to undergo an extra step of selection and enrichment after the transduction.

Next, DMD myoblasts which were transduced with LV-CK9-nFLDys at MOI 5 were transplanted into cryoinjured muscles of mdx nude mice[41] to evaluate their contribution to muscle regeneration and dystrophin restoration *in vivo*. Donor fibres (human lamin AC+/human spectrin+) were present in muscles 4 weeks after transplantation. However, only 44.3±4.31% donor-derived muscle fibres expressed dystrophin and this was at low levels (Figure 2C), indicating that further improvement of the lentiviral vector for better *in vivo* efficacy is necessary.

Sequence optimization to improve the full-length dystrophin expression *in vitro*

To investigate whether the expression of dystrophin could be improved by sequence optimization, we produced lentiviruses encoding either sequence optimized full-length dystrophin (soFLDys) or nFLDys, both driven by the CK9 promoter (Figure 3A) and transduced DMD myoblasts (del Ex52) at MOI 5. The viral copy numbers/cell in transduced cell population was determined by qPCR which showed 5.53±0.12 copies for DMD-nFLDys and 4.97±0.13 copies for DMD-soFLDys (supplementary Figure S9). After being induced to undergo myogenic differentiation, the fusion indices were 35.38±2.68% (DMD-NT), 37.65±2.74% (DMD-nFLDys) and 36.47±2.47% (DMD-soFLDys), respectively, with no statistically significant differences among these 3 groups ($p>0.05$, one-way ANOVA),

147 suggesting that the lentiviral transduction and the expression of dystrophin post-
148 differentiation had no adverse effect on the extent of differentiation (Figure 3B, C).
149 Next, we compared the full-length dystrophin expression at the transcriptional level, to
150 determine if the sequence optimization would improve the transgene expression at the mRNA
151 level. We performed qRT-PCR analysis of mRNA extracted from myotubes derived from
152 DMD myoblasts (del Ex52) transduced with LV-DMD-nFLDys and LV-DMD-soFLDys,
153 using primers specifically designed to recognize the common sequence of both nFLDys and
154 soFLDys, but not the *DMD* transcript produced by the non-transduced DMD myoblasts (del
155 Ex52), which lacks exon 52 (supplementary Figure S3). There was significantly higher
156 ($p=0.0286$, student t-test) *DMD* mRNA expression in cells transduced with LV-soFLDys than
157 LV-nFLDys (Figure 3E), suggesting that sequence optimization improved the full-length
158 dystrophin expression at transcriptional level.
159 Next, we investigated the expression of dystrophin in myotubes derived from transduced
160 myoblasts. Immunostaining of dystrophin and MF20 (an antibody recognizes myosin heavy
161 chain) showed that 88.58±1.96% and 93.58±2.17% myotubes from DMD-nFLDys and
162 DMD-soFLDys groups are positive for dystrophin, distributed in a punctate pattern along the
163 myotubes (Figure 3B, 3D). In myotubes *in vitro*, dystrophin expression is sometimes
164 punctate, especially when the dystrophin transgene is delivered to muscle precursor cells via
165 viral vectors[42]. This may be due to an uneven distribution of the dystrophin protein in
166 differentiated myotubes, which are formed by fusion of transduced and non-transduced cells.
167 Western blot showed that the 427KD full-length dystrophin protein was present in normal,
168 DMD-nFLDys and DMD-soFLDys groups, but was absent, as expected, in the DMD-NT
169 group (Figure 3F, 3H and 3I). The extent of myogenic differentiation was similar in all
170 groups, as indicated by the amount of MF20 expression in each group (Figure 3F, 3G). There
171 were significantly higher amounts (around 6 fold higher) of full-length dystrophin expressed

in DMD-soFLDys cells than DMD-nFLDys cells ($p<0.05$, one-way ANOVA), when normalized to either α-actinin or MF20 (Table 1). There were around 40% and 240% of normal levels of dystrophin protein in DMD-nFLDys and DMD-soFLDys groups, respectively.

In summary, our results show that DMD myoblasts transduced with LV-CK9-soFLDys resulted in higher dystrophin expression in myotubes at both mRNA level (3.5 folds) and protein level (around 6 fold) than the same cells transduced with same amount of LV-CK9-nFLDys, indicating sequence optimization of the full-length dystrophin increases the *in vivo* restoration of the protein.

Sequence optimization of the full-length dystrophin improves dystrophin restoration *in vivo*

We then investigated the contribution of the transduced cells to muscle regeneration and whether the sequence optimization could increase the amount of restored full-length dystrophin *in vivo*.

Dystrophin restoration in regenerated muscle fibres is greater in DMD-soFLDys transplanted muscles

First, we compared the transplantation efficiency within muscles that were transplanted with non-transduced, LV-CK9-nFLDys (MOI 5) or LV-CK9-soFLDys (MOI 5) transduced DMD myoblasts. There were no statistically significant differences in the number of either cells or myofibres of donor origin (number of human lamin AC+ nuclei, human spectrin+ fibres, or human spectrin+/human lamin AC+ fibres) between these groups (One-way ANOVA, $p>0.05$) (Table 2 and Figure 4A-C), indicating that the lentiviral transduction did not alter the engraftment capacity of the DMD myoblasts *in vivo*.

Next, we investigated the restoration of the full-length dystrophin in muscles that were transplanted with different cells. As expected, in muscle sections of the DMD-NT group,

197 there were no human dystrophin fibres (Figure 4B). There were significantly more ($p<0.05$, t-
198 test) human dystrophin+/hSpectrin+ fibres in muscles transplanted with DMD-soFLDys than
199 DMD-nFLDys cells, which represents a significantly higher percentage ($p=0.0087$, student t-
200 test) of dystrophin-expressing donor fibres in the DMD-soFLDys group than in the DMD-
201 nFLDys group (Table 3 and Figure 4B). There was a significantly stronger expression
202 (relative intensity) of human dystrophin in normal ($p<0.05$) or DMD-soFLDys ($p<0.01$)
203 groups than in the DMD-nFLDys group, and no difference ($p>0.05$) in the human dystrophin
204 intensity between normal and DMD-soFLDys groups (one-way ANOVA) (Table 3 and
205 Figure 4C, D and E).
206 Our data show that, in comparison to DMD-nFLDys myoblasts, DMD-soFLDys myoblasts
207 not only gave rise to a higher percentage of donor fibres which expressed dystrophin, but also
208 to approximately 2.8 times stronger dystrophin expression in these donor fibres, after their
209 transplantation into cryodamaged TA muscles of mdx nude mice (Table 3, Figure 4C and D).
210 ***Higher level of α-sarcoglycan was recruited to the sarcolemma of dystrophin+ fibres in***
211 ***DMD-soFLDys myoblast transplanted muscles***
212 In DMD muscle, lack of dystrophin leads to loss of components of the dystrophin
213 glycoprotein complex (DGC) in muscle fibres,[43-45] resulting in secondary pathological
214 changes of the muscle.[46-48] To achieve a better therapeutic outcome, both dystrophin and the
215 DGC are required to be restored at the sarcolemma.[49] In addition, recruitment of members of
216 the DGC also serves as a functional readout for the restored dystrophin isoform within treated
217 fibres.[50-53]
218 We investigated the recruitment of the DGC protein α-sarcoglycan (α-SG) in donor derived
219 dystrophin-expressing muscle fibres, by co-immunostaining with Mandys 106 and α-SG on
220 muscle sections of DMD-nFLDys, DMD-soFLDys or normal myoblast transplanted groups.

In the DMD-nFLDys group, the expression of α-SG was not increased in human dystrophin+ fibres (Figure 5A and B), and there was no statistically significant difference (paired t-test, $p>0.05$) in the intensity of α-SG between human dystrophin (Mandys106)+ (113.9± 7.46) and human dystrophin- fibres (106.8 ± 4.24) within the same section. In contrast, in the DMD-soFLDys group, the expression of α-SG was significantly higher ($p<0.001$, paired t-test) in human dystrophin+ fibres (110.8± 17.73) than in human dystrophin- fibres (78.24± 15.12) (Figure 5 A and C), similar to that of the normal group, where the relative intensity of α-SG was also significantly higher ($p=0.0038$, paired t-test) in human dystrophin+ fibres (128.3± 10.91) than in human dystrophin- fibres (84.7± 4.04) (Figure 5D).

The fold change of α-SG expression in human dystrophin+ fibres versus human dystrophin- fibres in the DMD-nFLDys group (1.067± 0.059) was significantly lower ($p=0.0062$, one-way ANOVA) than that of DMD-soFLDys group (1.551± 0.1817) and normal group (1.507 ± 0.095) (Figure 5E).

Our data show that, in myofibres derived from DMD-nFLDys myoblasts, the full length dystrophin was not expressed at high enough quantities to restore the α-SG to levels detectable using IHC. The sequence optimized dystrophin vector, however, restored α-SG to levels similar to those found in donor-derived myofibres in muscles transplanted with control (non-DMD) myoblasts. Similar to α-SG, γ-SG in human dystrophin+ fibres were also found in DMD-soFLDys and normal groups, but to a lesser extent in DMD-nFLDys transplanted muscles (supplementary Figure S4).

Utrophin is down-regulated in human dystrophin+ fibres in DMD-soFLDys myoblast transplanted muscles

Utrophin is an autosomal homologue of dystrophin which is upregulated in dystrophin-deficient mouse muscles, partially compensating for the missing dystrophin.[54, 55] The reduced expression of utrophin in mdx myofibres in which dystrophin has been restored is an

indication that the restored dystrophin is functional.[11] In order to determine to what extent the full-length dystrophin delivered via lentivirally-corrected DMD myoblasts could lead to utrophin reduction, we performed double immunostaining of utrophin (with an antibody that recognises both mouse and human utrophin), and human dystrophin in sections of DMD-nFLDys, DMD-soFLDys and normal myoblast transplanted muscles (Figure 6A-F and a'-f') and measured the intensity of the utrophin in human dystrophin+ or human dystrophin- fibres in each section. We found that utrophin expression (relative intensity) was similar in human dystrophin+ (62.68±5.99) and human dystrophin- (62.90±3.38) fibres, in DMD-nFLDys myoblast transplanted muscles (Figure 6G) ($p>0.05$, paired t-test). The ratio of the utrophin intensity in human dystrophin+ versus human dystrophin- fibres of this group is 0.9911± 0.06349 (mean±SEM, n=6), providing evidence that there was no reduction of utrophin in human dystrophin+ fibres, suggesting the dystrophin in this group is not restored at high enough levels to reduce the utrophin expression at the sarcolemma. In contrast, utrophin expression was significantly reduced in human dystrophin+ fibres (91.76±13.35) compared to human dystrophin- (127.8±11.27) fibres in the DMD-soFLDys group (Figure 6H) ($p<0.0001$, paired t-test), which is similar to the normal group (Figure 6I) ($p=0.012$, paired t-test) in which there was also significantly less utrophin in human dystrophin+ fibres (69.01±9.304) than human dystrophin- fibres (105.6±13.13). The ratio of the utrophin intensity in human dystrophin+ versus human dystrophin- fibres in DMD-soFLDys group was 0.6984± 0.04572 (mean±SEM, n=6), which is similar to the normal group 0.6736± 0.06212 (mean±SEM, n=6). This suggests that myoblasts transduced with DMD-soFLDys, but not DMD-nFLDys, could restore sufficient dystrophin at the sarcolemma of the donor-derived fibres to downregulate utrophin expression, to a similar extent as normal myoblasts.

Discussion

The overarching aim of this study was to develop an effective therapeutic strategy to restore fully functional, full-length dystrophin isoform Dp427, in dystrophic skeletal muscles. Ideally, any therapeutic strategy should be applicable to all patients, regardless of which mutation they have in their *DMD* gene. Although gene editing[56-58] can precisely correct a *DMD* mutation[59, 60] and give rise to full-length dystrophin expression,[61] this method is highly mutation-dependent and likely inefficient for full cDNA repair, meaning different designs need to be developed and validated for patients with different *DMD* mutations. In contrast, viral vectors introduce the dystrophin coding sequence into cells and are potentially applicable to the majority of the patients with a wide spectrum of mutations. However, the potential immune response against the full-length dystrophin will still need to be considered, especially for patients with large deletions, or with deletions removing specific protein domains.[62] This may be lessened by the incorporation of a muscle specific promotor to limit dystrophin expression to muscle.

AAV has been used to restore dystrophin *in vivo*,[3, 4] but it only accommodates small, partially functional dystrophin microdystrophin,[63] due to its small packaging capacity.[5, 64] As AAV has a low integration rate *in vivo*,[65] dystrophin expression may diminish over time and as a consequence of cell turnover. Furthermore, despite a plethora of pre-clinical studies to circumvent pre-existing immunity to AAV vectors and to achieve immune toleration permissive of AAV re-administration, utilising approaches such as plasmapheresis,[66] use of alternative and capsid engineered AAV serotypes[67, 68] and modulation of the immune response,[69-71] current AAV based gene therapies are limited to a single administration. In contrast, lentiviral vectors integrate into the host genome, giving sustained transgene expression. They can transduce muscle satellite cells *in vivo*[72, 73] that would provide long-term therapy for muscular dystrophies such as DMD that are characterised by ongoing

myofibre necrosis. Their safety and efficacy has been demonstrated in clinical trials,[74] either *ex vivo*[75-78] or *in vivo*.[79, 80] Also, lentiviral vectors can package full-length dystrophin.[13]

The bottleneck preventing the direct application of lentiviral vectors *in vivo* is the relatively low titre that can be achieved using current systems and the inefficient targeting of skeletal muscles after systemic delivery, due to the relative larger size (~100nm) for lentivirus particles[81, 82] versus ~20 nm for AAV particles,[3, 83] which negatively impacts the dissemination of viral particles through the vasculature. They may, however, be used in a cell-mediated strategy, by transducing autologous stem cells.[42, 84] Use of autologous stem cells should reduce immune rejection. We have shown that it is feasible to remove the GFP cassette from the lentiviral vector and by doing so; the transduced cells do not have to undergo *in vitro* manipulation such as FACS sorting, followed by extra rounds of cell expansion, which would reduce the myogenic capacity of the muscle stem cells and their engraftment efficiency.[85-87]

We did find some discrepancies in the expression of human spectrin and human dystrophin at the sarcolemma of regenerated muscle fibres in transplanted muscles (Supplementary Figure S5). In our model system, donor cells repair segments of host myofibres,[85] giving rise to mosaic fibres, containing myonuclei of both mouse and human origin. The finding of human spectrin, but not human dystrophin, within a myofibre is not surprising, as the grafted cells contained both transduced and non-transduced cells, so some myonuclei of human origin would produce dystrophin and others would not. The presence of human dystrophin, but not human spectrin, may be due to either the different sensitivity of these two human specific antibodies, or possibly because spectrin spreads further from the nucleus than does dystrophin.

When the SFFV promoter was replaced by the CK9 promoter to drive the native form of dystrophin (nFLDys) cDNA in the lentiviral vector, the restored dystrophin was at lower than

normal levels both *in vitro* and *in vivo*. However, when the insert was replaced by a sequence optimized full-length dystrophin (soFLDys), in comparison to nFLDys, there was a 6 fold increase in dystrophin protein expression *in vitro*, and a 2.8 fold improvement in the dystrophin intensity at sarcolemma in donor fibres *in vivo*. This was consistent with a previous report [36] that the sequence optimization of microdystrophin transgenes improves expression of dystrophin in mdx mouse muscles following AAV2/8 gene transfer.

By sequence optimization which largely introduces human codon bias, it was anticipated that the major increase in transgene expression would occur at the translational level. However, we showed that the dystrophin expression driven by soFLDys was improved not only at the translational level, but also at the transcriptional level, as evidenced by proximally 3 fold higher soFLDys transcripts than nFLDys transcripts in transduced cells. This is in line with the previous finding [88] that codon biases are results of genome adaptation to both transcription and translation machineries, and codon biases determine the transcription levels by affecting chromatin structures. Apparently the additional sequence modifications employed in our design of the cDNA also played a role. The GC content of the transgene was increased in order to improve the mRNA stability and subsequently, prolong the transcript half-life. Overall, due to the human codon bias, sequence alterations and increased GC content, an increase at the transcript level was anticipated[88, 89] and observed.

When comparing with normal myoblasts, the amount of dystrophin in myotubes derived from DMD-soFLDys myoblasts was 2.4 fold higher *in vitro*, while the intensity of dystrophin in host muscles that had been grafted with DMD-soFLDys myoblasts was equivalent to, not higher than, that in host muscles which had been transplanted with normal donor myoblasts (*in vivo*). The discrepancies in the extent of *in vitro* and *in vivo* upregulation of the dystrophin transgene require further investigation. There is no information in the literature on the activity of the CK9 promoter in muscles of different fibre types. But interestingly, the CK6 promoter,

346 although it drives dystrophin expression in most skeletal muscles, does not seem to be active
347 in the diaphragm[90] and the MHCK7 promoter is more active in the mouse soleus muscle than
348 the quadriceps and gastrocnemius, as it is more highly expressed in slow (MyHC type I) and
349 fast oxidative (MyHC type IIa) fibers.[91] It should be noted that the mouse TA muscle (the
350 recipient muscle for our cell injections) consists predominantly of type IIa and IIb fibres. A
351 possible drawback in the use of the CK9 promoter is that it does not drive dystrophin
352 expression prior to myogenic differentiation, which may compromise the function of donor-
353 derived satellite cells[25, 92] and limit long-term effectiveness of cell-mediated gene therapy.
354 The desmin promoter has been shown to be superior to mouse muscle creatine kinase or
355 human cytomegalovirus promoters when used in a lentivirus to drive EGFP expression in
356 mouse myoblasts or mouse muscle[93] and is also expressed in non-differentiated myoblasts.[94]
357 But its size (1.7Kb) presents additional challenges for packaging into a lentiviral vector that
358 also contains full-length dystrophin.
359 The level of the DGC proteins α-SG and γ-SG, as well as the utrophin expression in the
360 transduced myotubes, further confirm the advantages of sequence optimization of the full-
361 length dystrophin. Without optimization, the α-SG, γ-SG and utrophin levels were not
362 changed in donor-derived muscle fibres, most likely due to the insufficient restoration of
363 dystrophin at the sarcolemma, while in muscles transplanted with DMD-soFLDys, the α-SG
364 intensity was 1.5 fold higher in donor fibres, similar to the muscle group that was
365 transplanted with normal myoblasts, suggesting the effective recruitment of DGC proteins in
366 donor fibres corrected by sequence optimized lentivirus. Similarly, utrophin expression was
367 not changed in DMD-nFLDys transplanted muscles, while its expression was significantly
368 reduced on donor fibres derived from DMD-soFLDys and normal myoblasts.
369 We have used myoblasts rather than induced pluripotent cell (iPSC)-derived myogenic cells
370 as the donor cell in these experiments, as it has been previously shown that human satellite

371 cells or satellite cell -derived myoblasts transplanted into mouse muscle contribute to satellite
372 cells, as well as to regenerated muscle fibres.[95-104] Although there has been no direct
373 comparison of the engraftment efficiency of human iPSC-derived myogenic cells and human
374 myoblasts following their transplantation into the same mouse model, myoblasts give rise to
375 similar numbers of myofibres of donor origin (up to150) as do transplanted human iPSC-
376 derived myogenic cells,[105-108] after their injection into mouse muscle. This number of
377 myofibres expressing dystrophin would not be sufficient to give any functional benefit to the
378 transplanted muscle; for this, the host environment, cells or the transplantation method
379 would have to be optimised to give at least 5% of dystrophin throughout the majority of the
380 myofibres in the treated muscle.[27-33]
381 The number of myoblasts that we transplanted into each muscle (5×10^5) may limit the
382 number of myofibres of donor origin, but this number of cells in a final volume of 5-10
383 microlitres is the most that can be injected into a mouse tibialis anterior muscle. There is
384 evidence (from studies in mouse and monkey) that the number of transplanted myoblasts
385 does affect the number of regenerated muscle fibres to which they contribute [19, 109], but the
386 volume of cells that can be injected into muscle is a limitation.
387 In summary, our work demonstrates the efficacy of a novel lentiviral vector to restore full-
388 length dystrophin *in vivo*, mediated by autologous muscle stem cells. Such a strategy takes
389 advantage of autologous stem cells and lentiviral vector containing a tissue specific promoter
390 and soFLDys, which can be readily progressed to clinical application to treat key muscles of
391 DMD boys by intramuscular transplantation of autologous cells. Our strategy could also be
392 used as a supplementary to other treatment options, such as exon-skipping and AAV-
393 mediated gene therapy, to provide longer term protection of muscle fibres. Future work
394 should focus on comparing other promoters and optimized dystrophin sequences.
395

Materials and Methods

Ethics

The work was performed under the NHS National Research Ethics: Setting up of a rare diseases biological samples bank (biobank) for research to facilitate pharmacological, gene and cell therapy trials in neuromuscular disorders (REC reference number: 06/Q0406/33), and the use of cells as a model system to study pathogenesis and therapeutic strategies for Neuromuscular Disorders (REC reference 13/LO/1826).

Mice were bred and experimental procedures were carried out in the Biological Services Unit, University College London Great Ormond Street Institute of Child Health, in accordance with the Animals (Scientific Procedures) Act 1986. Experiments were performed under Home Office licence numbers 70/8389 and 2611161. Experiments were approved by the local University College London ethical committee prior to the licence being granted.

Maintenance and differentiation of human myogenic cell preparations

Three human myogenic cell preparations were used in this study. DMD myoblasts (delEx52)[11, 13] and normal myoblasts derived from a healthy donor were maintained on collagen I (1x, Sigma, Dorset, UK)-coated culture vessels in M10 medium, consisting of Megacell DMEM medium (Sigma, Dorset, UK) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher, Paisley, UK), 2mM glutamine (Thermo Fisher, Paisley, UK) and 5ng/ml bFGF (Peprotech, London, UK). Cells were kept at low density ($2.5x10^5$ cells/T75 flask) and split every 3-4 days. For myogenic differentiation, cells were seeded onto 0.1mg/ml Matrigel (vWR, Lutterworth, UK)-coated 4-well plates (Nunc, for immunostaining of myosin heavy chain and dystrophin) or 6-well plates (for western blot sample collection) at a density of $5x10^4$ cells/cm^2 in proliferation medium. Medium was changed into differentiation medium (M2, Megacell DMEM containing 2% FBS) 24 hours later, to initiate myogenic differentiation. 7 days after the onset of differentiation, cells in 4-well plates were

fixed with 4% paraformaldehyde for 15 min at room temperature and proceed for immunostaining. Cells in 6-well plates were used for protein sample collection for western blot analysis as described below.

Lentiviral transfer plasmids and viral production

EGFP expressing lentiviral vectors driven by either muscle specific promoters (Esyn[39] or CK9[40]) or ubiquitous promoters (SFFV or PGK) were generated using a previously described protocol.[42] These lentiviruses were transduced into DMD pericytes[41] or DMD myoblasts[13] at equivalent MOIs (MOI= 10), and the transduced cells were then induced to undergo myogenic differentiation. The expression of GFP was monitored by immunostaining or western blot analysis at D0 (non-differentiated) or D7 (differentiated) after differentiation.

The open reading frame (ORF) of either native full-length dystrophin (nFLDys) or codon-optimized full-length dystrophin (soFLDys) driven by the CK9 promoter was cloned into a 3rd generation lentiviral transfer plasmid pCCLsin.cPPT.WPRE using NEBuilder HiFi DNA assembly. To produce LV-CK9-nFLDys and LV-CK9-soFLDys, the transfer plasmid, packaging plasmids (pMDLg/pRRE and pRSV-Rev) and the envelope plasmid (pMD2.G) were co-transfected at a ratio of 4:2:1:1 in HEK293T cells. Supernatant was collected at 48 and 72 hours after transfection and concentrated by ultracentrifugation at 23,000g for 2 hours at 4°C. Lentiviral titre was determined in DMD myoblasts as described below.

Transduction of human DMD myoblasts and LV titration

Cells were plated in 24-well plates at a density of $1x10^4$ cells/ well and transduced with different amounts of virus. Cells were changed into fresh medium 6 hours after adding the virus. The transduced cells were then expanded in M10 medium for subsequent experiments.

For lentivirus titration, genomic DNA of the cells was extracted using DNeasy blood and tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instruction. Viral copy number within the transduced cells was determined using Primetime qPCR probe assay

(Integrated DNA Technologies, Leuven, Belgium). The primers and probes used for qPCR are: WPRE-forward primer: TGGATTCTGCGCGGGA, WPRE-reverse: GAAGGAAGGTCCGCTGGATT, WPRE-probe: CTTCTGCTACGTCCCTTCGGCCCT, β-actin-forward primer: CAGCGGAACCGCTCATTGCCAATGG, β-actin-reverse primer: TCACCCACACTGTGCCCATCTACGA, β-actin-probe: ATGCCCTCCCCCATGCCATCCTGCGT.

Immunofluorescent staining of cells

Differentiated cells fixed by 4% paraformaldehyde (PFA) were immunostained using antibodies against GFP (rabbit polyclonal, 1:2000, Thermo Fisher, Paisley, UK) or dystrophin (Rabbit polyclonal, 1:1000, Fisher Scientific, Loughborough, UK) and myosin heavy chain (MF20, mouse IgG 2b monoclonal antibody, 1:500, DSHB, Iowa, USA) at room temperature for 2 hours, followed by alexa-488 conjugated goat anti-rabbit IgG (H+L) (1:1000, Thermo Fisher, Paisley, UK) and alexa-594 conjugated goat anti-mouse IgG2b (1:1000, Thermo Fisher, Paisley, UK) antibodies at room temperature for one hour. Nuclei were stained with 10µg/ml 4,6-diamidino-2-phenylindole (DAPI). Images were taken by IX71 Olympus inverted fluorescent microscope. The fusion indices of the myotubes were calculated as the percentage of total nuclei within a field that was within an MF20+ myotube (containing at least 3 nuclei).

Western blot

Transduced cells either before (D0) or after (D7) myogenic differentiation were lysed with Radio-Immunoprecipitation Assay (RIPA) buffer (Sigma, Dorset, UK), supplemented with protease inhibitor (Roche, Welwyn Garden City, UK) on ice for 15 min. The cell lysate was boiled for 5 min and then centrifuged at 14,000 x g for 10 minutes at 4°C. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher, Paisley, UK). 30µg/well of each sample was loaded onto NuPAGE Novex 3-8% Tris-acetate

gel and run at a constant voltage of 150V for 1.5 hours, before being transferred to a nitrocellulose membrane using a constant current of 300mA for 2 hours. The membrane was then blocked with Odyssey block solution (LI-COR Biosciences, Cambridge, UK) for one hour, before being incubated with primary antibodies against GFP (rabbit polyclonal IgG, 1:2000, Thermo Fisher, Paisley, UK) or dystrophin (rabbit polyclonal IgG, 1:2000, Fisher Scientific, Loughborough, UK), MF20 (mouse monoclonal IgG2b, 1:1000, DSHB, Iowa, USA), using α-actinin (mouse monoclonal IgG1, 1:10,000, Sigma, Dorset, UK) or tubulin 2.1 (mouse monoclonal IgG1, 1:3000, Santa Cruz, Heidelberg, Germany) or β-actin (mouse monoclonal IgG1, 1:3000, Sigma, Dorset, UK) as housekeeping protein controls. After washing with PBS containing 0.1% Tween 20 (PBST) for 15 min x 3 times at room temperature, the membrane was incubated with biotinylated anti-rabbit secondary antibody (1:1000) for 2 hours, followed by IRDye 680RD Streptavidin and IRDye 800CW goat anti-mouse 2nd antibodies (1:15000, LI-COR Biosciences, Cambridge, UK) for 1 hour at room temperature. The image of the blotted membrane was acquired by Odyssey Clx infrared imaging system (LI-COR Biosciences, Cambridge, UK) using Image Studio Lite 5.2 software.

qRT-PCR to determine the transcript of nFLDys and coFLDys in transduced cells

LV-nFLDys or LV-soFLDys transduced DMD myoblasts (delEx52) with comparable VCN was induced to differentiate into myotubes. Total RNA was extracted from cells at D5 of differentiation, using RNeasy mini kit (Qiagen, Manchester, UK). This was DNAseI treated (Sigma-Aldrich, Dorset, UK) and subjected to a high capacity cDNA synthesis reaction (Thermo Fisher, Paisley, UK), in accordance with the manufacturers protocols.

Quantitative-PCR of the dystrophin expression was performed using Primetime qPCR probe assay (Integrated DNA Technologies, Leuven, Belgium). The dystrophin primers and probes were designed to recognize a common sequence of the nFLDys and soFLDys at exon 51

(forward) and exon 52 (reverse) of dystrophin gene. Due to the lack the exon 52 sequence in the myoblasts used in this study, the endogenous transcript will not be amplified, in this manner we are assessing only differences in transgene expression. The sequence of the dystrophin primers is: Dys/soDys forward: TGAAAAACAAGACCAGCAA, Dys/soDys reverse: GATATCAACGAGATGATCATCAAGCAGAA. However, different probes were used to detect the PCR product from DMD-nFLDys and DMD-soFLDys cells, respectively. nFLDys probe: TGGGCAGCGGTAATGAGTTCTTCC, soFLDys probe: AGCTGGAAGAACTGATCACAGCCG.

We also performed qRT-PCR using primer/probe against MYH1 as control to monitor the extent of myogenic differentiation, and primer/probe against β-actin as loading control. The sequences are: MYH1 forward: GGTCGCATCTCTACGCCAGG, MYH1 reverse: ACTTTCGGAGGAAAGGAGCAG, MHY1 probe: ATAACCTGCAGCCATGAGTTCCGA. The sequence of the primer/probe of β-actin is described above.

The relative amount of nFLDys transcripts in DMD-nFLDys cells is presented as the fold change between nFLDys and β-actin transcripts, calculated using the formula: 2^-(delCt). DelCt refers to the differences in the cycle numbers between nFLDys and β-actin. The relative amount of soFLDys transcripts in DMD-soFLDys cells were calculated in a similar manner, and compared with that of nFLDys transcripts in DMD-nFLDys cells.

Intramuscular transplantation

4-8 week-old mdx nude mice[11, 41] were used as recipients for cell transplantation. On the day of transplantation, mice were anesthetized with isoflurane and tibialis anterior (TA) muscles were exposed and cryodamaged with 3 freeze-thaw cycles using a cryo-probe pre-chilled in liquid nitrogen.[85, 110] $5x10^5$ cells in 5 µl medium were injected into each TA with a Hamilton syringe. Host muscles were cryoinjured immediately before cell transplantation, as we (and

521 others) find that human myoblasts,[111] human pericytes and CD133+ cells[110] and mouse
522 satellite cells[14, 112] transplanted into non-injured muscles of immunodeficient, dystrophin-
523 deficient mice do not engraft as well as they do following injection into injured muscles.
524 Grafted muscles were dissected 4 weeks after transplantation, mounted on corks in 6% gum
525 tradacanth (Sigma, Dorset, UK) and frozen in isopentane pre-chilled in liquid nitrogen.
526 **Immunofluorescent staining on muscle sections**
527 10 µm transverse cryosections were air dried and blocked with AffiniPure $F(ab')_2$ Fragment
528 Donkey Anti-Mouse IgG (H+L) (1:50, Jackson Immuno Research, Cambridge, UK) for one
529 hour at room temperature and stained with the following combination of antibodies: 1)
530 Antibodies against human spectrin (mouse IgG 2b, Vector labs, VP-S283, 1:100,
531 Peterborough, UK), human lamin A/C (mouse IgG2b, Vector labs, VP-L550, 1:500,
532 Peterborough, UK), and human dystrophin (Mandys 106, mouse IgG2a, 1:200, Millipore).
533 The number of human lamin A/C+ nuclei, human spectrin+ fibres, human spectrin+ fibres
534 containing at least one human lamin A/C+ nucleus (S+L) (as a confirmation that the
535 spectrin+ fibres were of donor origin)[113] were counted in representative transverse sections.
536 The number of dystrophin+/hSpectrin+ fibres was also quantified to evaluate the percentage
537 of dystrophin-expressing fibres of donor origin. The intensity of the dystrophin (Mandys 106,
538 red channel) on human spectrin+ fibres (green channel) was measured using MetaMorph
539 software, normalized by the background intensity (we measured the intensity of the red
540 channel on human spectrin- fibres as background intensity) within the same muscle section,
541 and compared among normal myoblast, DMD-nFLDys and DMD-coFLDys transplanted
542 groups. 2). Dystrophin (Mandys 106, mouse IgG2a, Millipore) and α-sarcoglycan (mouse
543 IgG1, 1:100, Leica biosystems). 3) Dystrophin (Mandys 106, mouse IgG2a, Millipore) and
544 γ-sarcoglycan (rabbit polyclonal, 1:500, Santa Cruz), 4). Dystrophin (Mandys 106, mouse
545 IgG2a) and utrophin (mouse IgG1, 1:200, Leica Biosystems). The results were acquired using

a Leica microscope and the intensity of α-sarcoglycan, γ-sarcoglycan or utrophin on dystrophin+ or dystrophin- fibres was quantified using MetaMorph software.

Statistical analysis

For two groups comparison, paired or un-paired student t-test was used. For comparison involved three or more groups, One-way ANOVA (Kruskal-Wallis test) followed by Dunn's Multiple Comparison Test were used to determine statistical significance. Results presented in this study are displayed as Mean ± Standard Error of the Mean (Mean ± SEM). GraphPad Prism 5.0 software was used for statistical analysis and graph design. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Acknowledgements

Funding: This work was funded by MDUK (grant number: 17GRO-PG36-0165) and in part by the Wellcome Trust (grant number:210774/Z/18/). For the purpose of open access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. The support of the MRC Centre for Neuromuscular Diseases Biobank is gratefully acknowledged. JEM was supported by Great Ormond Street Hospital Children's Charity. This research was supported by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Author Contributions Conceptualization and designing of the project, J.M., L.P. and J.E.M., methodology, J.M. and J.C., analysis and investigation, J.M., M.M., writing – original draft preparation, J.M. and J.E.M., writing – review and editing, J.E.M., M.M., J.C. ,L.P. and F.M., supervision, J.E.M., project administration, J.E.M., funding acquisition, J.E.M.

Disclosure of Interests

Royal Holloway University of London has a patent on the sequence optimized full length *DMD* cDNA. The remaining authors declare no competing financial interests.

Keywords lentivirus; Duchenne muscular dystrophy; native full-length dystrophin; sequence-optimized full-length dystrophin; myoblasts; muscle specific promoter.

References

1. Kunkel,L., Burns,G., Aldrige,J., & Latt,S. Genetic analysis of Duchenne dystrophy. Adv. Exp. Med. Biol. 182, 287-294 (1985).

2. Duan,D., Goemans,N., Takeda,S., Mercuri,E., & Aartsma-Rus,A. Duchenne muscular dystrophy. Nat. Rev. Dis. Primers. 7, 13 (2021).

3. Duan,D. Systemic AAV Micro-dystrophin Gene Therapy for Duchenne Muscular Dystrophy. Mol. Ther. 26, 2337-2356 (2018).

4. Kawecka,K.,Theodoulides,M., Hasoglu,Y., Jarmin,S., Kymalainen,H., Le-Heron,A., Popplewell,L., Malerba,A., Dickson,G., Athanasopoulos,T. Adeno-Associated Virus (AAV) Mediated Dystrophin Gene Transfer Studies and Exon Skipping Strategies for Duchenne Muscular Dystrophy (DMD). Curr. Gene Ther. 15, 395-415 (2015).

5. Lostal,W., Kodippili,K., Yue,Y., & Duan,D. Full-length dystrophin reconstitution with adeno-associated viral vectors. Hum. Gene Ther. 25, 552-562 (2014).

6. Morgan,J. & Muntoni,F. Changes in Myonuclear Number During Postnatal Growth - Implications for AAV Gene Therapy for Muscular Dystrophy. J. Neuromuscul. Dis.(2021).

7. DelloRusso,C., Scott,J.M., Hartigan-O'Connor,D., Salvatori,G., Barjot,C., Robinson,A.S., Crawford,R.W., Brooks,S.V., Chamberlain,J.S. Functional correction of adult mdx mouse muscle using gutted adenoviral vectors expressing full-length dystrophin. Proc. Natl. Acad. Sci. U. S. A 99, 12979-12984 (2002).

8. Clemens,P.R., Kochanek,S., Sunada,Y., Chan,S., Chen,H.H., Campbell,K.P., Caskey,C.T. In vivo muscle gene transfer of full-length dystrophin with an adenoviral vector that lacks all viral genes. Gene Ther. 3, 965-972 (1996).

9. Reay,D.P., Bilbao,R., Koppanati,B.M., Cai,L., O'Day,T.L., Jiang,Z., Zheng,H., Watchko,J.F., Clemens,P.R. Full-length dystrophin gene transfer to the mdx mouse in utero. Gene Ther. 15, 531-536 (2008).

10. Akkaraju,G.R., Huard,J., Hoffman,E.P., Goins,W.F., Pruchnic,R., Watkins,S.C., Cohen,J.B., Glorioso,J.C. Herpes simplex virus vector-mediated dystrophin gene transfer and expression in MDX mouse skeletal muscle. J. Gene Med. 1, 280-289 (1999).

11. Meng,J., Sweeney,N., Doreste,B., Muntoni,F., McClure,M., Morgan,J. Restoration of functional full-length dystrophin after intramuscular transplantation of Foamy Virus-transduced myoblasts. Hum. Gene Ther.(2019).

12. Sweeney,N.P., Meng,J., Patterson,H., Morgan,J.E., & McClure,M. Delivery of large transgene cassettes by foamy virus vector. Sci. Rep. 7, 8085 (2017).

13. Counsell,J.R., Asgarian,Z., Meng,J., Ferrer,V., Vink,C.A., Howe,S.J., Waddington,S.N., Thrasher,A.J., Muntoni,F., Morgan,J.E., et al. Lentiviral vectors can be used for full-length dystrophin gene therapy. Sci. Rep. 7, 79 (2017).

14. Collins,C.A., Olsen,I., Zammit,P.S., Heslop,L., Petrie,A., Partridge,T.A., Morgan,J.E. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122, 289-301 (2005).

15. Sacco,A., Doyonnas,R., Kraft,P., Vitorovic,S., & Blau,H.M. Self-renewal and expansion of single transplanted muscle stem cells. Nature 456, 502-506 (2008).

16. Patel,K. & Morgan,J. 185th ENMC International Workshop: stem/precursor cells as a therapeutic strategy for muscular dystrophies 3-5 June 2011, Naarden, The Netherlands. Neuromuscul. Disord. 22, 447-452 (2012).

17. Dellavalle,A., Sampaolesi,M., Tonlorenzi,R., Tagliafico,E., Sacchetti,B., Perani,L., Innocenzi,A., Galvez,B.G., Messina,G., Morosetti,R., et al. Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat. Cell Biol. 9, 255-267 (2007).

18. Skuk,D., Goulet,M., & Tremblay,J.P. Transplanted myoblasts can migrate several millimeters to fuse with damaged myofibers in nonhuman primate skeletal muscle. J. Neuropathol. Exp. Neurol. 70, 770-778 (2011).

19. Skuk,D., Goulet,M., & Tremblay,J.P. Intramuscular transplantation of myogenic cells in primates: importance of needle size, cell number, and injection volume. Cell Transplant. 23, 13-25 (2014).

20. Lafreniere,J.F., Mills,P., Tremblay,J.P., & El,F.E. Growth factors improve the in vivo migration of human skeletal myoblasts by modulating their endogenous proteolytic activity. Transplantation 77, 1741-1747 (2004).

21. Lafreniere,J.F., Caron,M.C., Skuk,D., Goulet,M., Cheikh,A.R., Tremblay,J.P. Growth factor coinjection improves the migration potential of monkey myogenic precursors without affecting cell transplantation success. Cell Transplant. 18, 719-730 (2009).

22. Min,Y.L., Bassel-Duby,R., & Olson,E.N. CRISPR Correction of Duchenne Muscular Dystrophy. Annu. Rev. Med. 70, 239-255 (2019).

23. Beitinjaneh,A., Saliba,R.M., Medeiros,L.J., Turturro,F., Rondon,G., Korbling,M., Fayad,L., Fanale,M.A., Alousi,A.M., Anderlini,P., et al. Comparison of survival in patients with T cell lymphoma after autologous and allogeneic stem cell transplantation as a frontline strategy or in relapsed disease. Biol. Blood Marrow Transplant. 21, 855-859 (2015).

24. Zhang,M. & McLennan,I.S. Use of antibodies to identify satellite cells with a light microscope. Muscle Nerve 17, 987-994 (1994).

25. Dumont,N.A., Wang,Y.X., von,Maltzahn J., Pasut,A., Bentzinger,C.F., Brun,C.E., Rudnicki,M.A. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. Nat. Med. 21, 1455-1463 (2015).

26. Meng,J., Counsell,J., & Morgan,J.E. Effects of Mini-Dystrophin on Dystrophin-Deficient, Human Skeletal Muscle-Derived Cells. Int. J. Mol. Sci. 21, (2020).

27. Wells,D.J. What is the level of dystrophin expression required for effective therapy of Duchenne muscular dystrophy? J. Muscle Res. Cell Motil. 40, 141-150 (2019).

28. van der Pijl,E.M., van,Putten M., Niks,E.H., Verschuuren,J.J.G.M., Aartsma-Rus,A., Plomp,J.J. Low dystrophin levels are insufficient to normalize the neuromuscular synaptic abnormalities of mdx mice. Neuromuscul. Disord. 28, 427-442 (2018).

29. Godfrey,C., Muses,S., McClorey,G., Wells,K.E., Coursindel,T., Terry,R.L., Betts,C., Hammond,S., O'Donovan,L., Hildyard,J., et al. How much dystrophin is enough: the physiological consequences of different levels of dystrophin in the mdx mouse. Hum. Mol. Genet. 24, 4225-4237 (2015).

30. Li,D., Yue,Y., & Duan,D. Preservation of muscle force in Mdx3cv mice correlates with low-level expression of a near full-length dystrophin protein. Am. J. Pathol. 172, 1332-1341 (2008).

31. Sharp,P.S., Jee,H., & Wells,D.J. Physiological characterization of muscle strength with variable levels of dystrophin restoration in mdx mice following local antisense therapy. Mol. Ther. 19, 165-171 (2011).

32. van,Putten M., Hulsker,M., Nadarajah,V.D., van Heiningen,S.H., van,Huizen E., van,Iterson M., Admiraal,P., Messemaker,T., den Dunnen,J.T., 't Hoen,P.A., et al. The effects of low levels of dystrophin on mouse muscle function and pathology. PLoS. One. 7, e31937 (2012).

33. van,Putten M., Hulsker,M., Young,C., Nadarajah,V.D., Heemskerk,H., van der Weerd,L., 't Hoen,P.A., van Ommen,G.J., Aartsma-Rus,A.M. Low dystrophin levels increase survival and improve muscle pathology and function in dystrophin/utrophin double-knockout mice. FASEB J. 27, 2484-2495 (2013).

34. Inouye,S., Sahara-Miura,Y., Sato,J., & Suzuki,T. Codon optimization of genes for efficient protein expression in mammalian cells by selection of only preferred human codons. Protein Expr. Purif. 109, 47-54 (2015).

35. Mauro,V.P. & Chappell,S.A. A critical analysis of codon optimization in human therapeutics. Trends Mol. Med. 20, 604-613 (2014).

36. Foster,H., Sharp,P.S., Athanasopoulos,T., Trollet,C., Graham,I.R., Foster,K., Wells,D.J., Dickson,G. Codon and mRNA sequence optimization of microdystrophin transgenes improves expression and physiological outcome in dystrophic mdx mice following AAV2/8 gene transfer. Mol. Ther. 16, 1825-1832 (2008).

37. Le,Guiner C., Servais,L., Montus,M., Larcher,T., Fraysse,B., Moullec,S., Allais,M., Francois,V., Dutilleul,M., Malerba,A., et al. Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. Nat. Commun. 8, 16105 (2017).

38. Mendell,J.R., Sahenk,Z., Lehman,K., Nease,C., Lowes,L.P., Miller,N.F., Iammarino,M.A., Alfano,L.N., Nicholl,A., Al-Zaidy,S., et al. Assessment of Systemic Delivery of rAAVrh74.MHCK7.micro-dystrophin in Children With Duchenne Muscular Dystrophy: A Nonrandomized Controlled Trial. JAMA Neurol. 77, 1122-1131 (2020).

39. Wang,B., Li,J., Fu,F.H., Chen,C., Zhu,X., Zhou,L., Jiang,X., Xiao,X. Construction and analysis of compact muscle-specific promoters for AAV vectors. Gene Ther. 15, 1489-1499 (2008).

40. Himeda,C.L., Chen,X., & Hauschka,S.D. Design and testing of regulatory cassettes for optimal activity in skeletal and cardiac muscles. Methods Mol. Biol. 709, 3-19 (2011).

41. Meng,J., Adkin,C.F., Xu,S.W., Muntoni,F., & Morgan,J.E. Contribution of human muscle-derived cells to skeletal muscle regeneration in dystrophic host mice. PLoS. One. 6, e17454 (2011).

42. Meng,J., Counsell,J.R., Reza,M., Laval,S.H., Danos,O., Thrasher,A., Lochmuller,H., Muntoni,F., Morgan,J.E. Autologous skeletal muscle derived cells expressing a novel functional dystrophin provide a potential therapy for Duchenne Muscular Dystrophy. Sci. Rep. 6, 19750 (2016).

43. Ervasti,J.M., Ohlendieck,K., Kahl,S.D., Gaver,M.G., & Campbell,K.P. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 345, 315-319 (1990).

44. Ohlendieck,K. & Campbell,K.P. Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. J. Cell Biol. 115, 1685-1694 (1991).

45. Ohlendieck,K., Matsumura,K., Ionasescu,V.V., Towbin,J.A., Bosch,E.P., Weinstein,S.L., Sernett,S.W., Campbell,K.P. Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. Neurology 43, 795-800 (1993).

46. Ehmsen,J., Poon,E., & Davies,K. The dystrophin-associated protein complex. J. Cell Sci. 115, 2801-2803 (2002).

47. Sancar,F., Touroutine,D., Gao,S., Oh,H.J., Gendrel,M., Bessereau,J.L., Kim,H., Zhen,M., Richmond,J.E. The dystrophin-associated protein complex maintains muscle excitability by regulating Ca(2+)-dependent K(+) (BK) channel localization. J. Biol. Chem. 286, 33501-33510 (2011).

48. Spence,H.J., Dhillon,A.S., James,M., & Winder,S.J. Dystroglycan, a scaffold for the ERK-MAP kinase cascade. EMBO Rep. 5, 484-489 (2004).

49. Cirak,S., Feng,L., Anthony,K., Arechavala-Gomeza,V., Torelli,S., Sewry,C., Morgan,J.E., Muntoni,F. Restoration of the dystrophin-associated glycoprotein complex after exon skipping therapy in Duchenne muscular dystrophy. Mol. Ther. 20, 462-467 (2012).

50. Cirak,S. et al. 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 (2011).

51. Janghra,N., Morgan,J.E., Sewry,C.A., Wilson,F.X., Davies,K.E., Muntoni,F., Tinsley,J. Correlation of Utrophin Levels with the Dystrophin Protein Complex and Muscle Fibre Regeneration in Duchenne and Becker Muscular Dystrophy Muscle Biopsies. PLoS. One. 11, e0150818 (2016).

52. Kinali,M., Arechavala-Gomeza,V., Feng,L., Cirak,S., Hunt,D., Adkin,C., Guglieri,M., Ashton,E., Abbs,S., Nihoyannopoulos,P., et al. 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 (2009).

53. Scaglioni,D., Ellis,M., Catapano,F., Torelli,S., Chambers,D., Feng,L., Sewry,C., Morgan,J., Muntoni,F., Phadke,R. A high-throughput digital script for multiplexed immunofluorescent analysis and quantification of sarcolemmal and sarcomeric proteins in muscular dystrophies. Acta Neuropathol. Commun. 8, 53 (2020).

54. Amenta,A.R., Yilmaz,A., Bogdanovich,S., McKechnie,B.A., Abedi,M., Khurana,T.S., Fallon,J.R. Biglycan recruits utrophin to the sarcolemma and counters dystrophic pathology in mdx mice. Proc. Natl. Acad. Sci. U. S. A 108, 762-767 (2011).

55. Banks,G.B., Combs,A.C., Odom,G.L., Bloch,R.J., & Chamberlain,J.S. Muscle structure influences utrophin expression in mdx mice. PLoS. Genet. 10, e1004431 (2014).

56. Doudna,J.A. & Charpentier,E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 346, 1258096 (2014).

57. Mali,P., Yang,L., Esvelt,K.M., Aach,J., Guell,M., DiCarlo,J.E., Norville,J.E., Church,G.M. RNA-guided human genome engineering via Cas9. Science 339, 823-826 (2013).

58. Ran,F.A., Hsu,P.D., Wright,J., Agarwala,V., Scott,D.A., Zhang,F. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281-2308 (2013).

59. Amoasii,L., Long,C., Li,H., Mireault,A.A., Shelton,J.M., Sanchez-Ortiz,E., McAnally,J.R., Bhattacharyya,S., Schmidt,F., Grimm,D., et al. Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. Sci. Transl. Med. 9, (2017).

60. Young,C.S., Hicks,M.R., Ermolova,N.V., Nakano,H., Jan,M., Younesi,S., Karumbayaram,S., Kumagai-Cresse,C., Wang,D., Zack,J.A., et al. A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells. Cell Stem Cell 18, 533-540 (2016).

61. Maino,E., Wojtal,D., Evagelou,S.L., Farheen,A., Wong,T.W.Y., Lindsay,K., Scott,O., Rizvi,S.Z., Hyatt,E., Rok,M., et al. Targeted genome editing in vivo corrects a Dmd duplication restoring wild-type dystrophin expression. EMBO Mol. Med.e13228 (2021).

62. Mendell,J.R., Campbell,K., Rodino-Klapac,L., Sahenk,Z., Shilling,C., Lewis,S., Bowles,D., Gray,S., Li,C., Galloway,G., et al. Dystrophin immunity in Duchenne's muscular dystrophy. N. Engl. J. Med. 363, 1429-1437 (2010).

63. Crudele,J.M. & Chamberlain,J.S. AAV-based gene therapies for the muscular dystrophies. Hum. Mol. Genet. 28, R102-R107 (2019).

64. Wang,B., Li,J., & Xiao,X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. Proc. Natl. Acad. Sci. U. S. A 97, 13714-13719 (2000).

65. Dhungel,B.P., Bailey,C.G., & Rasko,J.E.J. Journey to the Center of the Cell: Tracing the Path of AAV Transduction. Trends Mol. Med. 27, 172-184 (2021).

66. Chicoine,L.G., Montgomery,C.L., Bremer,W.G., Shontz,K.M., Griffin,D.A., Heller,K.N., Lewis,S., Malik,V., Grose,W.E., Shilling,C.J., et al. Plasmapheresis eliminates the

negative impact of AAV antibodies on microdystrophin gene expression following vascular delivery. Mol. Ther. 22, 338-347 (2014).

67. Buning,H. & Srivastava,A. Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors. Mol. Ther. Methods Clin. Dev. 12, 248-265 (2019).

68. Purohit,N., Jain,A., Mathews,V., & Jayandharan,G.R. Molecular characterization of novel Adeno-associated virus variants infecting human tissues. Virus Res. 272, 197716 (2019).

69. Hardet,R., Chevalier,B., Dupaty,L., NaÃ¯mi,Y., Riou,G., Drouot,L., Jean,L., Salvetti,A., Boyer,O., Adriouch,S. Oral-tolerization Prevents Immune Responses and Improves Transgene Persistence Following Gene Transfer Mediated by Adeno-associated Viral Vector. Mol. Ther. 24, 87-95 (2016).

70. Meliani,A., Boisgerault,F., Hardet,R., Marmier,S., Collaud,F., Ronzitti,G., Leborgne,C., Costa,Verdera H., Simon,Sola M., Charles,S., et al. Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. Nat. Commun. 9, 4098 (2018).

71. Mingozzi,F., Hasbrouck,N.C., Basner-Tschakarjan,E., Edmonson,S.A., Hui,D.J., Sabatino,D.E., Zhou,S., Wright,J.F., Jiang,H., Pierce,G.F., et al. Modulation of tolerance to the transgene product in a nonhuman primate model of AAV-mediated gene transfer to liver. Blood 110, 2334-2341 (2007).

72. Kimura,E., Li,S., Gregorevic,P., Fall,B.M., & Chamberlain,J.S. Dystrophin delivery to muscles of mdx mice using lentiviral vectors leads to myogenic progenitor targeting and stable gene expression. Mol. Ther. 18, 206-213 (2010).

73. MacKenzie,T.C., Kobinger,G.P., Louboutin,J.P., Radu,A., Javazon,E.H., Sena-Esteves,M., Wilson,J.M., Flake,A.W. Transduction of satellite cells after prenatal intramuscular administration of lentiviral vectors. J. Gene Med. 7, 50-58 (2005).

74. Milone,M.C. & O'Doherty,U. Clinical use of lentiviral vectors. Leukemia 32, 1529-1541 (2018).

75. Biffi,A., Montini,E., Lorioli,L., Cesani,M., Fumagalli,F., Plati,T., Baldoli,C., Martino,S., Calabria,A., Canale,S., et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341, 1233158 (2013).

76. Cartier,N., Hacein-Bey-Abina,S., Bartholomae,C.C., Veres,G., Schmidt,M., Kutschera,I., Vidaud,M., Abel,U., Dal-Cortivo,L., Caccavelli,L., et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. Science 326, 818-823 (2009).

77. Cavazzana-Calvo,M., Payen,E., Negre,O., Wang,G., Hehir,K., Fusil,F., Down,J., Denaro,M., Brady,T., Westerman,K., et al. Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467, 318-322 (2010).

78. Sessa,M., Lorioli,L., Fumagalli,F., Acquati,S., Redaelli,D., Baldoli,C., Canale,S., Lopez,I.D., Morena,F., Calabria,A., et al. Lentiviral haemopoietic stem-cell gene

therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial. Lancet 388, 476-487 (2016).

79. Milone,M.C. & O'Doherty,U. Clinical use of lentiviral vectors. Leukemia 32, 1529-1541 (2018).

80. Maude,S.L., Frey,N., Shaw,P.A., Aplenc,R., Barrett,D.M., Bunin,N.J., Chew,A., Gonzalez,V.E., Zheng,Z., Lacey,S.F., et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N. Engl. J. Med. 371, 1507-1517 (2014).

81. Ako-Adjei,D., Johnson,M.C., & Vogt,V.M. The retroviral capsid domain dictates virion size, morphology, and coassembly of gag into virus-like particles. J. Virol. 79, 13463-13472 (2005).

82. Papanikolaou,E., Kontostathi,G., Drakopoulou,E., Georgomanoli,M., Stamateris,E., Vougas,K., Vlahou,A., Maloy,A., Ware,M., Anagnou,N.P. Characterization and comparative performance of lentiviral vector preparations concentrated by either one-step ultrafiltration or ultracentrifugation. Virus Res. 175, 1-11 (2013).

83. Lai,Y., Yue,Y., & Duan,D. Evidence for the failure of adeno-associated virus serotype 5 to package a viral genome > or = 8.2 kb. Mol. Ther. 18, 75-79 (2010).

84. Quenneville,S.P., Chapdelaine,P., Skuk,D., Paradis,M., Goulet,M., Rousseau,J., Xiao,X., Garcia,L., Tremblay,J.P. Autologous transplantation of muscle precursor cells modified with a lentivirus for muscular dystrophy: human cells and primate models. Mol. Ther. 15, 431-438 (2007).

85. Brimah,K., Ehrhardt,J., Mouly,V., Butler-Browne,G.S., Partridge,T.A., Morgan,J.E. Human muscle precursor cell regeneration in the mouse host is enhanced by growth factors. Hum. Gene Ther. 15, 1109-1124 (2004).

86. Cooper,R.N., Thiesson,D., Furling,D., Di Santo,J.P., Butler-Browne,G.S., Mouly,V. Extended amplification in vitro and replicative senescence: key factors implicated in the success of human myoblast transplantation. Hum. Gene Ther. 14, 1169-1179 (2003).

87. Meng,J., Chun,S., Asfahani,R., Lochmuller,H., Muntoni,F., Morgan,J. Human skeletal muscle-derived CD133(+) cells form functional satellite cells after intramuscular transplantation in immunodeficient host mice. Mol. Ther. 22, 1008-1017 (2014).

88. Zhou,Z., Dang,Y., Zhou,M., Li,L., Yu,C.H., Fu,J., Chen,S., Liu,Y. Codon usage is an important determinant of gene expression levels largely through its effects on transcription. Proc. Natl. Acad. Sci. U. S. A 113, E6117-E6125 (2016).

89. Graf,M., Deml,L., & Wagner,R. Codon-optimized genes that enable increased heterologous expression in mammalian cells and elicit efficient immune responses in mice after vaccination of naked DNA. Methods Mol. Med. 94, 197-210 (2004).

90. Gregorevic,P., Blankinship,M.J., Allen,J.M., Crawford,R.W., Meuse,L., Miller,D.G., Russell,D.W., Chamberlain,J.S. Systemic delivery of genes to striated muscles using adeno-associated viral vectors. Nat. Med. 10, 828-834 (2004).

91. Salva,M.Z., Himeda,C.L., Tai,P.W., Nishiuchi,E., Gregorevic,P., Allen,J.M., Finn,E.E., Nguyen,Q.G., Blankinship,M.J., Meuse,L., et al. Design of tissue-specific regulatory cassettes for high-level rAAV-mediated expression in skeletal and cardiac muscle. Mol. Ther. 15, 320-329 (2007).

92. Morgan,J. & Partridge,T. Skeletal muscle in health and disease. Dis. Model. Mech. 13, (2020).

93. Talbot,G.E., Waddington,S.N., Bales,O., Tchen,R.C., & Antoniou,M.N. Desmin-regulated lentiviral vectors for skeletal muscle gene transfer. Mol. Ther. 18, 601-608 (2010).

94. Jonuschies,J., Antoniou,M., Waddington,S., Boldrin,L., Muntoni,F., Thrasher,A., Morgan,J. The human desmin promoter drives robust gene expression for skeletal muscle stem cell-mediated gene therapy. Curr. Gene Ther. 14, 276-288 (2014).

95. Bencze,M., Negroni,E., Vallese,D., Yacoub-Youssef,H., Chaouch,S., Wolff,A., Aamiri,A., Di Santo,J.P., Chazaud,B., Butler-Browne,G., et al. Proinflammatory macrophages enhance the regenerative capacity of human myoblasts by modifying their kinetics of proliferation and differentiation. Mol. Ther. 20, 2168-2179 (2012).

96. Cooper,R.N., Irintchev,A., Di Santo,J.P., Zweyer,M., Morgan,J.E., Partridge,T.A., Butler-Browne,G.S., Mouly,V., Wernig,A. A new immunodeficient mouse model for human myoblast transplantation. Hum. Gene Ther. 12, 823-831 (2001).

97. Ehrhardt,J., Brimah,K., Adkin,C., Partridge,T., & Morgan,J. Human muscle precursor cells give rise to functional satellite cells in vivo. Neuromuscul. Disord. 17, 631-638 (2007).

98. Garcia,S.M., Tamaki,S., Lee,S., Wong,A., Jose,A., Dreux,J., Kouklis,G., Sbitany,H., Seth,R., Knott,P.D., et al. High-Yield Purification, Preservation, and Serial Transplantation of Human Satellite Cells. Stem Cell Reports. 10, 1160-1174 (2018).

99. Laumonier,T., Bermont,F., Hoffmeyer,P., Kindler,V., & Menetrey,J. Human myogenic reserve cells are quiescent stem cells that contribute to muscle regeneration after intramuscular transplantation in immunodeficient mice. Sci. Rep. 7, 3462 (2017).

100. Marg,A., Escobar,H., Gloy,S., Kufeld,M., Zacher,J., Spuler,A., Birchmeier,C., IzsvÃ¡k,Z., Spuler,S. Human satellite cells have regenerative capacity and are genetically manipulable. J. Clin. Invest 124, 4257-4265 (2014).

101. Riederer,I., Negroni,E., Bencze,M., Wolff,A., Aamiri,A., Di Santo,J.P., Silva-Barbosa,S.D., Butler-Browne,G., Savino,W., Mouly,V. Slowing down differentiation of engrafted human myoblasts into immunodeficient mice correlates with increased proliferation and migration. Mol. Ther. 20, 146-154 (2012).

102. Silva-Barbosa,S.D., Butler-Browne,G.S., Di Santo,J.P., & Mouly,V. Comparative analysis of genetically engineered immunodeficient mouse strains as recipients for human myoblast transplantation. Cell Transplant. 14, 457-467 (2005).

103. Skuk,D., Paradis,M., Goulet,M., Chapdelaine,P., Rothstein,D.M., Tremblay,J.P. Intramuscular transplantation of human postnatal myoblasts generates functional donor-derived satellite cells. Mol. Ther. 18, 1689-1697 (2010).

104. Xu,X., Wilschut,K.J., Kouklis,G., Tian,H., Hesse,R., Garland,C., Sbitany,H., Hansen,S., Seth,R., Knott,P.D., et al. Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles. Stem Cell Reports. 5, 419-434 (2015).

105. Azzag,K., Ortiz-Cordero,C., Oliveira,N.A.J., Magli,A., Selvaraj,S., Tungtur,S., Upchurch,W., Iaizzo,P.A., Lu,Q.L., Perlingeiro,R.C.R. Efficient engraftment of pluripotent stem cell-derived myogenic progenitors in a novel immunodeficient mouse model of limb girdle muscular dystrophy 2I. Skelet. Muscle 10, 10 (2020).

106. Darabi,R., Arpke,R.W., Irion,S., Dimos,J.T., Grskovic,M., Kyba,M., Perlingeiro,R.C. Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. Cell Stem Cell 10, 610-619 (2012).

107. Hicks,M.R., Hiserodt,J., Paras,K., Fujiwara,W., Eskin,A., Jan,M., Xi,H., Young,C.S., Evseenko,D., Nelson,S.F., et al. ERBB3 and NGFR mark a distinct skeletal muscle progenitor cell in human development and hPSCs. Nat. Cell Biol. 20, 46-57 (2018).

108. Zhao,M., Tazumi,A., Takayama,S., Takenaka-Ninagawa,N., Nalbandian,M., Nagai,M., Nakamura,Y., Nakasa,M., Watanabe,A., Ikeya,M., et al. Induced Fetal Human Muscle Stem Cells with High Therapeutic Potential in a Mouse Muscular Dystrophy Model. Stem Cell Reports. 15, 80-94 (2020).

109. Montarras,D., Morgan,J., Collins,C., Relaix,F., Zaffran,S., Cumano,A., Partridge,T., Buckingham,M. Direct isolation of satellite cells for skeletal muscle regeneration. Science 309, 2064-2067 (2005).

110. Meng,J., Bencze,M., Asfahani,R., Muntoni,F., & Morgan,J.E. The effect of the muscle environment on the regenerative capacity of human skeletal muscle stem cells. Skelet. Muscle 5, 11 (2015).

111. Vallese,D., Negroni,E., Duguez,S., Ferry,A., Trollet,C., Aamiri,A., Vosshenrich,C.A., Fuchtbauer,E.M., Di Santo,J.P., Vitiello,L., et al. The Rag2 (-)/Il2rb(-)/Dmd (-) mouse: a novel dystrophic and immunodeficient model to assess innovating therapeutic strategies for muscular dystrophies. Mol. Ther. 21, 1950-1957 (2013).

112. Boldrin,L., Neal,A., Zammit,P.S., Muntoni,F., & Morgan,J.E. Donor satellite cell engraftment is significantly augmented when the host niche is preserved and endogenous satellite cells are incapacitated. Stem Cells 30, 1971-1984 (2012).

113. Rozkalne,A., Adkin,C., Meng,J., Lapan,A., Morgan,J.E., Gussoni,E. Mouse regenerating myofibers detected as false-positive donor myofibers with anti-human spectrin. Hum. Gene Ther. 25, 73-81 (2014).

932

Table 1. Relative expression of full-length dystrophin in transduced cells					
	Normal	DMD-NT	DMD-nFLDys	DMD-soFLDys	Statistical comparison
Normalized to α-actinin	1.14±0.37	0.15±0.025 (background level)	0.45±0.01	2.8±0.31	$p<0.05$, one-way ANOVA
Normalized to MF20	1.31± 0.49	0.16±0.03(background level)	0.53±0.017	3.14±0.46	$p<0.05$, one-way ANOVA

933

Table 2: Transplantation efficiency of cells in cryodamage TA muscles of mdx nude mice				
	DMD-NT n=5	DMD-nFLDys n=6	DMD-soFLDys n=6	Statistical significance
human lamin AC+ nuclei	45.6±12.66	47.17±16.13	108.7±31.12	No. ($p>0.05$)
Human Spectrin+ fibres	34.6±10.02	48.17±11.95	87.17±24	No. ($p>0.05$)
S+L fibres	28.8±9.7	40±12.32	68.5±18.26	No. ($p>0.05$)
Data were presented as Mean±SEM, statistical analysis using One-way ANOVA, Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. *$p<0.05$				

934

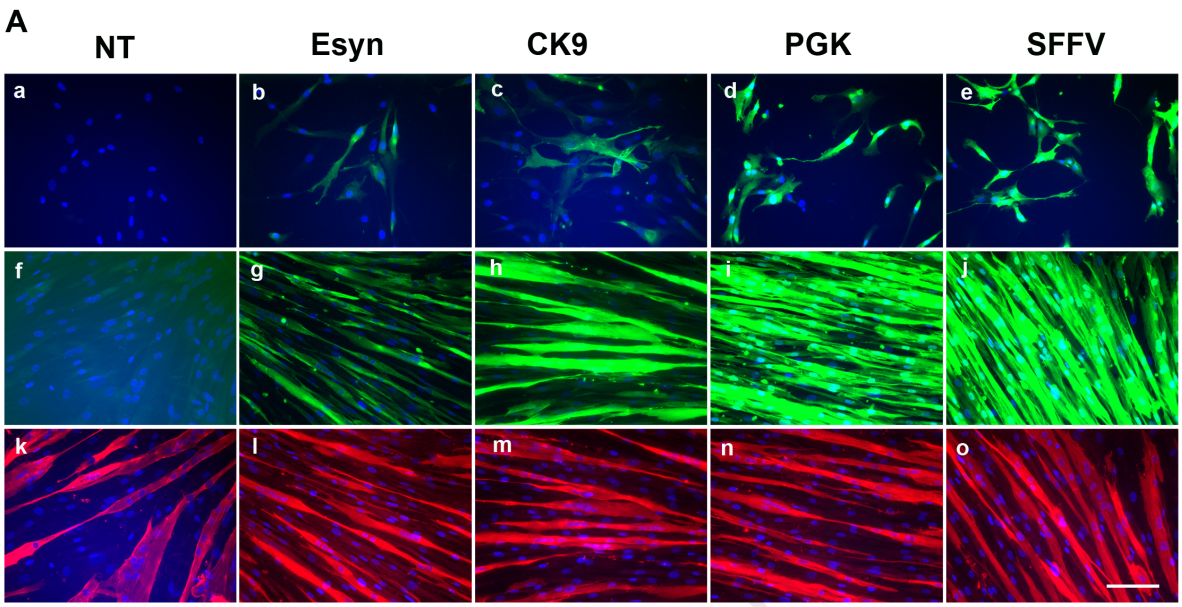
935

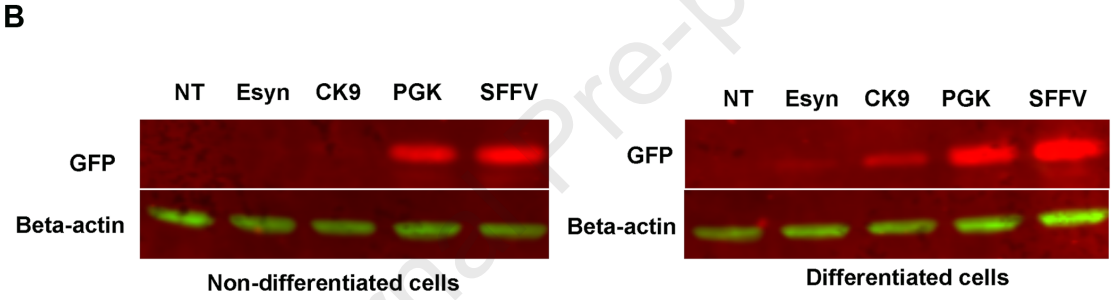
936

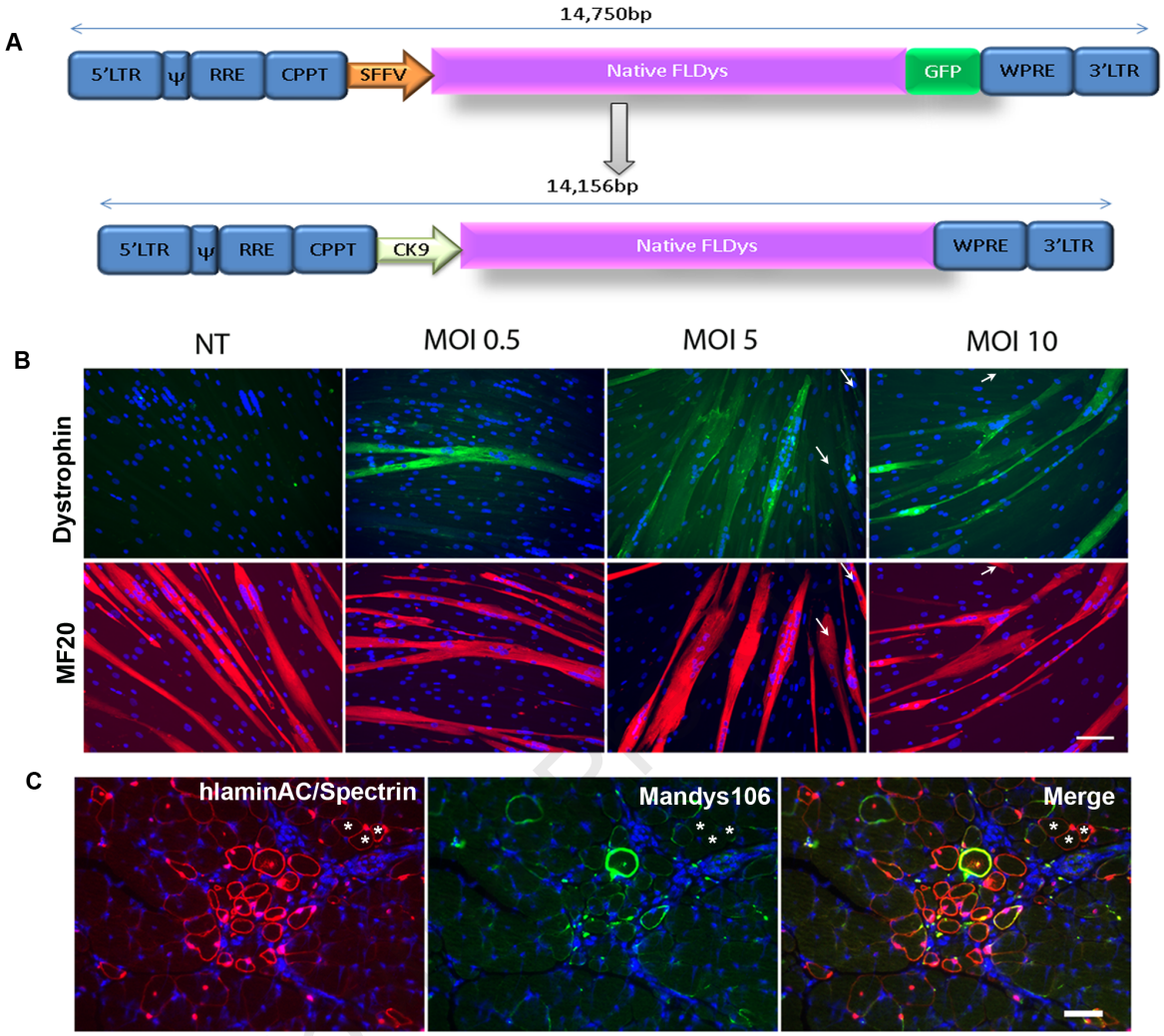
Table 3. Human dystrophin+ fibres in transplanted muscles				
	DMD-nFLDys (mean±SEM)	DMD-soFLDys (mean±SEM)	Normal (mean±SEM)	Statistical analysis
No. Human dystrophin+&human spectrin+ fibres	22.5 ±6.3	55.33±15.19	N.D.	Student t-test p<0.05
% Human dystrophin+/human spectrin+ fibres	44.35%±4.31%	64.44%±2.87%	N.D.	Student t-test p<0.01
Relative intensity of Human dystrophin	1.66±0.15	4.67±0.8	4.05±0.40	One-way ANOVA, p<0.05

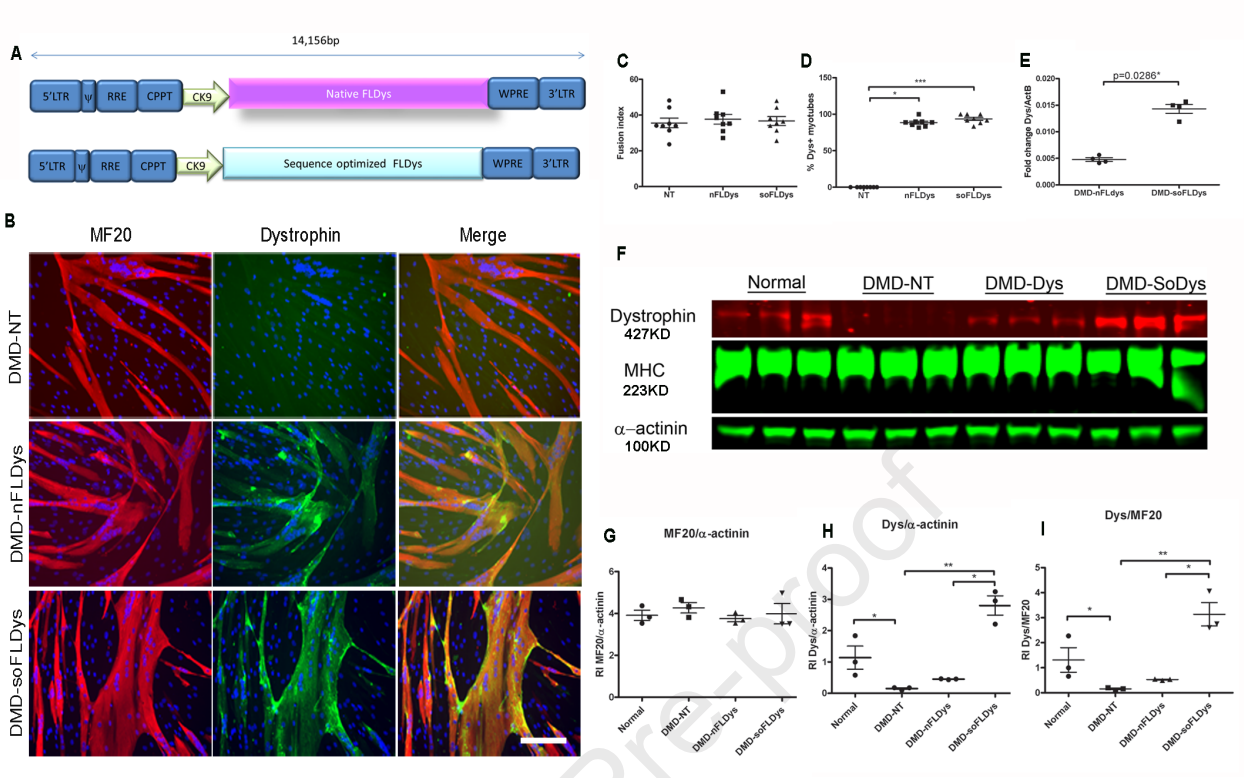
937 N.D. Not determined.

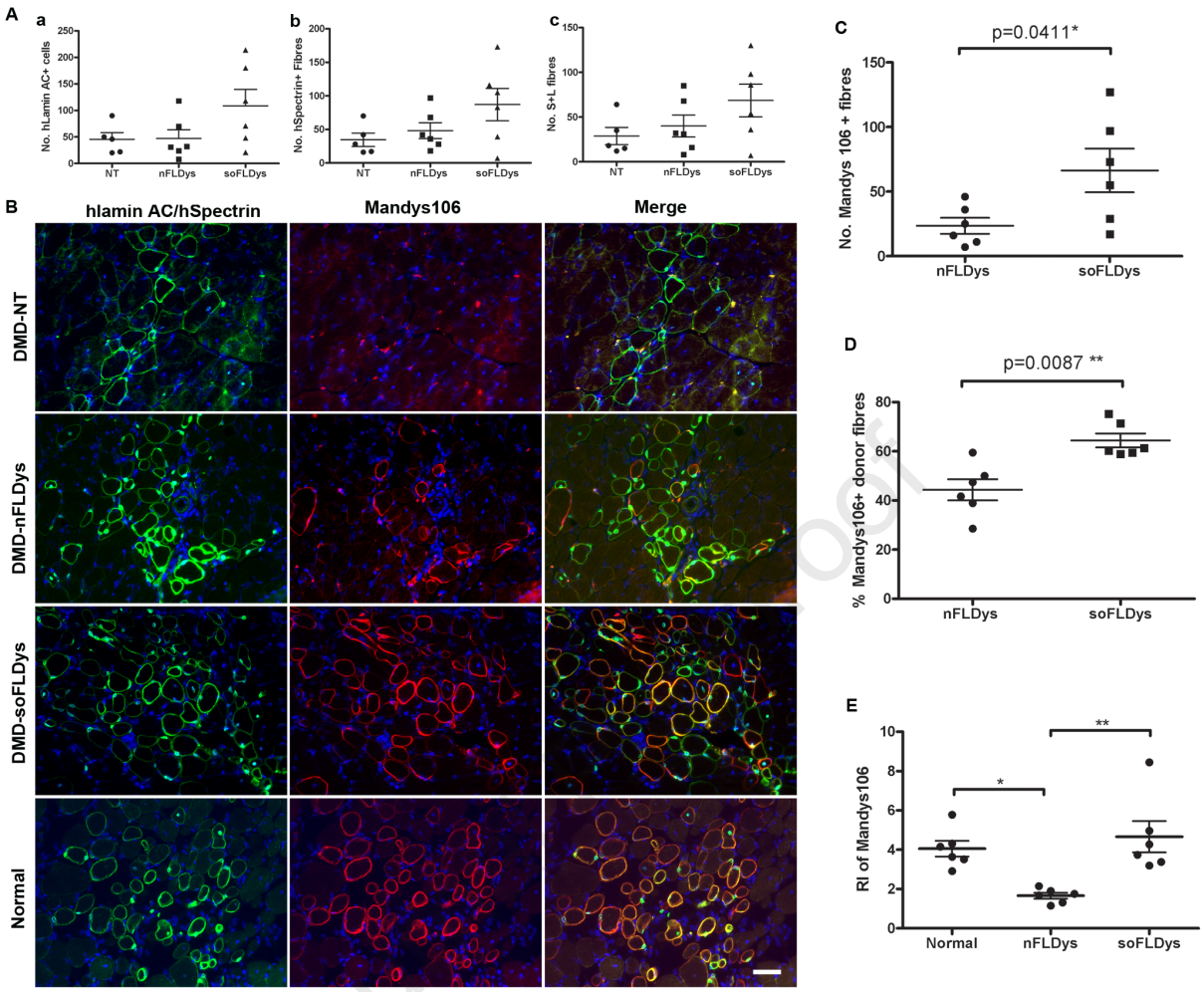
938

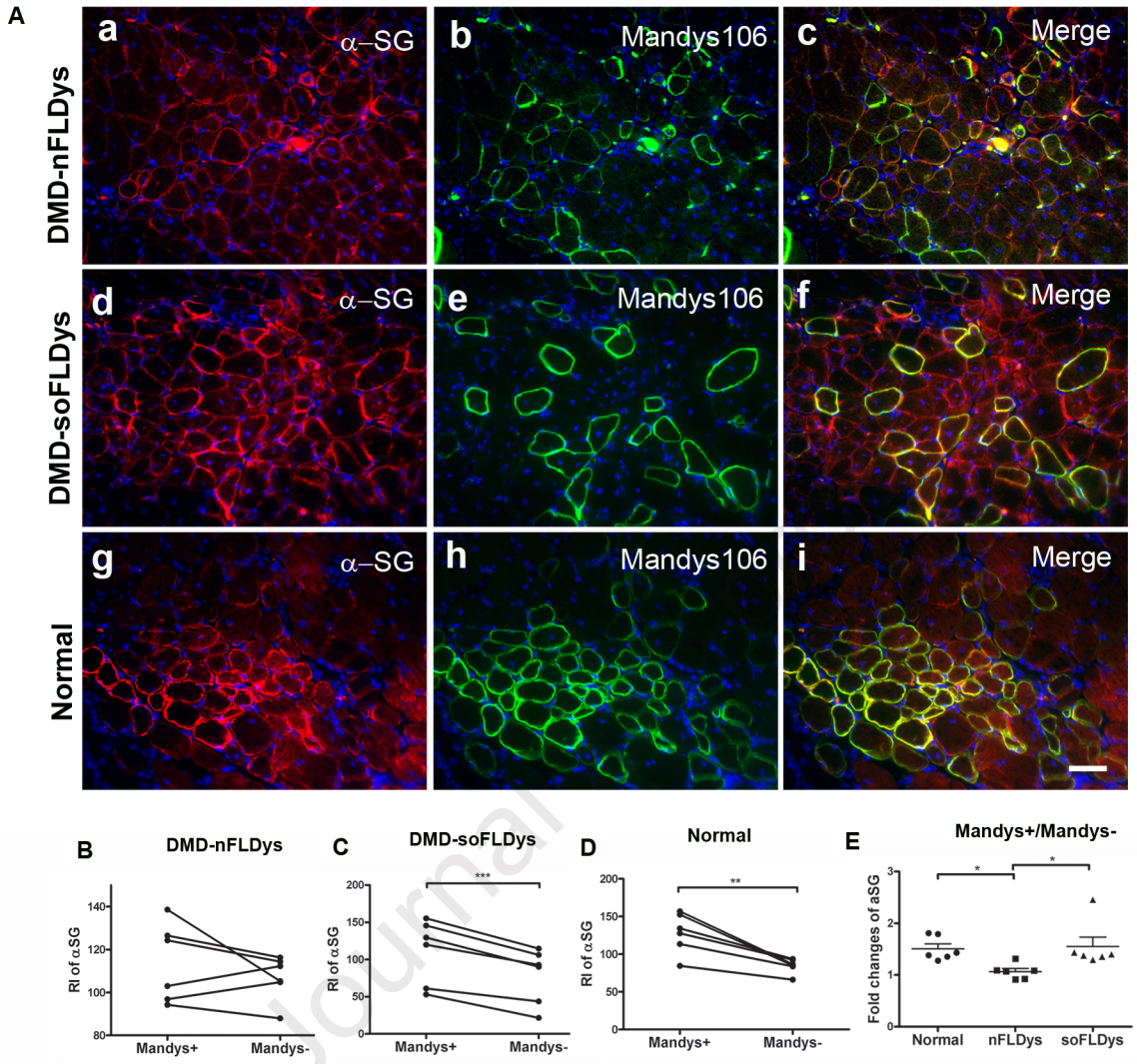


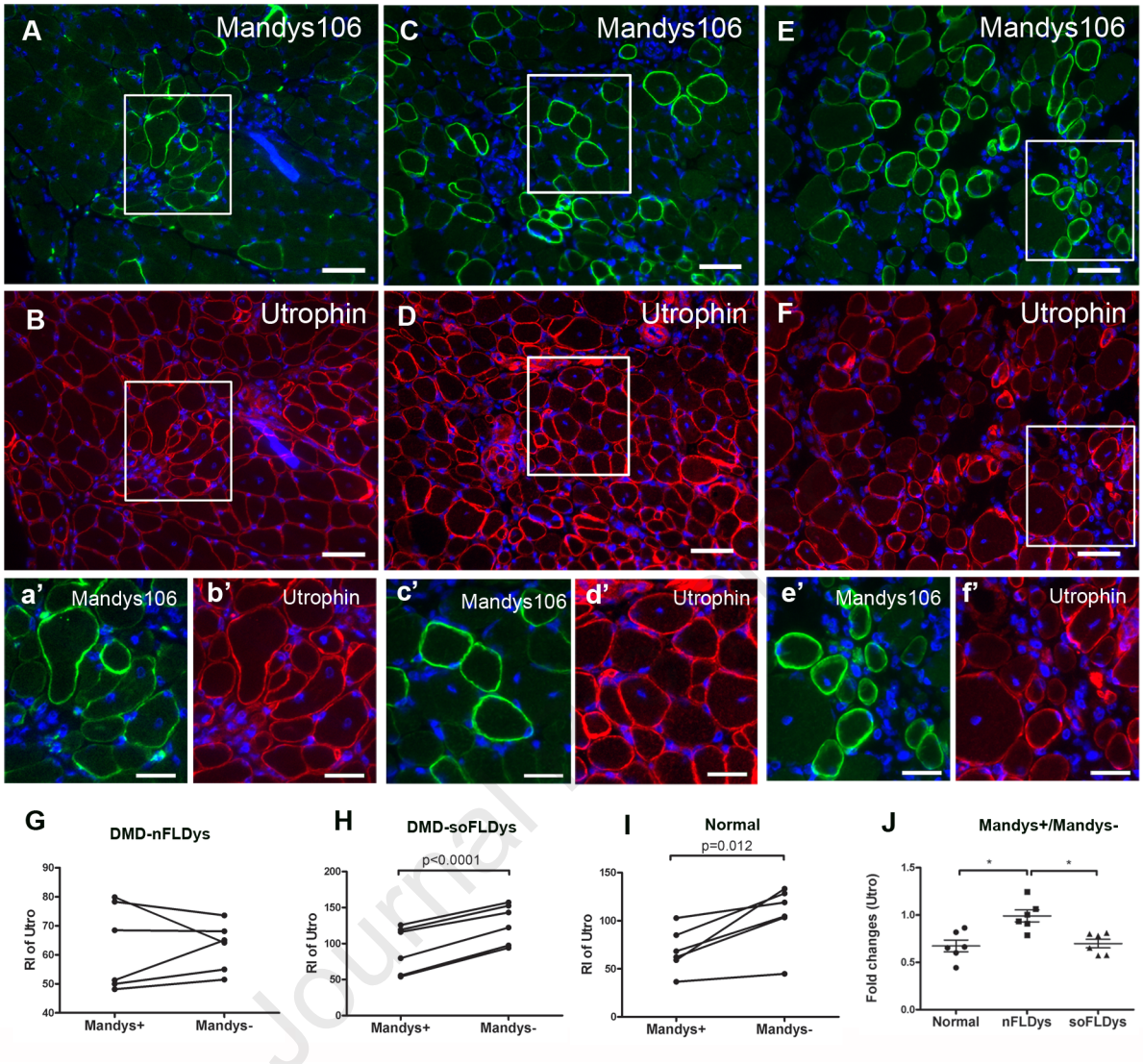



A
14,750bp
5'LTR
ψ
RRE
CPPT
SFFV
Native FLDys
GFP
WPRE
3'LTR
14,156bp
5'LTR
ψ
RRE
CPPT
CK9
Native FLDys
WPRE
3'LTR
B
NT
MOI 0.5
MOI 5
MOI 10
Dystrophin
MF20
C
hlaminAC/Spectrin
Mandys106
Merge




A
a
No. hLamin AC+ cells
NT
nFLDys
soFLDys
b
No. hSpectrin+ Fibres
c
No. S+L fibres
B
hlamin AC/hSpectrin
Mandys106
Merge
DMD-NT
DMD-nFLDys
DMD-soFLDys
Normal
C
p=0.0411*
No. Mandys 106 + fibres
D
p=0.0087 **
% Mandys106+ donor fibres
E
RI of Mandys106
Normal


A
a
α–SG
b
Mandys106
c
Merge
DMD-nFLDys
d
α–SG
e
Mandys106
f
Merge
DMD-soFLDys
g
α–SG
h
Mandys106
i
Merge
Normal
B
DMD-nFLDys
RI of αSG
Mandys+
Mandys-
C
DMD-soFLDys
***
RI of αSG
Mandys+
Mandys-
D
Normal
**
RI of αSG
Mandys+
Mandys-
E
Mandys+/Mandys-
*
*
Fold changes of aSG
Normal
nFLDys
soFLDys


A
Mandys106
C
Mandys106
E
Mandys106
B
Utrophin
D
Utrophin
F
Utrophin
a'
Mandys106
b'
Utrophin
c'
Mandys106
d'
Utrophin
e'
Mandys106
f'
Utrophin
G
DMD-nFLDys
RI of Utro
Mandys+
Mandys-
H
DMD-soFLDys
p<0.0001
RI of Utro
Mandys+
Mandys-
I
Normal
p=0.012
RI of Utro
Mandys+
Mandys-
J
Mandys+/Mandys-
Fold changes (Utro)
Normal
nFLDys
soFLDys

eTOC synopsis:

A lentiviral vector containing a muscle specific promoter and sequence optimized full-length *DMD* was developed. Functional full-length dystrophin is restored in within muscles of a DMD mouse model that had been transplanted with lentivirally corrected DMD myoblasts. This cell mediated gene therapy strategy would be applicable to treat DMD patients.