CRISPR/Cas9-Based Dystrophin Restoration Reveals a Novel Role for Dystrophin in Bioenergetics and Stress Resistance of Muscle Progenitors

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Key Words. CRISPR • Skeletal muscle • Somatic stem cells • Stem/progenitor cell • Stem cells • Tissue-specific stem cells

ABSTRACT

Although the lack of dystrophin expression in muscle myofibers is the central cause of Duchenne muscular dystrophy (DMD), accumulating evidence suggests that DMD may also be a stem cell disease. Recent studies have revealed dystrophin expression in satellite cells and demonstrated that dystrophin deficiency is directly related to abnormalities in satellite cell polarity, asymmetric division, and epigenetic regulation, thus contributing to the manifestation of the DMD phenotype. Although metabolic and mitochondrial dysfunctions have also been associated with the DMD pathophysiology profile, interestingly, the role of dystrophin with respect to stem cells dysfunction has not been elucidated. In the past few years, editing of the gene that encodes dystrophin has emerged as a promising therapeutic approach for DMD, although the effects of dystrophin restoration in stem cells have not been addressed. Herein, we describe our use of a clustered regularly interspaced short palindromic repeats/Cas9-based system to correct the dystrophin mutation in dystrophic (mdx) muscle progenitor cells (MPCs) and show that the expression of dystrophin significantly improved cellular properties of the mdx MPCs in vitro. Our findings reveal that dystrophin-restored mdx MPCs demonstrated improvements in cell proliferation, differentiation, bioenergetics, and resistance to oxidative and endoplasmic reticulum stress. Furthermore, our in vivo studies demonstrated improved transplantation efficiency of the corrected MPCs in the muscles of mdx mice. Our results indicate that changes in cellular energetics and stress resistance via dystrophin restoration enhance muscle progenitor cell function, further validating that dystrophin plays a role in stem cell function and demonstrating the potential for new therapeutic approaches for DMD.

SIGNIFICANCE STATEMENT

Lack of dystrophin in muscle myofibers is the central cause of Duchenne muscular dystrophy (DMD). The most common inherited muscular dystrophy. Accumulating evidence suggests that DMD may also be a stem cell disease. In this study, the authors restored dystrophin in muscle progenitors using gene editing (clustered regularly interspaced short palindromic repeats/Cas9) and enhanced their function, suggesting a novel role of dystrophin in muscle progenitor and potentially new therapeutic approaches for DMD.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common inherited form of muscular dystrophy. It is an X-linked genetic disorder that primarily affects males and results in the lack of expression of dystrophin, a structural myofiber sarclemma protein [1]. Dystrophin deficiency leads to progressive weakening and wasting of skeletal, cardiac, and respiratory muscles in DMD patients and, subsequently, premature death [2, 3]. Despite the lack of dystrophin at birth in DMD patients, the histopathological signs of muscle weakness do not typically become apparent until the patient reaches 4–8 years of age, which happens to coincide with depletion of the muscle progenitor cell (MPC) pool [4, 5]. Stem cell-based therapy is a promising treatment for muscular dystrophy due to its capability to restore dystrophin expression and reconstitute the stem cell pool. Over the last 30 years, multiple cell-based therapies have been used in an attempt to deliver the full-length DMD gene via the fusion of donor cells with host myofibers. Although some of these strategies have proceeded to clinical trials, their success has been limited due to...
poor cell survival [6–11]. Other current therapies for DMD include gene therapies, such as small molecules that target translation termination caused by nonsense mutations [12, 13] or delivery of truncated DMD genes to dystrophic muscle tissue using viral vectors [14–16]. However, various limitations have hindered the widespread application of gene therapy for DMD [17]. Exon-skipping oligonucleotides have been used successfully in a number of animal models and tested in several clinical trials [18–22]. Although these therapeutic designs are promising, the clinical translation of this work has been limited. Postnatal genome editing via clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) or CRISPR technology has emerged as a promising therapeutic approach for DMD and, in preclinical settings, CRISPR has been shown to restore dystrophin in dystrophic muscle and improve muscle function in mdx skeletal muscle [3, 23–25]. Although CRISPR technology represents a valuable therapeutic approach for DMD, it should be noted that most reports on gene editing using viral vectors describe studies performed in young animals and show limited efficiency in aged animals.

The deficiency of dystrophin in myofibers is a generally accepted cause underlying DMD histopathology. However, the muscle wasting observed in DMD patients is a complex process, with repetitive cycles of degeneration followed by regeneration, which consequently exhausts or depletes the functional muscle stem cell pool [4, 5]. Thus, DMD can also be considered a muscle stem cell disease. Indeed, a recent study showed dystrophin expression in satellite cells and revealed a novel role for dystrophin as a key regulator of asymmetric cell division and stem cell function [26, 27]. Dystrophin-null satellite cells exhibit a loss in cell polarity that causes a decrease in the number of myogenic progenitors, resulting in impaired regeneration of dystrophin-null myofibers and progressive muscle loss. In addition, multiple lines of evidence exist that highlight the role of MPC depletion/dysfunction in DMD progression. As mentioned above, the relatively late age of disease manifestation coincides with MPC depletion, despite the lack of dystrophin at birth in DMD patients. In a supporting mouse model, mdx/mTR mice (dystrophin-deficient with telomere dysfunction, specifically in their MPCs) develop a more severe dystrophic phenotype than that of standard mdx mice, which rapidly deteriorates with age due to depletion of MPCs [28]. Similarly, the dystrophin/utrophin double knockout (dKO) mouse, another severely affected model, also features a rapid dystrophic progression that correlates with a defective MPC pool [29, 30]. In addition, a dystrophic muscle microenvironment, such as hypoxia, oxidative and inflammatory stresses, and nutrient deficiency, might exacerbate stem cell depletion/dysfunction due to poor stem cell survival under these adverse conditions. Previous studies have indicated that apoptosis is increased in mdx mouse muscle and in cultured mdx muscle cells [31], and also suggested that cell death in mdx muscle may be initiated by apoptosis and followed by necrosis [32–34]. It has been reported that intracellular adenosine triphosphate (ATP) levels, hypoxia, and/or reactive oxygen species (ROS) can dictate whether a cell dies by a primarily necrotic or an apoptotic pathway [35] or direct muscle regeneration [36]. Taken together, these studies suggest that the occurrence of stem cell dysfunction due to the lack of dystrophin is a major contributing factor to the onset of the pathologic features of muscular dystrophy.

In the dystrophic cell, lack of dystrophin leads to complex pathologic changes that drive skeletal muscle weakness, atrophy, and eventually death [2]. The underlying mechanisms are believed to include calcium overload due to cellular and mitochondrial Ca2+ entry through tears in dystrophin-deficient sarcotubular or activation of calcium leak channels [37–39], as well as mitochondrial dysfunction due to Ca2+ influx through the activation of proteases [40, 41]. However, the effects of dystrophin deficiency on the fundamental aspects of mitochondrial function are not completely understood. Mitochondria play a major role in skeletal muscle energetics due to their primary function of synthesizing ATP by oxidative phosphorylation (oxphos). They also play a pivotal role in muscle cell signaling due to their handling of intramuscular Ca2+ and cell death [42]; studies of the role of mitochondria in Ca2+ handling and necrotic cell death have provided additional insights into abnormal mitochondrial function in DMD. Mitochondrial dysfunction manifests in both prolonged mitochondrial permeability transition pore opening and in the inhibition of mitochondrial ATP synthesis, which promotes cell death [43, 44]. Myoblasts derived from dystrophic (mdx) mice exhibit reduced oxygen consumption, increased mitochondrial membrane potential, and heightened ROS formation [45]. The mitochondrial function may also be impacted by dystrophin deficiency via the disruption of mitochondrial localization, which leads to the uncoupling of oxphos and reducing the maximal rate of ATP synthesis [42]. Several studies have shown an impaired mitochondrial function in the mdx mouse model [46], the dKO model [47], and DMD patients [48]. Dystrophin-mediated repair in DMD cardiomyocytes was shown to mitigate the mitochondrial deficiencies [49]. In addition, recent reports provide compelling evidence that mitochondria represent an important drug target for muscular dystrophy [41, 43, 50–52]. Taken together, these studies demonstrate the impairment of mitochondria in DMD myofibers and emphasize the need for mitochondrial studies in dystrophic MPCs. Fascinating new roles for dystrophin in muscle stem cells have recently emerged [26, 27]; however, these studies did not encompass the modulation of mitochondrial and metabolic processes or stress tolerance conferred by dystrophin.

Here, we hypothesized restoration of dystrophin in MPCs would influence their ability to survive, self-renew, and regenerate myofibers within the diseased microenvironment. We, therefore, tested whether dystrophin restoration using CRISPR/Cas9 editing can, in addition to improving the mechanical properties of myofibers, enhance the energetic properties of mdx MPCs. We found that dystrophin-restored mdx MPCs showed improvements in cell proliferation, differentiation, bioenergetics, and resistance to oxidative and endoplasmic reticulum (ER) stress in vitro. Our in vivo studies revealed improved transplantation efficiency of the dystrophin-restored mdx MPCs in the muscles of mdx mice. This study provides insight into the mitochondrial and metabolic dysfunction of dystrophic MPCs, which may assist in the identification of new therapeutic approaches for DMD.

**Materials and Methods**

**Cloning of sgRNA and CRISPR/Cas9 System Construction**

Four single-guide RNAs (sgRNAs), sgRNA1/2 and sgRNA3/4 (see Supporting Information Table S1), were cloned into a pSpCas9 (BB)-2A-green fluorescent protein (GFP) (PX458) plasmid containing the SpCas9 gene with 2A-enhanced green fluorescent protein (EGFP) and the backbone of the sgRNA (Addgene plasmid #48138). The sequences for sgRNA1/2 have been used.

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Proliferation, Stress Resistance, and Differentiation Assays

Cell proliferation and viability assays were performed using a CellTiter-Blue Cell Kit (Promega, Madison, WI). Cell cycle analysis was performed using flow cytometry cell cycle analysis with DAPI. The proliferation and differentiation potentials of MPCs were analyzed under 20% \( \text{O}_2 \) versus 1% \( \text{O}_2 \), in media containing 400 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (oxidative stress) for 3 hours, and in media containing 10 \( \mu \text{M} \) euyarezstatin for 6 hours to induce ER stress; and they were cultured in myogen, osteogenic, and chondrogenic induction media. Chondrogenic (StemPro Chondrogenesis Differentiation Kit, Gibco) and osteogenic (DMEM, 10% FBS, 50 \( \mu \text{g/ml} \) ascorbic acid, 10 mM \( \beta \)-glycerophosphate, 10 nM dexamethasone) induction media were used for the respective differentiation assays. Alkaline phosphatase (ALP) staining, computed tomography scanning of pellet cultures, and Alcian blue staining were used to confirm differentiation as described previously [56, 57].

Gene Expression Analysis by Quantitative Real-Time Polymerase Chain Reaction

Total RNA from GC muscle or cells was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA). Specific cDNA was synthesized using the iScript Reverse Transcription Kit (Bio-Rad, Hercules, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was performed using an iCycler Thermal Cycler (Bio-Rad). The gene-specific primer sequences used are listed in Supporting Information Table S2. Gapdh and \( \beta \)- actin were used as internal controls to normalize gene expression. All results are expressed as mean \( \pm \) SEM.

Mitochondrial Effects (JC-1 Staining)

Mitochondrial membrane potential was assessed by flow cytometry using tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a cationic, fluorescent dye, (Abcam; Cambridge, MA). Treated cells were loaded with JC-1 dye according to the manufacturer’s instructions. The JC-1 solution was added at equal volumes and incubated in the dark at 37°C for 15 minutes prior to analysis. For the positive control, MPCs were incubated with uncoupler FCCP (carbonyl cyanide 4-fluoromethoxy phenylhydrazone) before the addition of the JC-1 solution. Monomeric (green) and JC-1-aggregate (red) fluorescence was measured by flow cytometry using green (Ex488/Em530) and red (Ex488/Em585) fluorescence spectra, and analyzed following compensation for spectral overlap.

ATP and Adenosine Diphosphatase/ATP Measurements

An adenosine diphosphate (ADP)/ATP ratio bioluminescence assay was used to measure ATP levels and the ADP/ATP ratio (Sigma–Aldrich, St. Louis, MO).

Gas Chromatography–Mass Spectrometry Metabolic Analysis

Gas chromatography–mass spectrometry metabolic analysis (GC–MS) metabolite extraction and analysis were performed at the Metabolomics Core Facility at the University of Utah, as previously described, with minor modifications [55]. Peak intensities of 38 metabolites were obtained for three replicates for each experimental condition.
Western Blotting

Protein extracts from tissues and cultured cells were prepared using a RIPA buffer (Cell Signaling). Protein concentration was determined using the BCA assay (Pierce). Fifty micrograms of total protein per lane were used for differentiated MPCs and muscles from injected adult mice. Samples were denatured at 95°C for 5 minutes before being loaded on to 3%–8% TA precast gels (Invitrogen). Dystrophin and vinculin (loading control) were detected by primary antibodies Mandra1 (1:100, Abcam) and V4505 (1:1,000, Sigma), respectively, followed by horse anti-mouse IgG HRP-conjugated secondary antibody (1:1,000, Cell Signaling Technology). A ChemiDoc imaging system (Bio-Rad) was used to detect chemiluminescence after using a SuperSignal West Dura ECL kit (Thermo Fisher). Intensities of dystrophin and vinculin bands were quantified using ImageJ (NIH) and the gel analysis function.

MPC Transplantation

MPC transplantsations were carried out as previously described [58]. One- to 3-month-old mice were used for the MPC transplantation experiments (n = 4 per group). Mice were housed and maintained in the barrier facility at the University of Texas Health Science Center at Houston (UTHealth) Center for Laboratory Animal Medicine and Care. All experimental studies were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at UTHealth.

Statistical Analysis

Statistical analysis was performed using the statistical software package GraphPad Prism (La Jolla, CA). Statistical differences between groups were determined using an analysis of variance (ANOVA) or a Student’s t test. The number of animals used was determined by power analysis based on a two-sample t test or ANOVA using the type I error (α = 0.05) and type II error (β = 0.8). All data were expressed as the mean ± SD or SEM as indicated. A p-value of <0.05 was considered statistically significant. For GC–MS metabolic data, statistical analysis was performed using the open source software MetaboAnalyst [59] and GraphPad Prism. The values were averaged, normalized by the sum, log-transformed, and scaled by subtracting the mean and dividing by the SD. The p-values were calculated by two-way ANOVA with Tukey’s post hoc test.

RESULTS

Excision of Mutated Exon 23 of the Dmd Gene Restores Dystrophin Expression

Adult somatic mdx MPCs were isolated using the preplate technique based on their slow adherence to collagen-coated flasks, as described previously [53, 60]. The MPCs are multipotent as they form single-cell clones that have been shown to differentiate into muscle, nerve-related cells, bone, and cartilage [53, 61–65]. We restored dystrophin in the mdx MPCs using the CRISPR strategy and following the methodology previously demonstrated as being effective in mdx myofibers and satellite cells [3, 23, 24]. We cloned four sgRNAs (Supporting Information Table S1) that target introns next to the mutated exon 23 of the Dmd mouse gene. All constructs were tested for their ability to produce double-stranded genomic DNA breaks in 3 T3 mouse fibroblasts, both alone by the Surveyor assay and in pairs for deletion of exon 23 by genomic PCR (Fig. 1B and Supporting Information Fig. S1A–S1C). The mdx MPCs were transiently transfected with the CRISPR/Cas9 system constructs that express SpCas9 nuclease, specific sgRNA, and GFP as a reporter. The tested sgRNA pairs produced the anticipated deletion with comparable efficiencies. Thus, for further analysis, we selected edited MPCs that were corrected by the pair of sgRNAs described previously [23], sgRNA1 and sgRNA2 (Supporting Information Table S1). Off-target analysis has previously been performed for these sgRNAs, and no significant off-target mutations were reported [23]. We showed that similar to previously published results [3, 23, 24], the selected sgRNA pair efficiently guided the excision of mutated exon 23 in mdx MPCs (Fig. 1). After sorting MPCs for GFP, we confirmed the efficacy and accuracy of the exon 23 deletion of the Dmd gene using genomic PCR analysis with primers flanking the sgRNA binding sites (Fig. 1B). The amplicon was also sequenced to demonstrate the irreversible genomic correction and the site of non-homologous end joining (Supporting Information Fig. S2). We induced differentiation of the control and corrected mdx MPCs into myotubes, which are known to express dystrophin at high levels, and tested the edits at the transcriptional level by quantitative RT-PCR. Our results showed that ~65% of the transcripts had exon 23 excised. Control RT-PCR using primers amplifying a nonmutated region of exon 45 showed the Dmd transcript at similar levels in the mdx and dystrophin-restored myotubes (Fig. 1C). The transcription levels of the myogenic regulatory factor Myf5 were used as a positive control for myogenesis (Fig. 1C). Furthermore, to determine whether genomic deletion led to the restoration of the dystrophin protein expression, we induced myogenic differentiation of the edited MPCs and measured dystrophin expression. Western blot analysis revealed expression of the restored dystrophin protein, which migrated at ~430 kDa (as expected; Fig. 1D). Immunostaining also confirmed comparable dystrophin expression in WT and dystrophin-restored mdx myotubes (Fig. 1E). The genomic editing of MPCs using sgRNA3 and sgRNA4 produced similar results (Supporting Information Fig. S2).

CRISPR/Cas9-Mediated Dystrophin Restoration Improves Multilineage MPC Differentiation

We sought to evaluate how dystrophin restoration affects the multilineage differentiation of mdx MPCs. We performed semi-quantitative RT-PCR on modified and control MPCs after 24-hour cultivation in myogenic differentiation media. Analysis of gene expression revealed increased mRNA levels in dystrophin-restored MPCs for multiple myogenesis markers, such as Pax3 (p = .017), Pax7 (p = .02), and Myf5 (p = .02), indicating that the myogenic differentiation potential was increased after dystrophin restoration (Fig. 2A). Myod and Myog, late markers of myogenesis, were not significantly upregulated (Fig. 2A). In addition, we evaluated the chondrogenic and osteogenic potential of dystrophin-restored MPCs. After mdx and dystrophin-restored mdx MPCs were cultured in chondrogenic and osteogenic differentiation induction media for 3 days, both chondrogenesis and osteogenesis were increased in dystrophin-restored mdx MPCs when compared with unmodified mdx MPCs. Gene expression analysis revealed increased mRNA levels for multiple chondrogenic markers, such as aggrecan (Acan, p = .038), collagen type II α 1 (Col2a1, p = .001), Sox5 (p = .0175), and Sox9 (p = .0007), and osteogenic markers, such as Alp (p = .01), collagen type I α 1 (Col1a1,
p = .03), Col2a1 (p = .0013), Osterix (OSX, p = .02), osteocalcin (OC, p = .002), runt-related transcription factor 2 (Runx2, p = .0005), and sirtuin 1 (Sirt1, p = .002). We further confirmed our results using in vitro staining for myosin heavy chain (MyHC) and ALP as myogenic and osteogenic markers, respectively. Immunofluorescence microscopy scoring showed a 10% increase in the fusion of myotubes with dystrophin-restored mdx MPCs compared with unmodified mdx MPCs after a 5-day myogenic induction in vitro (Fig. 2B). ALP staining following a 5-day osteogenic induction in vitro confirmed a significant increase in osteogenic differentiation (Fig. 2C). We have confirmed the enhanced differentiation potential using osteogenic (Fig. 2D) and chondrogenic (Fig. 2E) pellet cultures. Together, these results indicate that dystrophin-restored mdx MPCs via CRISPR/Cas9
editing have improved multipotent differentiation potentials for myogenic, osteogenic, and chondrogenic lineages.

**Increased Stress Resistance and Proliferation of Dystrophin-Restored mdx MPCs**

To further understand the changes associated with dystrophin restoration in mdx MPCs, we investigated their proliferative and stress resistance properties and compared the results with those of unmodified (control) mdx MPCs. In particular, we were interested in characterizing cell survival and proliferation under stress conditions that are typical of dystrophic muscle, such as conditions of low oxygen, oxidative stress, and protein synthesis stress. The cell viability assay showed no changes in growth under normal growth conditions with 21% oxygen. Cell viability assay showed no changes in growth under normal growth conditions with 21% oxygen.
growth for dystrophin-restored MPCs was also improved by roughly 40% under hypoxic conditions (O₂ 1%), when compared with control mdx MPCs (Fig. 3A). In addition, following exposure to oxidative (hydrogen peroxide, H₂O₂) and ER stress in dystrophin-restored mdx MPCs, the viability was higher than that of unmodified mdx MPCs by 36% (p = .02) (Fig. 3C) and 28%
Dystrophin restoration improves 
mdx MPCs' resistance to stress (Fig. 3C, 3D). Notably, dystrophin-restored 
mdx and WT MPCs showed no statistically significant differences in viability after the stress
treatments (Fig. 3C, 3D). Flow cytometry cell cycle analysis showed an increased percentage of G2/M cells in dystrophin-
restored 
mdx MPCs compared with control 
mdx MPCs, indicating faster cell growth and a higher rate of proliferation (Fig. 3B).

Figure 4. Dystrophin restoration repressed oxidative stress in 
mdx muscle progenitor cells (MPCs). Analysis of mitochondrial membrane potential (ΔΨm) measured by flow cytometry (JC-1 aggregate emits red fluorescence) showed notable decreases in ΔΨm (~40%, ****, p < .0001) for dystrophin-restored (mdx + clustered regularly interspaced short palindromic repeats [CRISPR]) MPCs compared with control 
mdx MPCs. JC-1 monomer mean fluorescence intensity (MFI) measurements (emits green fluorescence) were not significantly different for 
mdx versus 
mdx + CRISPR MPCs. The mean fluorescence intensity (MFI) of samples treated with a known uncoupler (FCCP) was used as a positive control for ΔΨm loss.

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Figure 5. Dystrophin restoration improved mitochondrial function in mdx muscle progenitor cells (MPCs). (A): A bioluminescence assay was used to measure adenosine diphosphate (ADP) and adenosine triphosphate (ATP) levels (relative light units per well) in mdx and mdx + clustered regularly interspaced short palindromic repeats (CRISPR) cells. Intracellular ATP content was increased by 20% in dystrophin-restored cells relative to mdx MPCs; **, p < .01. The ADP/ATP ratio was significantly lower in mdx + CRISPR cells, indicating lower apoptosis; ****, p < .0001. (B): To characterize the effects of dystrophin restoration on respiration, oxygen consumption rate (OCR) was measured using a Seahorse Bioscience XF24 extracellular flux analyzer. Dystrophin restoration increased basal OCR, ATP production, and maximal respiratory capacity in MPCs. Spare respiratory capacity and mitochondrial coupling were improved as well. Four replicate wells (50–60 x 10^5 cells per well) were analyzed. OCR values were normalized to the number of cells per well. (C): Gas chromatography–mass spectrometry-based metabolomics analysis of mdx, dystrophin-corrected, and wild-type MPCs. Semiquantitative analysis revealed that levels of the tricarboxylic acid cycle intermediates citrate (p = .03) and malate (p = .0069) were increased in dystrophin-restored MPCs. Peak intensities of 38 metabolites were obtained from each of three replicates per metabolite. The values were averaged for each metabolite, normalized by the sum and log-transformed, and then scaled by subtracting the mean and dividing by the SD. The p-values were calculated by one-way analysis of variance with Tukey’s post hoc test; SD is shown with bars.
Improved Mitochondria Function and Repressed Oxidative Stress in Dystrophin-Restored mdx MPCs

The dystrophic microenvironment is characterized by a high level of oxidative stress that is known to cause damage to mitochondria due to ROS, thus disrupting overall energy generation in the cell. In addition, dysregulated mitochondrial respiration might contribute to higher levels of ROS as well as promote further damage in the dystrophic cell. To investigate whether CRISPR/Cas9-mediated gene editing can boost the cell survival properties of mdx MPCs in the dystrophic microenvironment, we measured the ROS levels in dystrophin-restored mdx MPCs, mdx MPCs, and WT control MPCs. The mdx cells had significantly higher levels of total reactive oxygen/nitrogen species (ROS/RNS) than did WT MPCs (p = .0001, Fig. 3E). However, the observed higher level of
ROS/RNS production by mdx MPCs was decreased by ~10% in dystrophin-restored mdx MPCs (p = .0019, Fig. 3E). Although, the levels of intracellular H₂O₂, a type of ROS, were not significantly different in mdx MPCs compared with dystrophin-restored mdx MPCs (data not shown).

To further examine mitochondrial function, we assessed the transmembrane potential (ΔΨm). Mitochondrial ΔΨm is critical for maintaining the physiological function of the respiratory chain necessary to generate ATP. In mdx myoblasts, the observed mitochondrial ΔΨm is known to be adversely elevated when compared with WT myoblasts [45]. Analysis of mitochondrial ΔΨm measured by flow cytometry (aggregate red fluorescence) showed a notable decrease in dystrophin-restored mdx MPCs compared with control mdx MPCs, with a substantial decrease in MFI by ~40% compared with control mdx MPCs. The MFI of samples treated with a known uncoupling agent, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), was used as a positive control for ΔΨm loss (Fig. 4). In summary, we observed a significant normalization of mitochondrial ΔΨm in mdx MPCs after dystrophin restoration.

To corroborate the improvements in mitochondrial function after dystrophin restoration in mdx MPCs, we measured ADP and ATP levels in control mdx and dystrophin-restored mdx MPCs. First, we used a bioluminescence assay and showed a 20% increase in ATP levels in dystrophin-restored MPCs (p = .0048). Control mdx MPCs had a dramatically higher ADP/ATP ratio, which may be indicative of increased levels of apoptosis (Fig. 5A). Lower ATP levels in mdx MPCs reinforce the idea that the lack of dystrophin compromises overall energy production; our results support this and indicate that dystrophin restoration reverses the effect.

Based on the effects of dystrophin restoration on bioenergetic characteristics, such as ΔΨm, ROS and ATP production, and changes in cellular growth, we further investigated modulations of mitochondrial function, particularly mitochondrial respiration (Fig. 5B). We evaluated the oxygen consumption rate (OCR), a measure of overall mitochondrial respiration, and characterized the effect of dystrophin restoration on OCR. We observed that dystrophin restoration caused a significant increase in basal mitochondrial respiration (p = .0012). In addition, ATP production (p < .0001; which was calculated as the difference between the basal level and OCR after oligomycin treatment) and maximal respiratory capacity (p = .0013; which was calculated after FCCP was added) were restored to levels closer to those measured in WT control MPCs. The spare respiratory capacity, calculated as the difference between basal and maximal respiration, was improved (p = .0044), along with the mitochondrial coupling efficiency (p < .0001) in the dystrophin-corrected mdx MPCs. (Fig. 5B). To investigate whether restoration of dystrophin in MPCs can improve bioenergetics properties in differentiated MPCs (myotubes), we measured OCR in differentiated myotubes (Supporting Information Fig. S3) and observed similar results as mdx undifferentiated MPCs (Fig. 5).

To corroborate our findings, we performed GC–MS-based metabolomics analysis by collecting peak intensities of 38 metabolites from three replicates of cultured mdx, dystrophin-restored, and WT MPCs. The semiquantitative analysis showed that levels of Krebs cycle intermediates, such as citrate (p = .0124) and malate (p = .0328), were significantly decreased in mdx MPCs compared with the WT control cells, which indicates lower levels of tricarboxylic acid (TCA) cycle activity. Dystrophin restoration appeared to repair the TCA cycle and restore levels of these important Krebs cycle metabolites, as levels of citrate (p = .03) and malate (p = .0069) were increased in dystrophin-restored mdx MPCs compared with WT MPCs (Fig. 5C). Collectively, our data show a significant improvement in mitochondrial function associated with dystrophin restoration in mdx MPCs, as determined by metabolic measurements.

**Improved Engraftment of Dystrophin-Restored mdx MPCs**

Finally, we evaluated the regenerative potential of gene-corrected mdx MPCs in vivo. Either the corrected or uncorrected mdx MPCs, each labeled with a Lenti-LacZ marker, were intramuscularly injected into the GC muscle. The GC muscles were harvested 3 weeks following transplantation, and frozen sections were evaluated for engraftment using beta-galactosidase and anti-dystrophin antibody staining. Although no dystrophin expression was detected in control-injected muscles (mdx), except for occasional revertant fibers, muscles that had been injected with CRISPR/Cas9-corrected cells (mdx + CRISPR) generated engrafted areas with dystrophin-positive myofibers (Fig. 6A). Histomorphometric analysis based on the LacZ-positive area of grafts confirmed the significantly greater cell engraftment for dystrophin-restored mdx MPCs (Fig. 6B). The numbers of Pax7+ satellite cells were dramatically increased proximal to the modified dystrophin-positive myofibers relative to mdx control myofibers (0.6 Pax7+ cells per myofiber vs. 0.06 in controls; p = .0036). These data (Fig. 6C, 6D and Supporting Information Fig. S4) indicate that gene-corrected mdx MPCs transplanted into mdx skeletal muscle may contribute to satellite cell compartment directly or indirectly.

**DISCUSSION**

Myofiber dystrophin deficiency is generally accepted as a cause of DMD histopathology [2]. The muscle wasting process observed in DMD patients is complex; in addition to causing muscle fragility, DMD is also a muscle stem cell disease. A recent study showed dystrophin expression in satellite cells and revealed a novel role of dystrophin in stem cell function [26, 27]. Herein, we provide additional evidence and fresh insight into the autonomous defects of dystrophic stem cells in muscular dystrophy. Our findings provide further details and previously undescribed effects of dystrophin deficiency in MPCs with respect to bioenergetics and stress resistance. Here, we demonstrate improvements in dystrophin-restored mdx MPC characteristics, such as cell proliferation, differentiation, and stress resistance in vitro, as well as enhanced survival of these modified MPCs upon transplantation in vivo and their ability to regenerate dystrophic muscle. To our knowledge, the only published study of dystrophin restoration in muscle stem cells derived from an mdx mouse model showed successful restoration, but did not describe the effects of such restoration on the intrinsic properties of the stem cells [38]. It has been reported that, for human DMD cardiomyocytes, some of the dystrophin corrections by CRISPR-Cpf1 also led to improved mitochondrial function [49]. Further research is necessary to determine if the approach for dystrophin restoration used in the current study will confer similar benefits to human MPC properties.

Metabolic dysregulation and mitochondrial dysfunction are important components of the DMD phenotype. However, previous studies have primarily focused on skeletal muscle as a whole,
or only on myoblasts or myofibers at the cellular level [47]. Mitochondrial dysfunction has been attributed to several mechanisms, such as mislocalization of mitochondria within muscle fibers and the functional aberration of mitochondria due to calcium overload [46]. Even though our in vitro studies revealed no obvious changes in mitochondrial localization between the mdx, dystrophin-restored, and WT MPCs, future studies in vivo are necessary to confirm the unchanged mitochondria localization in dystrophin-restored MPCs. Interestingly, our in vitro studies clearly demonstrated improvement in mitochondrial function following dystrophin restoration, such as increased ATP production and both basal and maximal OCR, as well as higher concentrations of TCA cycle metabolites.

We demonstrated that dystrophin-restored MPCs exhibit an improved growth curve, enhanced proliferation, and lower levels of cell death, especially when cultivated under stressful conditions. The improvement in growth under conditions of stress, such as oxidative and ER stress, underlines the importance of dystrophin for MPC survival and self-renewal upon transplantation to dystrophic muscle. Dystrophic muscle is characterized by a micromilieu with high levels of inflammation and oxidative stress. The gene-edited MPCs appeared to possess an enhanced capability for handling ROS as well. One might speculate that dystrophin may be involved in the regulation of mitochondrial function through improved calcium channeling by mitochondria. This could be the result of the restored mitochondrial capacity to buffer Ca++ or a reduction in the activity of Ca++-dependent processes, which are analogous to mechanisms proposed in prior studies [59]. However, in satellite cells, dystrophin does not function in its typical fashion as a stabilization protein on the intracellular side of the cell membrane. Notably, the dystrophin-glycoprotein complex of satellite cells has been demonstrated to have only basal location [26]. Thus, the underlying mechanisms by which dystrophin impacts mitochondrial processes are beyond the scope of this work and need to be elucidated. Nevertheless, our in vivo results provide strong evidence to support our in vitro findings—that is, restoring dystrophin in MPCs improves their ability to survive, self-renew, and regenerate myofibers within the diseased micromilieu.

CONCLUSION

In summary, the current study provides proof-of-concept evidence supporting the efficacy of ex vivo genome editing to correct detrimental mutations in dystrophic MPCs and fosters the apparent need for further investigation of new approaches for stem cell therapy. Despite being a heavily explored approach, stem cell therapy has failed during clinical translation due to many limitations, including poor cell survival. Progress in this direction will have a direct impact on improving current clinical therapeutic modalities and outcomes for DMD patients, potentially by adding novel dystrophin restoration methods using CRISPR/Cas9 to therapeutic approaches to reduce stem cell depletion. The demonstrated improvements in survival, proliferation, and differentiation of dystrophin-restored mdx MPCs are remarkable and should have significant impact not only on the development of new therapies for the treatment of DMD, but also on our efforts to further understand the role of dystrophin in muscle stem cells and stem cell biology.

ACKNOWLEDGMENTS

This work was supported, in part, by startup funding from the University of Texas Health Science Center at Houston, Houston, TX, and by a philanthropic gift from the Shear Family Foundation to the Steadman Philippon Research Institute, Vail, CO. The authors also thank Dr. Aiping Lu, Dr. Chieh Tseng, and Ms. Haiying Pan for scientific expertise and technical support.

AUTHOR CONTRIBUTIONS

P.R.M.: study concept and experimental design, and contributed experiments, data analysis and interpretation, writing of the manuscript, and review of the manuscript and concur with the content; X.M., J.W., D.D.: experiments, data analysis, scientific consultation, and review of the manuscript and concur with the content; M.A.H.: manuscript writing, editing, scientific consultation, and review of the manuscript and concur with the content; M.G.K., R.D., J.H.: experimental design, scientific consultation, and review of the manuscript and concur with the content.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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