An ex vivo gene therapy approach to treat muscular dystrophy using inducible pluripotent stem cells

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Duchenne muscular dystrophy is a progressive and incurable neuromuscular disease caused by genetic and biochemical defects of the dystrophin–glycoprotein complex. Here we show the regenerative potential of myogenic progenitors derived from corrected dystrophic induced pluripotent stem cells generated from fibroblasts of mice lacking both dystrophin and utrophin. We correct the phenotype of dystrophic induced pluripotent stem cells using a Sleeping Beauty transposon system carrying the micro-utrophin gene, differentiate these cells into skeletal muscle progenitors and transplant them back into dystrophic mice. Engrafted muscles displayed large numbers of micro-utrophin-positive myofibers, with biochemically restored dystrophin–glycoprotein complex and improved contractile strength. The transplanted cells seed the satellite cell compartment, responded properly to injury and exhibit neuromuscular synapses. We also detect muscle engraftment after systemic delivery of these corrected progenitors. These results represent an important advance towards the future treatment of muscular dystrophies using genetically corrected autologous induced pluripotent stem cells.
Pluripotent stem cells are particularly attractive for therapeutic application as these cells are endowed with robust expansion potential, enabling the generation of large quantities of tissue-specific stem/progenitor cell preparations. This is extremely beneficial as cell number has been one of the main barriers to implementing cell-based therapies. Induced pluripotent stem (iPS) technology\textsuperscript{1–4} further allows for the derivation of patient-specific pluripotent stem cell preparations without the ethical and immunological concerns associated with human embryonic stem (ES) cells. In the case of muscular dystrophies, either allogeneic or autologous cell transplantations have the potential to lead to an effective treatment. For autologous transplantation, one would utilize iPS-derived myogenic progenitors obtained from a healthy HLA-matched donor, which following transplantation would give rise to new healthy myofibers, as well as fuse to the recipient’s myofibers, to generate hybrid myofibers that express dystrophin. The autologous approach would require \textit{ex vivo} genetic correction of dystrophic iPS cells before transplantation. Despite extensive research in iPS technology, to date there are only two reports on the therapeutic potential of disease-specific iPS cells that have been genetically corrected, transplanted back into an appropriate mouse model and shown to provide therapeutic benefit: for sickle cell anemia\textsuperscript{5} and β-thalassemia\textsuperscript{6}. Genetic correction for muscular dystrophies has been tested with some success in mesangioblasts\textsuperscript{7–9}; however, these somatic cells have reported limited expansion potential\textsuperscript{10}. The deficit in translational studies involving genetically corrected iPS cells is most likely because of the difficulty in coaxing pluripotent stem cells to become lineage-specific stem cells that are able to produce functional tissue \textit{in vivo}.

We developed and continued to improve a method to efficiently generate skeletal muscle stem/progenitor cells endowed with significant regeneration potential through Pax3 or Pax7\textsuperscript{+} induction in control ES and iPS cells\textsuperscript{11–14}. In the present study, we show proof-of-principle for the \textit{ex vivo} gene correction of mouse dystrophic iP\textsubscript{S} cells. We inserted the micro-utrophin (\textmu UTRN) gene into iPS cells obtained from dystrophin/utrophin double knockout (dKO) mice using the \textit{Sleeping Beauty} transposon (Tn) system, and demonstrated that skeletal myogenic progenitors derived from gene-corrected iPS cells have the capacity to promote substantial muscle regeneration \textit{in vivo} when transplanted back in dKO mice, and that engraftment is accompanied by functional improvement.

**Results**

Characterization and correction of dystrophic iPS cells. To address the feasibility of using disease-specific iP\textsubscript{S} cells and genetic correction in the context of Duchenne muscular dystrophy (DMD), we utilized the dystrophin/utrophin dKO mouse model as the source of tail-tip fibroblasts (TTFs), as well as recipients of iPS-derived therapeutic myogenic cell preparations (Fig. 1a). This choice was based on the fact that \textit{mdx} mice, although a favourite model for DMD\textsuperscript{15}, present a mild phenotype, attributed to compensatory overexpression of the dystrophin-related protein, utrophin\textsuperscript{16}. The dKO mice, which lack both dystrophin and utrophin, present a severe phenotype characterized by progressive muscle wasting, impaired mobility, abnormal breathing pattern, cardiomyopathy and premature death\textsuperscript{17,18}, which more closely resembles DMD in human patients. To restore the dystrophin-glycoprotein complex (DGC), we chose to re-express a µUTRN transgene, which has been shown to ameliorate the dystrophic phenotype\textsuperscript{19–22}. This approach tests a vector that would potentially be preferred in human patients because it would avoid the immune response that is elicited by the dystrophin-naive immune system (the mouse model tests function of µUTRN in this context, but not immune aspects). As summarized in Fig.1a, the therapeutic strategy applied in the present study involved (1) reprogramming of dystrophic donor fibroblasts into iP\textsubscript{S} clones, (2) genetic repair of selected iP\textsubscript{S} clones with µUTRN using the non-viral \textit{Sleeping Beauty} Transposon (Tn) System, (3) \textit{in vitro} differentiation of corrected iP\textsubscript{S} cell clones into myogenic progenitors and (4) transplantation of corrected myogenic precursors into dystrophic dKO mice (mdx; \textit{utrn}\textsuperscript{−/−}).

Dystrophic iP\textsubscript{S} cells were generated by retroviral transduction of TTF cells using Oct4, Klf4 and Sox2 (ref. 23). iP\textsubscript{S} clones were initially screened by morphology, SSEA-1 expression and alkaline phosphatase activity (Supplementary Fig. S1a,b). Based on these markers, a subset of clones was selected for additional analysis, which included immunofluorescence staining to pluri potency markers (Supplementary Fig. S1c), as well as assessment of their ability to form embryonic mesoderm following \textit{in vitro} differentiation into embryoid bodies (EBs), as evidenced by Fk-1 and PDGFrα expression (Supplementary Fig. S1d–e). After this analysis, one iP\textsubscript{S} clone (C3) was chosen for the studies presented here. These cells displayed normal karyotype and exhibited the ability to develop typical teratomas (Supplementary Fig. S1f,g). Importantly, following EB differentiation, iP\textsubscript{S} cells showed downregulation of pluripotency markers and normal
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Control iPS

IresGFP

Pax3 Ires Cherry

μUTRN Ires GFP

μUTRN

Uncorrected Flk-1

PDGFαR

Ref 1

D5 EBs

Dox (D3-D5)

(3)

iPax3 corrected-iPS

Pax3 Ires Cherry

Corrected iPS cells

μUTRN Ires GFP

Corrected myogenic precursors

μUTRN

Dif

Proliferation

Differentiation

250 kDa

150 kDa

50 kDa

37 kDa
expression of imprinted genes (Supplementary Fig. S1h–i, respectively), which has been previously shown to distinguish fully reprogrammed iP5 cells.24

Dystrophic iP5 cells were then corrected with the μUTRN transgene (R4–R21). This gene is a contracted version of UTRN-lacking sequences encoding spectrin-like repeats 4 through 21, but containing the N-terminal domain that binds to F-actin, and the C-terminal domain (C/C cysteine-rich domain) that interacts with the dystrophin–glycoprotein complex. This gene complements both the loss of dystrophin and utrophin.20 The Sleeping Beauty Tn system has several advantages including efficient gene transfer and stable gene expression in mouse and human ES cells25–26, and reduced likelihood of insertional mutagenesis.27 We developed a T2-inverted terminal repeat Tn vector (pKR2/μUTRN) carrying a 7.3-kb engineered transgene containing μUTRN and an iresGFP reporter (Fig. 1b), which allows for FACS selection of μUTRN-corrected iP5 cells. SB100X (Fig. 1b) is a recently engineered hyperactive variant that yields high levels of Tn integration, leading to efficient and stable gene transfer.28 We observed a stable gene-transfer frequency of 0.3%, 1 week after microinjection with Tn and transposase vectors (Fig. 1c). Green fluorescent protein (GFP+) cells were sorted, expanded and re-sorted, giving rise to a homogeneous and stable population of μUTRN-corrected iP5 cells (>90% GFP+) (Fig. 1d, right panel). The expression of μUTRN was confirmed by quantitative PCR (Supplementary Fig. S2a). Corrected iP5 cells maintained a normal karyotype (Supplementary Fig. S2b).

Skeletal myogenic progenitors from gene-corrected iP5 cells. To enable the efficient generation of skeletal myogenic progenitors, both μUTRN-corrected and control (uncorrected) iP5 cells (Fig. 1d) were modified to allow doxycycline-regulated conditional Pax3 expression, which we have previously shown allows the derivation of repopulating myogenic progenitors from ES cells12 (Supplementary Fig. S3a). Pax3+ cells (mCherry+) were detected only when dox was added to the culture medium (Supplementary Fig. S3b). This has been confirmed by western blot analysis (Supplementary Fig. S3c). To isolate skeletal myogenic progenitors, Pax3 was induced from day 3 to day 5 of EB differentiation, at which point paraxial mesoderm progenitors were isolated based on the expression of PDGFRα and lack of Flk-1 (Fig. 1e, left panels).12 In the case of corrected iP5-derived EBs, cells were further purified based on positivity for GFP, which is co-expressed with μUTRN (Fig. 1e, lower middle panel). Because Pax3 is co-expressed with mCherry, both control and corrected dox-induced EBs were mCherry+ (Fig. 1e, right panels), whereas non-induced control counterparts were mCherry− (Supplementary Fig. S3d).

PDGFRα+Flk-1−GFP−-sorted cell preparations from corrected iP5-derived EBs (Fig. 1e) were expanded in the presence of basic fibroblast growth factor (bFGF) and doxycycline. Proliferating myogenic progenitors emerged from these cultures (Fig. 1f, left panels). When differentiation was induced in vitro, these had the ability to undergo final maturation, giving rise to multinucleated myotubes (Fig. 1f, right panels) that exhibited elevated fusion index (82±8%; Supplementary Fig. S3e) (details in methods). Importantly, abundant and stable expression of μUTRN was observed in corrected myogenic progenitors and their derivative myotubes, as shown by immunofluorescence staining (Fig. 1g), quantitative PCR (Fig. 1h) and western blot analysis (Fig. 1i). No utrophin was detected in control uncorrected myogenic preparations (Fig. 1i).

As anticipated, proliferating myogenic progenitors were characterized by abundant expression of Pax3 as well as MyoD (Supplementary Fig. S4a). Under these conditions, MyoD was observed in fewer nuclei, whereas terminal differentiation markers, such as MyHC, were barely detected (Supplementary Fig. S4a). This profile changed upon induction of myogenic differentiation, as multinucleated myotubes expressed high levels of MyHC and MyoD, and displayed significant downregulation of Pax3 and Myf5 (Supplementary Fig. S4b). These results were further confirmed by real-time RT–PCR (Supplementary Fig. S5). Flow-cytometric analysis of μUTRN-corrected myogenic progenitors showed homogeneous expression of M-cadherin (99%), CD56 (82%), VCAM1 (85%), SYN-D (98%) and CXCR4 (98%) (Supplementary Fig. S4c), surface markers characteristic of satellite and myogenic progenitor cells.29

Regenerative potential of gene-corrected myogenic progenitors. To assess whether these corrected autologous cells would engraft and differentiate into muscle in vivo, μUTRN-corrected iP5-derived myogenic progenitors were transplanted into the left tibialis anterior (TA) muscles of 3-week-old dKO mice, whereas the contra-lateral TA (right) was injected with PBS. Because the dKO strain presents a much more severe phenotype, we did not pre-injure with cardiotoxin (CTX) to enhance engraftment, as previously described for transplantsations in mdx mice.12 Rice received immunosuppression daily (Tacrolimus) as both dystrophin and utrophin are foreign proteins to these mice, in addition to GFP. Three weeks following transplantation, cryosections of TA muscles were evaluated for engraftment by immunofluorescence staining for utrophin using a polyclonal antibody that specifically recognizes the amino-terminal epitope preserved in the μUTRN transgene. As expected17,18, no expression of utrophin was detected in PBS-injected control muscles of the contra-lateral leg (Fig. 2a). On the contrary, TA muscles that had been transplanted with μUTRN-corrected iP5-derived myogenic precursors demonstrated substantial engraftment, as showed by the clear expression of utrophin in recipient muscles (Fig. 2b). Quantification of μUTRN+ myofibers showed engraftment average levels of 19±7.6% in transplanted mice (Fig. 2c). Analyses for β-dystroglycan, α7-syntrophin and neuronal nitric oxide synthase in consecutive sections revealed contiguous expression of these proteins across long sections of engrafted μUTRN+ myofibers (Fig. 2b). These data show that μUTRN-corrected iP5-derived myogenic precursors are able to engraft in vivo and, more importantly, μUTRN expression was able to restore other components of the DGC, which are missing in the absence of dystrophin (Fig. 2a)27,18,30,31. Another critical aspect is that no tumour was detected in any of the transplanted mice (total of 20 dKO mice) or in NOD/SCID mice injected with these cell preparations up to 3 months post transplantation. We have previously shown that by incorporating positive selection for paraxial mesodermal markers, teratoma-forming cells can be eliminated from mouse ES-derived cell preparations.12

Engrafted corrected cells reestablish functional properties. To investigate whether engraftment was accompanied by improve-ment in muscle function, we evaluated in a blinded manner the contractile properties of transplanted muscles compared with their contra-lateral leg controls. Engrafted muscles showed markedly superior isometric tetanic force (Fig. 3a) and increased absolute and specific force (Fig. 3b,c, respectively) when compared with their respective contra-lateral PBS-injected TA muscles. No changes were observed in cross-sectional area (CSA) (Supplementary Fig. S6c) or weight (Supplementary Fig. S6d), as previously observed for ES-derived myogenic progenitors using this delivery route.12 Data from the fatigue test showed no differences between transplanted and control groups (Fig. 3d), suggesting that levels of engraftment were not sufficient to restore this parameter.
Transplanted cells seed the satellite cell compartment. Next, we examined whether µUTRN-corrected myogenic precursors have the ability to seed the satellite cell compartment, and therefore to respond to injury (Supplementary Fig. S6d). To facilitate the detection of donor-derived satellite cells, corrected myogenic precursors were labelled with a lentiviral vector encoding GFP (Supplementary Fig. S6d). Three weeks following transplantation, we could clearly identify the presence of Pax7⁺ GFP⁺ cells beneath the basal lamina (Fig. 3e), indicative of donor-derived satellite cells. This was confirmed by another experimental cohort, in which we promoted CTX injury in dKO mice that had been transplanted 3 weeks prior with µUTRN-corrected myogenic precursors (not labelled with GFP) (Supplementary Fig. S6d). One week after injury, we detected the presence of donor-derived newly formed corrected myofibers, as shown by the presence of µUTRN⁺ /embryonic MHC⁺ myofibers (Fig. 3f). Recipient-derived newly formed myofibers (µUTRN⁻ /embryonic MHC⁻) were found in transplanted mice (Fig. 3f) as well as in PBS-injected controls (contra-lateral leg; Supplementary Fig. S6e), as expected. The donor-derived newly formed myotubes (eMHC⁺ µUTRN⁻; Fig. 3f) could be derived from iPS-derived satellite cells (most likely because there are donor-derived satellite cells in engrafted muscles; Fig. 3e), but could also form from a non-satellite myogenic precursor population that did not seed the satellite cell compartment and did not differentiate into myofibers. These results are of particular interest, as activation of corrected satellite cells could provide new µUTRN⁺ myofibers during the progress of the muscular dystrophy, slowing the progression of the disease.

Engrafted myofibers exhibit synaptic connections. To address whether engrafted µUTRN⁺ myofibers exhibited direct connection with motoneurons, we stained muscle sections with z-bungarotoxin (z-BTX). Our results clearly show the presence of nicotinic acetylcholine receptors at the neuromuscular junction of engrafted fibres (Fig. 4). In wild-type mice, as expected, utrophin was detected solely at the neuromuscular junction, along with z-BTX (Fig. 4c), whereas in PBS-injected controls, only z-BTX was present (Fig. 4b). All together, these data suggest that µUTRN-corrected iPS-derived myogenic precursors integrate with the neuromuscular system.

Systemic cell transplantation in dKO mice. Because reaching disparate muscle groups is critical when considering clinical application of cell therapy for DMD, we assessed engraftment levels following the systemic delivery of µUTRN-corrected iPS-derived myogenic precursors. Intravenous injection of these cell preparations resulted in distribution of donor-derived myofibers in several muscles, including TA, gastrocnemius lateralis and peroneal (Fig. 5a–c; Supplementary Table S1), of all the five mice that had been transplanted. In one of these mice, we were able to detected µUTRN⁺ donor-derived myofibers in the diaphragm (Supplementary Fig. S7a). No utrophin signal was detected in any muscles from the PBS-injected mice (Supplementary Figs S7b and S8; Supplementary Table S1). In this cohort of transplanted mice, no engraftment was observed in non-skeletal muscle tissues including heart, lung and liver (Supplementary Fig. S9). This was also the case following the systemic injection of mouse ES cells, as previously reported12.

Discussion
Muscular dystrophies are a genetically and clinically heterogeneous group of neuromuscular diseases characterized by progressive skeletal muscle weakening that are commonly associated with paralysis and cardiopulmonary complications. Although no effective treatment is available at present, one attractive therapeutic approach is to use cell-based therapies to promote muscle regeneration. Skeletal muscle stem cells represent the ideal cell population to be utilized because transplantation of...
these cells results not only in efficient muscle regeneration but also in long-term maintenance through engraftment of the satellite stem cell pool; however, their therapeutic potential so far has been limited because of their scarcity in adult muscle, and the fact that in vitro expansion of these cells results in reduced engraftment ability.

Pluripotent stem cells are unique in terms of proliferation and differentiation potential, and therefore they represent an advantageous option for therapeutic application. We have demonstrated that following introduction of Pax3 or Pax7, transcription factors that have a critical role in skeletal myogenesis, both ES and control iPS cells give rise to myogenic cells. This is supported by the presence of donor-derived satellite cells in engrafted dKO mice, as evidenced by the presence of Pax7⁺ and GFP⁺ cells under the basal lamina (indicated by arrows). Arrowheads denote endogenous Pax7⁺ and GFP⁺ satellite cells. (f) Engrafted cells respond to CTX injury, giving rise to new myofibers as evidenced by the co-expression of μUTRN (red) and embryonic MHC (green). White arrows indicate μUTRN⁺/eMHC⁺ donor-derived newly formed myofibers, and arrowheads point to μUTRN⁻/eMHC⁺ host-derived newly formed myofibers. Scale bar, 50 μm. DAPI, 4,6-diamidino-2-phenylindole.
Figure 4 | Engrafted μUTRN⁺ myofibers exhibit direct synaptic connections to motoneurons. Representative images show AChRs labelled with α-BTX in TA muscles that had been injected with corrected iPS-derived myogenic progenitors (a) or PBS (b). TA muscles from wild-type (WT) mice are also shown as a reference (c). Utrophin is indicated in red and α-BTX is shown in green. 4,6-Diamidino-2-phenylindole (DAPI) is shown in blue. Scale bar, 50 μm.

Figure 5 | Effective systemic delivery of μUTRN-corrected myogenic progenitors in dKO mice. Representative images reveal engraftment of TA (a), gastrocnemius lateralis (b) and peroneal (c) muscles of dKO mice that had received intravenous injection of corrected iPS-derived myogenic progenitors, as evidenced by staining with anti-utrophin and anti-α1-syntrophin (α1-SYN) antibodies. Roman numbers within these images indicate the order of serial sections. 4,6-Diamidino-2-phenylindole (DAPI) is shown in blue. Systemic delivery was performed in five dKO mice. Scale bar, 50 μm.
progenitors endowed with the ability to promote muscle regeneration, to improve the contractility of engrafted muscles and to seed the satellite cell compartment following transplantation in dystrophic mice. This encouraging data suggested that this strategy confers long-term expression of 

**RT-PCR and western blot analyses.** Real-time PCR for pluripotency and muscle-specific genes was performed using probes sets from Applied Biosystems. In the case of the 

**FACS analysis.** EB-derived cells were collected with trypsin (2 min at 37°C), washed in staining buffer (PBS, 2% FBS), suspended in the same buffer containing 0.25 µg per 106 cells of Fc block (ebioscience) and incubated on ice for 5 min. Cells were then incubated on ice for 30 min with specific antibody (1 µg per 106 cells). For 

**Immunofluorescence of cultured cells and tissue sections.** Immunofluorescence staining of 

**Cell culture.** ES and iPSCs were maintained as previously described and differentiated, as follows: iPSCs were trypsinized and plated at 50,000 cells per cm² on a monolayer of mouse embryonic fibroblasts. At day 3, a single-cell suspension of pluripotent cells was obtained by pre-plating on gelatin in the presence of EB medium for 1 h. These cells were then cultured as cell suspension at 10,000 cells per ml of EB medium and incubated on a slowly swirling table rotator at 70 rpm (set up inside of the tissue culture incubator). At day 3 of EB differentiation, 0.8 µg ml⁻¹ of doxycycline was added to the medium to induce Pax3. At day 5, EBs were trypsinized and stained for 

**Plasmids and generation of iPax3-µUTRN iPSCs.** We developed a T2-inverted terminal repeat Tn vector (pK72i/µUTRN) carrying a 7.3 kb engineered transgene containing 

**Mice and transplantation studies.** Animal experiments were carried out according to the protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. Dystrophin/utrophin-deficient (mdx/mdx

**Muscle preparation for mechanical studies.** For the measurement of contractile properties, mice were anesthetized with avertin (250 mg kg⁻¹ intraperitoneal).
and intact TA muscles were dissected and placed in an experimental organ bath as previously described in detail.13 Muscles were stimulated by an electric field generated between two platinum electrodes placed longitudinally on either side of the muscle (square wave pulses 25 V, 0.2 ms in duration, 150 Hz). Muscles were adjusted to the optimum length (Lo) for the development of isometric twitch force, and a 5-minute recovery period was allowed between stimulations. Optimal muscle length (Lo) and stimulation voltage (25 V) were determined from micromanipulation of muscle length and a series of twitch contractions that produced maximum isometric twitch force. For measuring fatigue time, muscles were stimulated for 1 min, and the time for force to decline to 30% of Fo was measured. In brief, after determination of optimal muscle length (Lo) and measurement of maximum isometric tetanic force, total muscle CSA was calculated by dividing muscle mass (mg) by the product of muscle length (mm) and 1.06 mg mm^{-3}, the density of mammalian skeletal muscle. Specific force was determined by normalizing maximum isometric tetanic force to CSA.

Statistical analysis. Differences between samples were assessed by using one-way analysis of variance. All data are presented as mean ± s.e.m.

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Author contributions

A.F. designed and conducted experiments, performed final analysis of the data and contributed to writing the paper. S.P., R.D., I.B., M.I., T.S. and T.M. performed experiments. J.S.C., J.M.E., R.S.M. and M.K. provided reagents, interpreted the data and contributed to writing the paper. R.C.R.P. supervised the overall project, designed experiments, analysed the data and wrote the paper.

Additional information

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