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Short Article

Human ES- and iPS-Derived Myogenic Progenitors Restore DYSTROPHIN and Improve Contractility upon Transplantation in Dystrophic Mice

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SUMMARY

A major obstacle in the application of cell-based therapies for the treatment of neuromuscular disorders is obtaining the appropriate number of stem/progenitor cells to produce effective engraftment. The use of embryonic stem (ES) or induced pluripotent stem (iPS) cells could overcome this hurdle. However, to date, derivation of engraftable skeletal muscle precursors that can restore muscle function from human pluripotent cells has not been achieved. Here we applied conditional expression of *PAX7* in human ES/iPS cells to successfully derive large quantities of myogenic precursors, which, upon transplantation into dystrophic muscle, are able to engraft efficiently, producing abundant human-derived DYSTROPHIN-positive myofibers that exhibit superior strength. Importantly, transplanted cells also seed the muscle satellite cell compartment, and engraftment is present over 11 months post-transplant. This study provides the proof of principle for the derivation of functional skeletal myogenic progenitors from human ES/iPS cells and highlights their potential for future therapeutic application in muscular dystrophies.

INTRODUCTION

Muscle wasting affects millions of individuals worldwide and is caused by a variety of conditions, including cachexia, sarcopenia, and muscular dystrophies (MDs). The latter comprises more than 30 genetically distinct disorders that culminate in paralysis and, in many instances, cardiopulmonary complications (Emery, 2002). Current treatment options are only palliative, and thus far there is no cure for any type of MD. Therapeutic strategies that focus on the replacement of the diseased muscle tissue with stem cells that can give rise to healthy myofibers, as well as self-renew, are particularly attractive. This strategy has been used in the hematopoietic system for the past 40 years with great success. A major caveat with muscle tissue is the impossibility of obtaining enough skeletal muscle stem cells (satellite cells) without causing severe and permanent damage to the muscle of the donor, in contrast to hematopoietic stem cells (HSCs), which can be harvested from mobilized peripheral blood or marrow with minimal harm to the donor. Small muscle biopsies allow for the ex vivo expansion of satellite cell progeny; however, as observed for HSCs (Guenechea et al., 1999), ex vivo expansion of myoblasts from satellite cells results in loss of engraftment ability (Montarras et al., 2005). Consistently, early clinical trials involving the transplantation of ex-vivo-expanded myoblasts failed to improve strength in patients with Duchenne's MD (Mendell et al., 1995; Vilquin, 2005). Therefore, alternate sources of early skeletal muscle progenitors are required for the feasibility of a stem cell therapy approach for MD.

One of the major advantages of pluripotent stem cells is the prospect of generating large quantities of specific cell populations for regenerative purposes. In particular, with the recent breakthrough of reprogramming somatic cells (Park et al., 2008; Takahashi et al., 2007; Yu et al., 2007), ethical concerns associated with human ES cells are eliminated, and the possibility of generating patient-specific iPS cells for autologous therapies is enabled. Whereas safety issues still need to be carefully addressed before these cells can be used in the clinical setting, a critical prerequisite for a potential therapeutic application is the generation of abundant engraftable tissue-specific cell preparations. Although the use of mouse iPS-derived cells to correct a disease phenotype has been documented for several models of disease through derivation of hematopoietic (Hanna et al., 2007), endothelial (Xu et al., 2009), neural (Wernig et al., 2008), pancreatic (Alipio et al., 2010), liver (Espejel et al., 2010), and myogenic (Darabi et al., 2011a; Mizuno et al., 2010) precursor cells, the human iPS field lags far behind in this regard. To date, there is only one study documenting functional improvement from human iPS cells, using a rat model of Parkinson's disease (Hargus et al., 2010). Thus, there is clearly a huge gap between transplantation studies involving mouse and human pluripotent stem cells. Proof-of-principle studies using human iPS cells are required in order to begin seriously considering potential therapeutic applications of these cells.

Here we describe the efficient derivation of a proliferating population of human skeletal myogenic progenitors from both ES

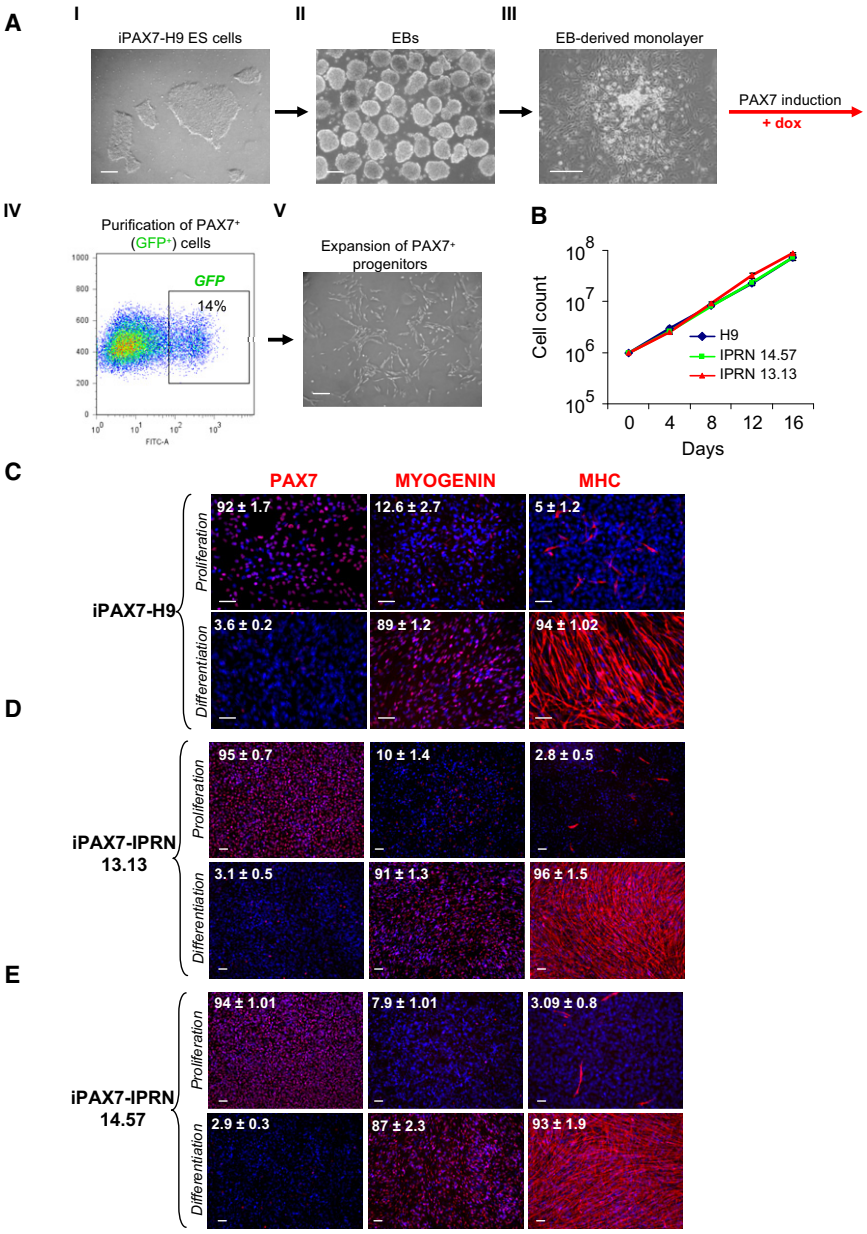

Figure 1. Myogenic Induction of Human ES/iPS Cells by PAX7

(A) Schematic of differentiation protocol with representative morphological aspects of iPAX7 H9: in the undifferentiated state as ES cell colonies in mTeSR medium (I), and in the EB stage (II). At day 7 of differentiation, EBs are collected and plated on a gelatinized flask to grow as a monolayer (III). PAX7 induction is initiated at day 10 of differentiation by adding dox to the myogenic medium. GFP$^+$ (PAX7$^+$) cells emerge in these cultures and begin to proliferate. GFP$^+$ cells are purified by FACS (IV). Representative FACS profile shows PAX7 (GFP) expression after 4 days of dox induction in H9 differentiating ES cells. The percentage indicated represents the fraction of GFP$^+$ cells (IV). PAX7$^+$ myogenic progenitors are expanded in myogenic induction medium supplemented with dox and human bFGF (V). Scale bars represent 100 μm.

(B) Growth curve of PAX7-induced ES- and iPS-derived myogenic progenitors during in vitro expansion. Data represent mean ± SE of four independent experiments.

(C–E) Immunostaining of PAX7-induced human ES-derived (C) and iPS-derived (D and E) myogenic cells for PAX7, MYOGENIN, and MHC in proliferation (top) and differentiation (bottom) conditions. With PAX7 induction under proliferation conditions, most cells express PAX7 and only a few express markers of terminal differentiation (top panels), whereas under differentiation conditions (and dox withdrawal), almost all of the cells become positive for MYOGENIN and MHC, forming multinucleated myotubes (bottom panels). Cells were costained with DAPI (blue). Numbers on each panel represent the percentage of cells expressing PAX7, MYOGENIN, or MHC. Data are mean ± SE. For each condition, four slides were used for quantification. Scale bars represent 100 μm. See also Figure S1.

and iPS cells, which, upon transplantation into dystrophin-deficient mice, promote extensive and long-term regeneration that is accompanied by functional improvement.

RESULTS

PAX7 Induces the Myogenic Program in Differentiating Human ES and iPS Cells

To assess whether *PAX7*, a paired-box transcription factor well known for its role in the maintenance of the adult satellite cell compartment (Oustanina et al., 2004; Seale et al., 2000), can efficiently induce the myogenic program in human ES- and iPS-derived embryoid bodies, as observed in mouse cultures (Darabi et al., 2011a; Darabi et al., 2011b), we modified the human H9 ES cell line and two well-characterized human iPS cell lines, IPRN13.13 and IPRN14.57 (see Figures S1A–S1F available online), generated from fibroblasts from normal donors, with a doxycycline-inducible lentiviral vector encoding *PAX7* (iPAX7). Expression of the transgene was detected by incorporating an ires-GFP reporter downstream of the *PAX7* gene (Figure S1G). Further confirmation of *PAX7* induction in these cells was provided by immunofluorescence analyses, which showed coexpression of PAX7 and GFP upon doxycycline (dox) induction (Figure S1H). Genetic modification did not alter the morphology of the pluripotent cells or their ability to differentiate into embryoid bodies (EBs) (Figure 1A).

In embryogenesis, *PAX7* and its homolog *PAX3* act to confer myogenic fate within paraxial mesoderm. We therefore differentiated iPAX7 human (h) ES and iPS cells for 7 days as EBs followed by 3 days in monolayer before inducing *PAX7* with dox (Figure 1A). This time point is well into the peak of mesoderm

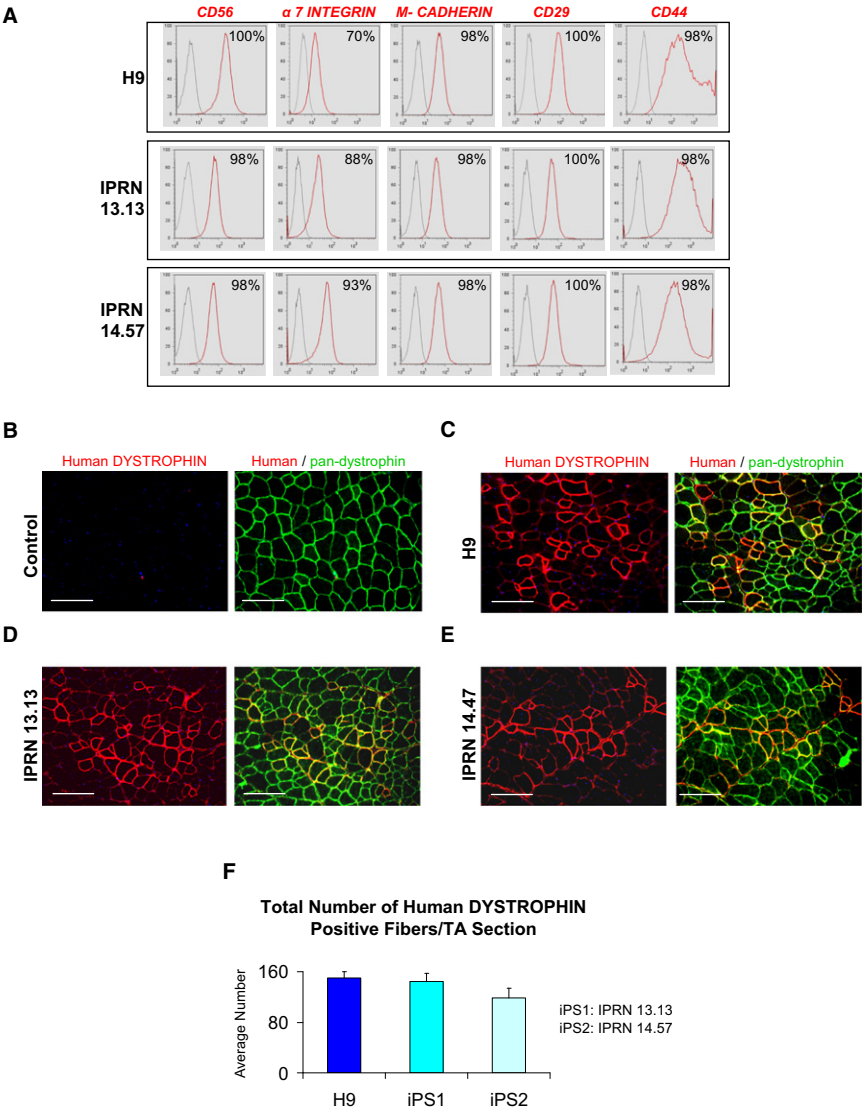

Figure 2. Phenotypic Profile and Regenerative Potential of Human ES/iPS-Derived Myogenic Cells

(A) Representative FACS profile of PAX7-induced human ES- and iPS-derived proliferating myogenic progenitors. Histogram plots show isotype control staining profile (gray line) versus specific antibody staining profile (red line). Percentages represent the fraction of cells that express a given surface antigen. See also Figure S2.

(B–F) Transplantation of myogenic progenitors into cardiotoxin-injured NSG mice. (B) PBS-injected control muscles show no staining for human-specific DYSTROPHIN (right, in red), but, as expected, do show uniform expression of mouse dystrophin (left, in green), as evidenced by the use of a pandystrophin antibody. (C–E) Engraftment of proliferating myogenic progenitors obtained from PAX7-induced human ES-derived (C) and iPS-derived (D and E) cells in TA muscles of NSG (n = 4 for each cell line) 2 months after intramuscular transplantation. Immunofluorescence staining with anti-human (in red) and anti-pandystrophin (in green) antibodies reveals presence of donor-derived myofibers expressing human DYSTROPHIN in recipient muscles (in red). Scale bars represent 100 μm. (F) Quantification of human DYSTROPHIN$^+$ fibers in engrafted muscles shows similar engraftment of human ES- versus iPS-derived myogenic progenitors. For this, the total number of human DYSTROPHIN$^+$ fibers in cross-sections of TA muscles (sections spanned entire muscles) was counted. Data are shown as mean ± SE.

generation, as indicated by Brachyury expression (Figure S1I). Following 4 days of induction, PAX7$^+$GFP$^+$ cells were purified by fluorescence-activated cell sorting (FACS) and expanded in secondary monolayer culture in proliferation medium containing dox and bFGF (Figure 1A and Figure S1J). Both ES- and iPS-derived myogenic progenitors demonstrated notable expansion potential, averaging 86-fold by week 2 (Figure 1B), with a total of six to seven doublings during this period. Under these proliferation conditions, iPAX7 hES and hiPS cells expressed PAX7 abundantly (Figures 1C–1E and Figures S1K and S1L). MYOGENIN and MYOSIN HEAVY CHAIN (MHC), markers of terminal muscle differentiation, were barely detectable (Figures 1C–1E). This profile changed when iPAX7 hES and hiPS cells were subjected to differentiation (5% horse serum and withdrawal of dox and bFGF; differentiation medium). In these culture conditions for 2 weeks, human myogenic progenitors differentiated into multinucleated myotubes, with abundant expression of MYOGENIN and MHC, while rare cells expressed PAX7 (Figures 1C–1E). These results were confirmed by gene expression analyses, which showed high levels of *PAX7* expression solely under proliferation conditions (in the presence of dox) (Figure S1M) and upregulation of *MYOD* and late skeletal muscle-specific markers, *MYOGENIN*, *DYSTROPHIN*, and *MHC*, when these myogenic progenitors had undergone final maturation (Figure S1M).

Human ES- and iPS-Derived Myogenic Progenitors Display Similar Surface Marker Profile

We characterized surface marker expression of these myogenic progenitors by FACS using a panel of antibodies. Our results show a remarkable similarity between hES- (Figure 2A and Figure S2A) and hiPS-derived myogenic progenitors (Figure 2A and Figures S2B and S2C). Cells in each preparation showed homogenous expression of CD56, CD29, CD44, M-CADHERIN, and α7-INTEGRIN. Although most of these markers are associated with murine satellite cells and early myogenic progenitors (Cornelison and Wold, 1997; Sacco et al., 2008; Sherwood et al., 2004), the human satellite cell has not yet been defined by flow cytometry. Only CD56 has been considered to be a reliable marker of human satellite cells (Péault et al., 2007). These cells were also found to express high levels of CD63, CD146, CD105, CD90, and CD13; the

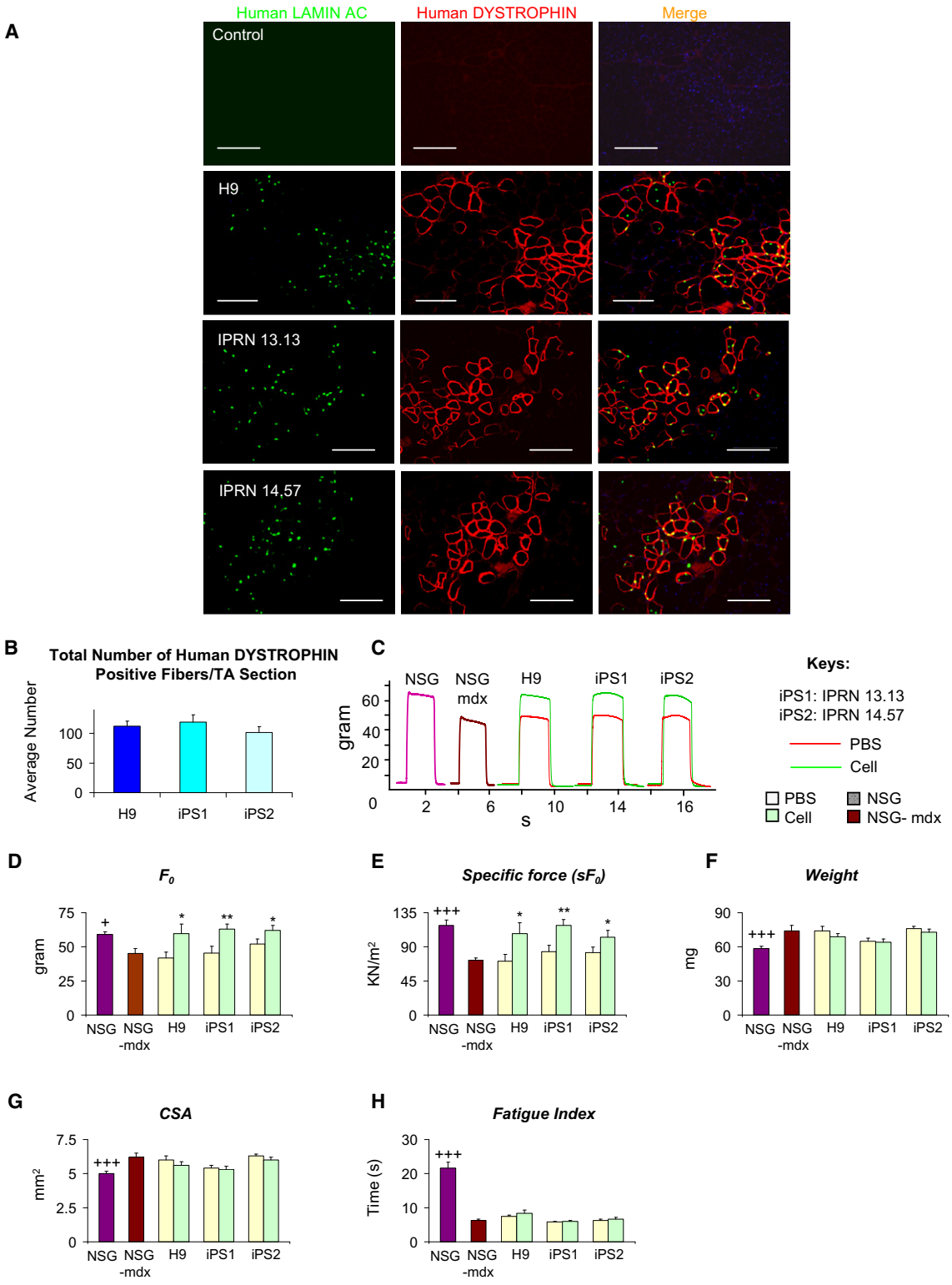

Figure 3. Efficient Engraftment and Functional Recovery after Transplantation of Human ES/iPS-Derived Myogenic Progenitors into Dystrophic Mice

(A) While no staining for human LAMIN AC or DYSTROPHIN is detected in PBS-injected control TA muscles of NSG-*mdx*^{4Cv} mice (top), abundant expression for human LAMIN AC (in green) and DYSTROPHIN (in red) is observed (bottom) in dystrophic muscles treated with human ES/iPS-derived myogenic progenitors 1 month after the transplantation (n = 5 for H9, n = 6 for IPRN13.13, and n = 7 for IPRN 14.47). Note that nuclear LAMIN AC staining occurs predominantly within human DYSTROPHIN^{+} myofibers. Scale bars represent 100 μm. See also Figure S3.

last three are antigens known to be present in mesenchymal stem cells (Pittenger and Martin, 2004). CD34 labeled a discrete subfraction of these cells. Other screened antigens, including CD45, CD33, KDR, and CD31, were undetectable in these myogenic progenitor populations (Figure S2), indicating the absence of hematopoietic and endothelial cells. The adhesion molecules CXCR4 and CD106 were also not detected. We examined MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) expression because the lack of MHC class I expression on other embryonic and ES-derived cells has limited engraftability, even in immunodeficient mice, due to NK cell-mediated responses (Rideout et al., 2002; Tabayoyong et al., 2009). This analysis revealed that, regardless of ES or iPS origin, proliferating myogenic progenitors express MHC class I molecules (Figure S2). This pattern is beneficial from the perspective of avoiding an NK-mediated lack of self-MHC response but indicates the importance of HLA matching.

In Vivo Regenerative Potential of Human ES/iPS-Derived Myogenic Progenitors

Next we examined the in vivo skeletal muscle regenerative potential of iPAX7 hES- and hiPS-derived myogenic progenitors by transplanting these cells directly into the tibialis anterior (TA) muscles of NOD/SCID gamma-c (NSG) mice, an immune-deficient strain commonly used as a recipient of human hematopoietic cells. The gamma-c mutation (*IL2Rg*) ablates NK cells, rendering NSG mice unable to reject human cells due to lack of self-MHC presentation, resulting in better hematopoietic engraftment than in mice bearing the NOD/SCID mutation alone (Shultz et al., 2005). NSG mice were injured with cardiotoxin (CTX) 24 hr prior to cell transplantation. The contralateral TA muscle, which served as a control, was also preinjured with CTX but injected only with PBS. Two months after transplantation, muscle sections were harvested and evaluated for engraftment by immunostaining with both pandystrophin and human-specific DYSTROPHIN antibodies. No expression of human DYSTROPHIN could be detected in PBS-injected control muscles (Figure 2B); staining was only observed with a pandystrophin antibody (Figure 2B). On the other hand, muscles that had been treated with iPAX7 hES- (Figure 2C) and hiPS-derived (Figures 2D and 2E) myogenic progenitors demonstrated engraftment of human-derived myofibers, as evidenced by the clear expression of human-specific DYSTROPHIN in recipient muscles (Figures 2C–2E). We did not observe major differences in terms of engraftment between ES- and iPS-derived myogenic progenitors (Figure 2F). No tumor formation was observed in transplanted mice, even in a long-term (46 weeks) cohort.

Functional Improvement in Dystrophic Mice

To determine the regenerative potential of these myogenic progenitors in the context of muscular dystrophy, we transplanted them into mdx mice engineered to lack B, T, and NK cells. These mice were generated by crossing mice carrying the *mdx*^{4Cv} mutation, an ENU-induced stop codon in exon 53 (Im et al., 1996) with very low reversion frequency (Danko et al., 1992), to NSG mice. Recombinant X chromosomes bearing both *mdx*^{4Cv} and *IL2Rg*$^{\Delta}$ were brought to homozygosity with the NOD/SCID mutation, and the stock was maintained by sibmating. The genetic background is thus mixed-inbred, distinct from either C57BL/6Rox (of *mdx*^{4Cv}) or NOD/ShiLtJ (of NSG). These NSG-*mdx*^{4Cv} mice, similarly to conventional *mdx* mice (Coulton et al., 1992; Durbeej and Campbell, 2002), lack dystrophin (Figure S3) and are characterized by extensive regeneration, as evidenced by the presence of centrally nucleated myofibers (Figure S3).

Intramuscular transplantation of TA muscles with ES- or iPS-derived myogenic progenitors resulted in considerable engraftment, as clearly shown by the large number of myofibers expressing human DYSTROPHIN (Figure 3A), whereas PBS-injected muscles lacked DYSTROPHIN (Figure 3A). This engraftment was confirmed by the use of a second human-specific antibody, LAMIN AC (Figure 3A). Human nuclei were exclusive to cell-transplanted muscle and mainly found within human DYSTROPHIN^{+} fibers (Figure 3A). We observed comparable engraftment between iPAX7 hES- versus hiPS-derived myogenic cells (Figure 3B), suggesting similar regenerative potential between ES- and iPS-derived myogenic progenitors. As controls, we transplanted dermal fibroblasts and myoblasts using the same cell number into the TA muscles of NSG-*mdx*^{4Cv} mice. No engraftment was detected following injection of fibroblasts (Figure S4A), whereas DYSTROPHIN^{+} myofibers could be observed in myoblast-transplanted mice (Figure S4A), although at a much lower level (Figure S4B) than ES- and iPS-derived myogenic progenitors using our protocol ($p < 0.001$).

Next we investigated whether muscle contractile parameters were altered following transplantation. Similarly to conventional *mdx* mice (Darabi et al., 2008), TA muscles from immunodeficient dystrophic mice were weak and hypertrophic, as shown in untreated or PBS-injected controls (Figures 3C–3G). On the other hand, dystrophic muscles that had been transplanted with human ES- and iPS-derived myogenic progenitors demonstrated significant functional improvement, as demonstrated by superior isometric tetanic force (Figure 3C), increased absolute force (Figure 3D), and specific force (Figure 3E), when compared

(B) Quantification of human DYSTROPHIN^{+} fibers in NSG-mdx^{4Cv} engrafted mice shows comparable engraftment of human ES- versus iPS-derived myogenic progenitors. For this, the total number of human DYSTROPHIN^{+} fibers in cross-sections of TA muscles (sections spanned entire muscles) was counted. Data are shown as mean ± SE.

(C) Representative example of force tracings in TA muscles of nontreated, noninjured NSG (purple line) and NSG-*mdx*^{4Cv} (brown line) mice, as well as CTX-injured NSG-*mdx*^{4Cv} mice that had been injected with PBS (control, red line) or human ES/iPS-derived myogenic progenitors (green line).

(D and E) Effect of iPAX7 human ES/iPS-derived myogenic cell transplantation on absolute and specific (sF_0: F_0 normalized to CSA) force, respectively. Values for nontreated, noninjured NSG (purple) and NSG-mdx^{4Cv} (brown) mice are shown for reference. Data are shown as mean ± SE.

(F and G) Weight and CSA of control and transplanted muscles, respectively. Values for nontreated, noninjured NSG (purple) and NSG-mdx^{4Cv} (brown) mice are shown for reference. See also Figure S4. Data are shown as mean ± SE.

(H) Fatigue index: time for force to decline to 30% of its maximal value shows no significant recovery with cell treatment compared to PBS control. Data are shown as mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to its PBS control. $^{+}p < 0.05$, $^{+++}p < 0.001$ compared to NSG-*mdx*^{4Cv}.

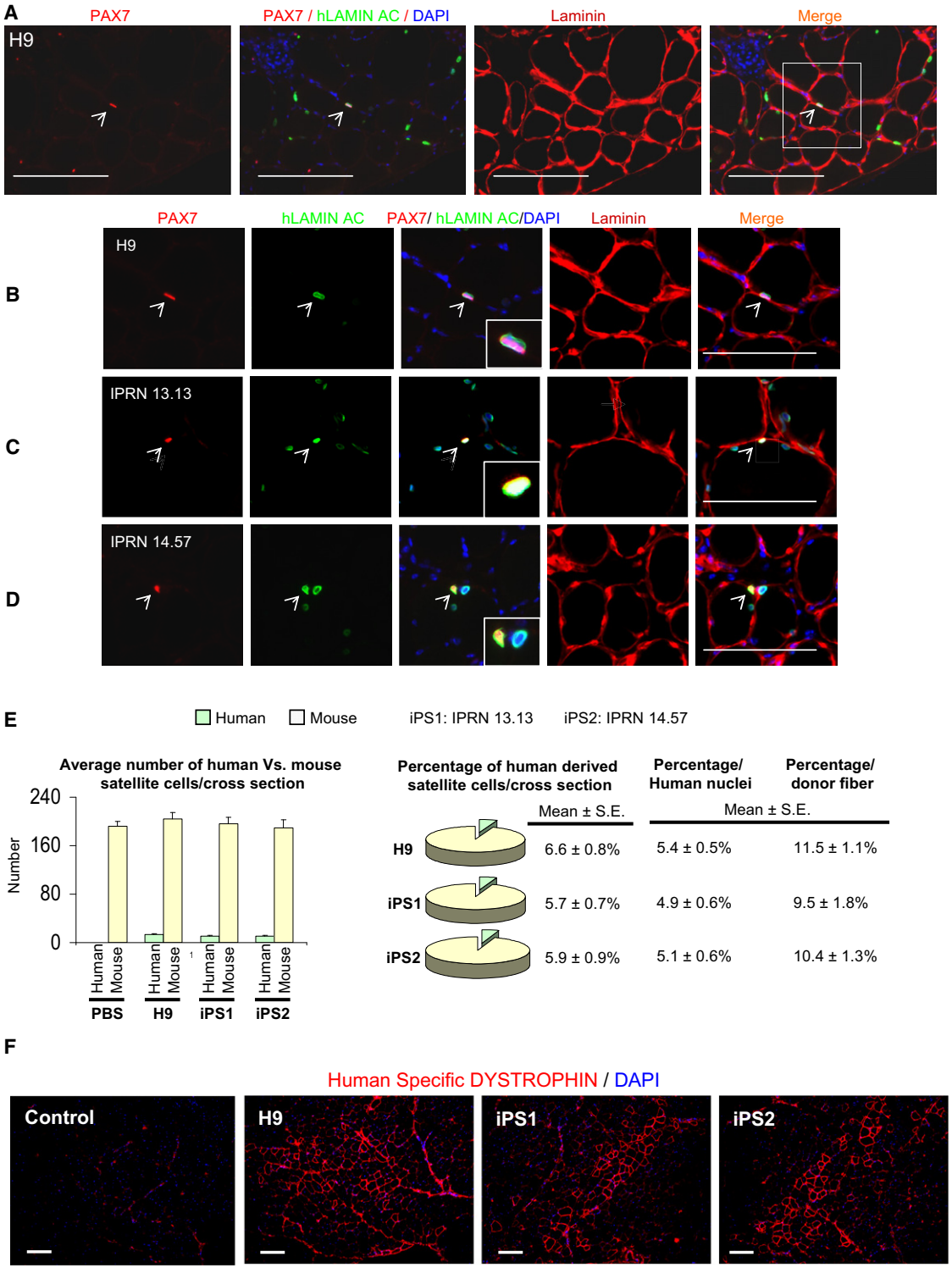

Figure 4. Satellite Cell Engraftment by Human ES/iPS-Derived Myogenic Cells

(A and B) Representative images show staining for satellite cells in muscle sections from NSG-*mdx*^{4Cv} mice that had been transplanted with H9 human ES-derived myogenic progenitors. Images are shown at lower (A) and higher (B) magnification. Immunostaining shows the presence of human LAMIN AC$^+$ (in green) cells in engrafted regions. Arrows shows the presence of human-derived satellite cells in engrafted muscles, as evidenced by the presence of PAX7$^+$ (in red) LAMIN AC$^+$ (in green) double-positive cells under the basal lamina. Scale bars represent 100 μm.

(C and D) Similar satellite cell engraftment was observed upon transplantation of human iPS-derived myogenic progenitors, IPRN 13.13 (C) and IPRN 14.57 (D). Scale bars represent 100 μm.

to their respective contralateral PBS-injected TA muscle. Weight and cross-sectional area (CSA) parameters remained unchanged (Figures 3F and 3G). No changes were observed when transplanted muscle was subjected to fatigue test (Figure 3H), suggesting that levels of engraftment were not sufficient to restore this parameter. Meanwhile, transplantation of fibroblasts or myoblasts did not result in improvement of any of these functional parameters (Figures S4E–S4G).

Engraftment of the Satellite Cell Compartment

Finally, we investigated whether ES- and iPS-derived myogenic progenitors have the ability to seed the satellite cell compartment following their transplantation into NSG-*mdx*^{4Cv}. We performed these analyses by staining muscle cryosections with Pax7 (satellite cell marker), human LAMIN AC (specific antibody to track human cells), Laminin (to identify position within the sarcolemma), and DAPI. The majority of human nuclei were PAX7$^-$ and were within human DYSTROPHIN$^+$ myofibers. This is expected, because the majority of transplanted human myogenic progenitors differentiate into myofibers. However, we also detected a significant number of PAX7$^+$ human LAMIN AC$^+$ cells, representing donor-derived satellite cells. These results were quantified and are shown in Figure 4E. The data clearly show that human ES- and iPS-derived myogenic progenitors are able to seed the satellite cell compartment (Figures 4A–4D). As expected, because muscles were not previously irradiated, the majority of the satellite cell pool was of recipient origin, with only a small fraction being donor derived (Figure 4E).

To determine whether the engraftment of ES- and iPS-derived myogenic progenitors was durable, we assessed the presence of human DYSTROPHIN$^+$ myofibers at 46 weeks after transplantation into NSG mice. Immunostaining of this long-term experimental cohort revealed significant engraftment in muscles of NSG mice (n = 9) that had been transplanted with human ES/iPS-derived skeletal myogenic progenitors (11 months post-transplantation) (Figure 4F and Figure S4H). Quantification of human DYSTROPHIN$^+$ myofibers demonstrated that the engraftment level at 46 weeks was about 60%–80% (Figure S4I) of the levels at 8 weeks (Figure 2F). This sustained long-term engraftment data is remarkable, because, to our knowledge, no study has followed engraftment for such a long period with human myogenic cells.

DISCUSSION

There has been increasing enthusiasm about the possibility of applying iPS technology to generate autologous cells for therapeutic purposes. Some of the advantages associated with these pluripotent stem cells include (1) the absence of ethical concerns, because cells are derived from adult tissue, (2) the potential for an off-the-shelf supply of HLA-matched or patient-specific stem cell preparations, (3) the possibility of correcting genetic defects by homologous recombination, and (4) the possibility of immunosuppression being dispensable, in the case of autologous cell transplantations. Although significant progress has been made in terms of generating integration-free iPS cells through the use of safer transient vectors (Kaji et al., 2009; Okita et al., 2008; Stadtfeld et al., 2008; Woltjen et al., 2009; Yu et al., 2009), transduction of recombinant proteins (Kim et al., 2009; Zhou et al., 2009), or the use of synthetic modified mRNA (Warren et al., 2010), proof-of-principle studies demonstrating functional recovery following transplantation of human iPS-derived stem cell preparations into animal models of disease are still lacking. Although one study has previously documented evidence of skeletal muscle differentiation after intramuscular transplantation of human ES cells (Barberi et al., 2007), this was very limited, and only a few myogenic cells were observed in vitro and in vivo. Moreover, these were not performed in a dystrophic mouse model, making it difficult to assess therapeutic relevance.

In this study we demonstrate the feasibility of generating large quantities of human ES- and iPS-derived early skeletal myogenic progenitors that are endowed with the ability to promote regeneration in vivo, not only restoring DYSTROPHIN expression in an immunodeficient model of Duchenne muscular dystrophy but also improving the force generation of engrafted muscles. We also show that PAX7-induced human ES- and iPS-derived myogenic progenitors contribute to the satellite cell pool and that engraftment is durable, being sustained for around half the life span of the animal, and most likely longer. It will be interesting to determine whether engraftment levels can be increased with different delivery or conditioning strategies. Because irradiation was not used in these studies, in addition to the human ES/iPS-derived regeneration, there was also ongoing regeneration by host satellite cells. Thus, it might be possible to improve engraftment levels by preconditioning muscles with irradiation (Skuk et al., 2010). Moreover, these cell preparations demonstrate significant scalability in response to maintained PAX7 expression. In the experiments described here, >80-fold expansion was achieved over 2 weeks, and much greater expansion is likely possible, potentially facilitating delivery of much larger numbers of cells. It will then be interesting to determine whether, in addition to improved contractility, ES-/iPS-myogenic transplantation can improve more complex functional parameters such as resistance to eccentric exercise-induced injury, general motility, or, in more severe models such as *mdx*/mTR mice (Sacco et al., 2010), life span.

In the system we describe, the in vitro expansion potential and the in vivo functional regeneration of PAX7-derived myogenic progenitors allows one to envision producing therapeutic quantities of myogenic progenitor cells for clinical evaluation in muscular dystrophies. However, before this is attempted, it will

(E) Quantification of PAX7$^+$LAMIN AC$^+$ and Pax7$^+$LAMIN AC$^-$ cells in transplanted muscles, representative of donor human-derived and host satellite cells, respectively. The total number of PAX7$^+$LAMIN AC$^+$ and Pax7$^+$LAMIN AC$^-$ cells in cross-sections of TA muscles was counted. Sections spanned entire muscles. Data are shown as mean ± SE. Left panels show absolute numbers; right panels indicate respective percentages. Data are also shown as percentage per human nuclei, as well as percentage per donor fiber.

(F) Assessment of long-term engraftment at 46 weeks after transplantation in NSG mice. Immunofluorescence staining with anti-human DYSTROPHIN antibody reveals the presence of donor-derived myofibers expressing human DYSTROPHIN (in red) in NSG recipient muscles. Scale bars represent 100 μm. See also Figure S4.

be necessary to establish nongenetic methods of delivering *PAX7* to generate equivalent myogenic progenitors. These could include the utilization of safer transient vectors, the transduction of recombinant proteins, or the use of synthetic modified mRNA, approaches that have been used with success to generate integration-free iPS cells.

EXPERIMENTAL PROCEDURES

Human iPS Induction

Human iPS cells were generated from adult human fibroblasts, as previously described (Dimos et al., 2008). Detailed information on the generation and full characterization of the two iPS clones studied here, IPRN13.13 and IPRN14.57, is provided in the Supplemental Experimental Procedures.

Generation of Human Inducible PAX7 ES and iPS Cell Lines

Human H9 ES cells and the iPS clones referred above were grown in feeder-free conditions using mTeSR medium on human ESC qualified Matrigel (BD Biosciences)-coated plates. To generate iPAX7 pluripotent cells, we transduced ES and iPS cells with a lentiviral vector expressing the reverse tet-transactivator (rtTA) (Bosnakovski et al., 2008). The full-length human *PAX7* cDNA (clone ID 40121582, Open Biosystems) was subcloned into pSAM2, a lentiviral construct containing the transactivator, a second-generation tet-responsive element (sgTRE) that allows the expression of the target gene upon doxycycline (dox) induction, and IRES-EGFP, which allows confirmation of integration and inducible expression (Bosnakovski et al., 2008). Vectors were cotransfected with packaging and coat protein constructs Δ8.91 and pVSVG into 293T cells using the FuGENE 6 transfection reagent (Roche). Virus-containing supernatant was collected 48 hr after transfection, filtered through a 0.45 μm filter, and used for infection. Human ES cells and iPS cells were coinfected with rtTA and pSAM2-*PAX7* simultaneously. ES/iPS cells containing the *PAX7* insert were purified by FACS based on GFP expression following an overnight incubation with dox (Sigma) at 0.75 μg/ml.

Differentiation of Human ES/iPS Cells into Myogenic Progenitors

Detailed information is provided in the Supplemental Experimental Procedures.

Real-Time PCR Analysis

Real-time PCR for muscle-specific genes was performed using probe sets from Applied Biosystems.

FACS Characterization

A detailed description is provided in the Supplemental Experimental Procedures.

NSG-*mdx*^{4Cv} Mice

Mdx^{4Cv} (B6Rox.Cg-*Dmd*$^{mdx\text{-}4Cv}$/J) and NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice were purchased from Jackson Laboratories (stock numbers 002378 and 005557, respectively). The *dystrophin* and *IL2Rg* genes are both X-linked: recombinant X chromosomes were isolated by crossing females bearing these mutations in *trans* with wild-type males. One male and one female recombinant were identified. The line was then established by sibmating and selecting for homozygosity of the *Prkdc*^{scid} mutation and kept as mixed-inbred.

Transplantation Studies

Animal experiments were carried out according to protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. We used 5- to 8-week-old male NSG mice from Jackson Laboratories (stock number 002378) and NSG-*mdx*^{4Cv} mice (above) for these in vivo studies. Before intramuscular cell transplantation, mice were preinjured with cardiotoxin, as previously described (Darabi et al., 2008). At 24 hr after cardiotoxin damage into both TA muscles, myogenic progenitors from iPAX7 ES or iPS cells (3 × 10^5 cells/15 μl PBS) were injected into left TA muscles, whereas the right leg received the same volume of PBS as the negative control.

Muscle Preparation for Mechanical Studies

For the measurement of contractile properties, mice were anaesthetized with avertin (250 mg/kg intraperitoneal), and intact TA muscles were dissected and placed in an experimental organ bath, as previously described in detail (Darabi et al., 2008). Detailed information is provided in the Supplemental Experimental Procedures.

Immunofluorescence Staining of Cultured Cells and Tissue Sections

Two months after transplantation, muscles were harvested and frozen in isopentane cooled in liquid nitrogen. Serial 8 to 12 μm cryosections were collected. For immunofluorescence staining, cells cultured on slides and tissue cryosections were either fixed using cold acetone or 4% PFA or unfixed (in the case of human DYSTROPHIN staining), permeabilized with 0.3% Triton X-100 (Sigma), blocked with 3% bovine serum albumin and 0.01% Triton X-100 in PBS, and then incubated with appropriate antibodies. For satellite cell quantification, slides were stained with DAPI, Pax7, human LAMIN AC, and Laminin (Skuk et al., 2010), and the number of mouse-derived (Pax7$^+$LAMIN AC$^-$) versus human-derived (PAX7$^+$LAMIN AC$^+$) satellite cells were quantified in cross-sections of TA muscles (two cross-sections/mice; ten per group). Absolute numbers and respective percentages were calculated and plotted. All antibodies are listed in the Supplemental Experimental Procedures.

Statistical Analysis

Differences between samples were assessed by using the Student's two-tailed t test for independent samples.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2012.02.015.

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