Myogenic Differentiation of Muscular Dystrophy-Specific Induced Pluripotent Stem Cells for Use in Drug Discovery

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Key Words. Induced pluripotent stem cells • Differentiation • Skeletal muscle • Muscular dystrophy

ABSTRACT

Human induced pluripotent stem cells (iPSCs) represent a scalable source of potentially any cell type for disease modeling and therapeutic screening. We have a particular interest in modeling skeletal muscle from various genetic backgrounds; however, efficient and reproducible methods for the myogenic differentiation of iPSCs have not previously been demonstrated. Ectopic myogenic differentiation 1 (MyoD) expression has been shown to induce myogenesis in primary cell types, but the same effect has been unexpectedly challenging to reproduce in human iPSCs. In this study, we report that optimization of culture conditions enabled direct MyoD-mediated differentiation of iPSCs into myoblasts without the need for an intermediate step or cell sorting. MyoD induction mediated efficient cell fusion of mature myocytes yielding multinucleated myosin heavy-chain-positive myotubes. We applied the same approach to dystrophic iPSCs, generating 16 iPSC lines from fibroblasts of four patients with Duchenne and Becker muscular dystrophies. As seen with iPSCs from healthy donors, within 36 hours from MyoD induction there was a clear commitment toward the myogenic identity by the majority of iPSCs in culture (50%–70%). The patient iPSC-derived myotubes successfully adopted the skeletal muscle program, as determined by global gene expression profiling, and were functionally responsive to treatment with hypertrophic proteins insulin-like growth factor 1 (IGF-1) and wingless-type MMTV integration site family, member 7A (Wnt7a), which are being investigated as potential treatments for muscular dystrophy in clinical and preclinical studies, respectively. Our results demonstrate that iPSCs have no intrinsic barriers preventing MyoD from inducing efficient and rapid myogenesis and thus providing a scalable source of normal and dystrophic myoblasts for use in disease modeling and drug discovery. Stem Cells Translational Medicine 2014;3:149–160

INTRODUCTION

Muscular dystrophies are a collection of progressive myopathies with diverse genetic bases and clinical manifestations. Several experimental therapies, mainly focused on the most prevalent X-linked diseases, are being investigated; however, no treatment developed specifically for a muscular dystrophy is currently available. One of the challenges of therapeutic discovery and translation for the muscular dystrophies is the lack of effective human cellular systems for drug screening and validation. Mature skeletal myocytes cannot be expanded in culture systems, and the self-renewing stem cell population in muscle, satellite stem cells, becomes activated and differentiates when introduced to in vitro culture, preventing the derivation of a scalable, disease-specific cellular system [1–3].

Human induced pluripotent stem cells (iPSCs) can be generated from a wide variety of somatic cells by the introduction and expression of transcription factors (POU class 5 homeobox 1 [POU5F1/Oct4], SRY [sex determining region Y]-box 2 [SOX2], Kruppel-like factor 4 [Klf4]), endowing them with the potential for unlimited self-renewal and subsequent differentiation into any cell type, depending on the availability of robust differentiation protocols [4]. With their ability to capture the genetic diversity of muscular dystrophies in a scalable culture system, iPSCs represent an attractive source for the generation of myoblasts for drug screening. However, the efficient differentiation of iPSCs into a homogenous myogenic cell population has yet to be demonstrated. Current methods for the myogenic differentiation of iPSCs are inefficient, as they rely on the lengthy process of differentiating iPSCs first into an intermediate stage such as embryoid bodies (EBs), mesenchymal cells, or mesoangio- blasts before switching on the skeletal program, and most methods require the subsequent enrichment of differentiated cells by fluorescence-activated cell sorting (FACS) [5–7].

In this study, we examined the ability of the myogenic regulatory factor myogenic differentiation 1 (MyoD) to induce direct myogenesis in patient-specific iPSCs. Exogenous expression of...
MyoD has been shown to induce myogenesis in multiple cell types, including mouse embryonic stem cells [8–10], but its ability to induce direct myogenesis in human pluripotent stem cells has not been demonstrated. Using a lentiviral system expressing MyoD under the control of Tet-inducible promoter, and under optimized culture conditions, we achieved efficient infection of iPSCs from healthy donors leading to their rapid and efficient myogenic differentiation. We applied the same approach to generate dystrophic myoblasts: deriving 16 iPSC lines from four patients with either Duchenne or Becker muscular dystrophies under feeder cell-free conditions. The induction of exogenous MyoD expression in these disease backgrounds led to rapid (36-hour) and efficient (50%–70%) commitment toward the myogenic identity. We tracked genome-wide expression profiles of the iPSC cultures over time following induction of MyoD expression. These studies confirmed the conversion of iPSCs into muscle cells, with expression profiles that were similar to those of primary myoblasts and that diverged greatly from their parental undifferentiated iPSCs. In addition, treatment of myoblasts derived from three different iPSC lines (from three patients) with the hypertrophy-inducing factor insulin-like growth factor 1 (IGF-1) and wingless-type MMTV integration site family, member 7A (Wnt7A), resulted in significant hypertrophic effects similar to those observed in primary myoblasts. Our direct and efficient approach offers a scalable and renewable source of normal and dystrophic myoblasts that could be used as tools for drug discovery, disease modeling, and potentially cell therapy.

**Materials and Methods**

**Cell Culture**

All cells were cultured at 37°C with humidified air with 5% CO2. Human iPSCs were cultured on Matrigel-coated plates (BD Biosciences, San Diego, CA, http://wwwbdbiosciences.com) in iPSC growth medium containing Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Mediatech, Manassas, VA, http://www.cellgro.com), 20% vol/vol knockout serum replacement (SR; Life Technologies, Rockville, MD, http://www.lifetech.com), 1% vol/vol nonessential amino acids (Mediatech), 2 mM l-glutamine (Mediatech), 100 mM β-mercaptoethanol (Life Technologies), and 10 ng/ml basic fibroblast factor (bFGF; Life Technologies). To enable single-cell feeder-free culture of iPSCs, growth media was supplemented with small molecule 4 (SMC4) cocktail, as previously described [11, 12]. SMC4 media additive contains 0.4 µM PD0325901 (Biovision, Milpitas, CA, http://www.biovision.com), 1 µM CHIR99021 (Biovision), 5 µM Thiazovivin (synthesized at Fate Therapeutics), and 2 µM SB431542 (Biovision). Healthy donor human primary myoblasts (Lonza, Walkersville, MD, http://www.lonza.com) were maintained in F10 (Mediatech), 15% vol/vol fetal bovine serum (FBS), 0.5% vol/vol chick embryo extract (VWR, Radnor, PA, https://us.vwr.com), 0.4 µg/ml dexamethasone (Sigma-Aldrich, St. Louis, MO, http://www.sigmahemrich.com), and 1 ng/ml bFGF on collagen-coated plates. To induce terminal differentiation of primary or iPSC-derived myoblasts, medium was changed to low-glucose DMEM with 2% vol/vol horse serum (HS; Life Technologies). Patient-specific fibroblasts were cultured in DMEM with 10% vol/vol FBS (Life Technologies), 1× GlutaMAX (Life Technologies), and 1× nonessential amino acids (Mediatech).

**Generation of Dystrophy-Specific iPSCs**

Fibroblasts derived from patients with Duchenne muscular dystrophy (DMD) (GM05169 and GM05112) and Becker muscular dystrophy (BMD) (GM05081 and GM02299) were obtained from the Coriell Institute for Medical Research (Camden, NJ, http://www.coriell.org). Generation and feeder-free culture of human iPSCs were performed as described previously [11, 12]. Briefly, the fibroblasts were infected with a lentivirus containing a polycistronic cassette expressing OCT4, SOX2, and KLF4 in the presence of 4 µg/ml polybrene (Millipore, Billerica, MA, http://www.millipore.com) and transferred to 37°C and 5% CO2 for 8–12 hours. After the completion of the incubation, the cells were washed three times with phosphate-buffered saline (PBS) and fed with fibroblast medium. Two days following infection, iPSC growth medium supplemented with SMC4 chemical cocktail was added. Upon confluence the cells were passaged (usually between days 4 and 6).

**Myogenic Differentiation of iPSCs Using Inducible MyoD Overexpression**

The MyoD lentivirus was generated by synthesizing the coding sequence for the human MYOD1 gene (GenScript) and inserting the fragment into doxycycline (Dox)-inducible lentiviral system (derived at Fate Therapeutics). To generate stable iPSC lines with integrated Dox-inducible MYOD1 transgene, iPSCs were infected with the MyoD lentivirus and another lentivirus expressing the transactivator and puromycin-resistance gene in iPSC media supplemented with SMC4 and 4 µg/ml polybrene. Uninfected cells were removed by 2-day incubation with 2 µg/ml puromycin. Following selection, iPSCs were pooled and expanded in growth media without Dox. For differentiation, MyoD-infected iPSCs were seeded on Matrigel- or collagen IV-coated plates in iPSC culture media without basic fibroblast growth factor (FGF) and supplemented with the β-associated kinase (ROCK) inhibitor Thiazovivin. Unless otherwise indicated, the next day medium was changed to induction medium (DMEM and 15% FBS) containing 1 µg/ml Dox. Medium was changed 4 days later to Dox-containing differentiation media (low-glucose DMEM and 5% horse serum).

**Immunofluorescence Staining and In Vitro Hypertrophy Assay**

For immunostaining, cells were fixed using 4% paraformaldehyde vol/vol (Alfa Aesar, Ward Hill, MA, http://www.alfa.com) for 10 minutes, permeabilized with 0.1% Triton X-100 vol/vol in PBS, and blocked with 10% goat serum vol/vol and 0.1% Triton X-100 vol/vol in PBS. For myosin-heavy chain (MYHC) staining, cells were incubated with a mixture of antibodies for slow and fast MYHC (dilution at 1:400; Sigma-Aldrich). Cells were also stained as indicated using antibodies for MyoD (dilution at 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), Myogenin (dilution at 1:100; Santa Cruz Biotechnology), NCAM (dilution at 1:50; ebioscience, San Diego, CA, http://www.ebioscience.com), Nanog homeobox (Nanog) (dilution at 1:100; Abcam, Cambridge, U.K., http://www.abcam.com), Oct4 (dilution at 1:250; Santa Cruz Biotechnology), tumor-related antigen-1-60 (TRA-1-60) (dilution at 1:50; BD Biosciences), and TRA-1-81 (dilution at 1:50; BD Biosciences). Nuclei were stained with 4',6-diamidino-2-phenylindole in PBS. Images of the stained cells were captured using the Zeiss fluorescence microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com) and charge-coupled device camera. For in vitro hypertrophy assays, myotube cultures were treated with either formulation control alone (PBS with 1% 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate...
vol/vol) or 100 ng/ml Wnt7a (Fate Therapeutics) or 100 ng/ml IGF-1 (Sigma-Aldrich) for an additional 2 days. Following staining with MYHC to visualize multinucleate fibers, images were captured for each treatment in the center of the well. Fiber diameter was quantified by manually measuring the fiber width at its thickest point within a field of a captured image of MYHC staining. For each treatment condition, 100 fibers were measured using Axiovision software. To calculate fusion index, images were taken for MYHC-stained cells from at least three random fields in three independent wells (n > 100 per well). Nuclei in myotubes (≥ 2 nuclei) were counted and plotted as percentage of total number of nuclei.

**Flow Cytometry**

Isolation of successfully reprogrammed human iPSCs using FACS has been described previously [1, 2]. Briefly, 3 weeks postinfection, cells were dissociated with Accutase (Millipore) and stained with cell surface pluripotency markers stage-specific embryonic antigen-4 (SSEA4), TRA-1-81, and tumor necrosis factor receptor superfamily, member 8 (TNFRSF8; CD30), and triple-positive cells were seeded into Matrigel-coated 96-well plates in SMC4-containing media. iPSC clones were expanded on Matrigel-coated plates in SMC4-supplemented media. Cell sorting was done using the FACSaria II (BD Biosciences). The primary antibodies used for cell sorting and flow cytometry analyses were SSEA4–fluorescein isothiocyanate (FITC; BD Biosciences), TRA-1-81–Alexa Fluor 647 (BD Biosciences), CD30 Alexa Fluor 555 (BD Biosciences), Myosin (fast and slow; Sigma-Aldrich), CD56 allophtocytocynin (APC; NCAM; eBioscience), CD29 FITC (eBioscience), and CD44 APC (eBioscience). For intracellular flow cytometry, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences; 557414). Briefly, cells were dissociated, fixed, and permeabilized in the Cytofix/Cytoperem solution for 30 minutes at 4°C. Cells were then washed three times in the perm/wash solution followed by staining with the slow and fast MYHC antibodies for 20 minutes at 4°C. Cells were once again washed in perm/wash buffer three times, stained with secondary antibody conjugated with Alexa Fluor 488 (Life Technologies) for 20 minutes at 4°C, and followed by three additional washes in perm/wash solution. For analyses that included surface staining, cells were first stained by surface antibodies and then processed for intracellular staining. Flow cytometry analysis was performed on the Guava EasyCyte 8HT (Millipore).

**Affymetrix Gene Expression Analysis**

Biotinylated amplified RNA (aRNA) was prepared from 100 ng total RNA for all samples involving the Message Amp II kit (Life Technologies) following the standard two-round amplification protocols. Fragmented and biotinylated aRNA was then hybridized to Affymetrix U133 plus 2.0 GeneChips for 16 hours at 45°C, according to the manufacturer’s instructions. Arrays were washed and stained with streptavidin-phycocerythrin in the GeneChip Fluidics Station 450 and scanned on the 3000 7G Scanner (Affymetrix, Santa Clara, CA, http://www.affymetrix.com). Intensity values were determined using GeneChip Expression Console software (Affymetrix). Probe intensities were normalized according to a log scale robust multiarray analysis (RMA) method (Affymetrix). The Log2 normalized intensities of the top 100 expressed probes upregulated in primary myotubes were visualized in a heat map with Spotfire for Genomics 4.5 (Tibco Spotfire, Boston, MA, http://spotfire.tibco.com). The Pearson correlation coefficient of the RMA probe set intensities of all possible sample pairs was visualized in a correlation matrix as a heat map using Expression console software (Affymetrix). The gene networks, pathway association, and functional analyses were generated through the use of IPA (Ingenuity Systems, Redwood City, CA, http://www.ingenuity.com).

**Real-Time Reverse Transcription Polymerase Chain Reaction Analysis**

RNA was isolated using the PicoPure RNA Isolation kit (Life Technologies) and used to generate first-strand cDNA using the Script cDNA Synthesis kit (Bio-Rad, Hercules, CA, http://www.bio-rad.com). Relative gene expression levels were determined using the TaqMan Fast Universal PCR Master Mix (Life Technologies) and the 6-carboxyfluorescein-labeled TaqMan probes listed in supplemental online Table 3.

**Karyotype Analysis**

Cytogenetic analysis was performed on 20 G-banded metaphase cells by WiCell Research Institute (Madison, WI, http://www.wicell.org).

**Statistical Analysis**

Student’s t test (two-tailed distribution) was used to calculate p values. For real-time reverse transcription polymerase chain reaction, StepOne Software v2.2 (Life Technologies) was used to determine relative quantification minimum and maximum values (error bars).

**Accession Numbers**

The Gene Expression Omnibus accession number for the Affymetrix profiling reported in this paper is GSE46633.

**RESULTS**

**Efficient and Direct MyoD-Mediated Myogenic Differentiation of iPSCs**

Our goal in this study was to establish an efficient method of human iPSC differentiation into myoblasts to model neuromuscular diseases for drug discovery. To explore the possibility of using MyoD to induce the direct myogenic conversion of human iPSCs, we constructed a Dox-inducible MyoD expression cassette in a lentiviral system (supplemental online Fig. 1A). This resulted in efficient delivery and controllable expression in the iPSC target cell population. We cultured iPSCs in growth media supplemented with the SMC4 small molecule cocktail that we have previously reported to enhance cell survival and enable single-cell cloning of human iPSCs [11, 12]. The single-cell culture system enabled high infection rate and high level of MyoD expression ( supplemental online Fig. 1B). We used the MyoD lentivirus to infect human iPSC lines generated from healthy donors (FTc01-C1 and FTc01-C2). These lines have previously been fully characterized and shown to possess the potential to efficiently differentiate into cell types representative of the three germ layers [12]. To optimize differentiation, different conditions were tested, including variable cell densities, media composition (FBS, serum replacement, and growth factors), and surface matrices (Matrigel and collagen IV). The conditions that best supported the myogenic differentiation of iPSCs were determined by...
Figure 1. Expression of MyoD induces efficient myogenic differentiation of iPSCs. (A): Schematic sketch of iPSC generation and myogenic differentiation protocols. (B): Phase images of MyoD-infected FTc01-C2 iPSC line (derived from healthy donor) on indicated days following Dox treatment. (C): MyoD-infected FTc01-C2 iPSC line stained for MYHC (green) and MyoD (red) untreated (lower panels) or on day 6 post-Dox treatment (upper panels). Nuclei were stained with DAPI (blue). Scale bar = 100 μm. (D): Top panel shows highlighted area in (C) at higher resolution. Lower panel shows highlighted area above. Arrows point to examples of striated pattern of staining for MYHC. Scale bar = 50 μm. (E): iPSCs stained for MYHC (green) and Myogenin (red) untreated (lower panels) or on day 6 post-Dox treatment (upper panels). Nuclei were stained with DAPI (blue). Scale bar = 200 μm. (F): Efficiency of myogenic differentiation without induction of MyoD (−Dox) or 6 days post-MyoD induction (+Dox) determined as percentage of Myogenin+ nuclei of total number of nuclei. Average of triplicate samples (three wells; n ≥ 100 each from at least 3 fields) and standard deviations are shown from a representative experiment. (G): Fusion index determined as the percentage of nuclei occurring in myotubes (>2 nuclei) stained with MYHC. Average of triplicate samples (three wells; n ≥ 100 each from at least 3 fields) and standard deviations are shown from a representative experiment. Abbreviations: bFGF, basic fibroblast factor; DAPI, 4',6-diamidino-2-phenylindole; Dox, doxycycline; FBS, fetal bovine serum; HS, horse serum; iPSCs, induced pluripotent stem cells; MYHC, myosin-heavy chain; MyoD, myogenic differentiation 1; RF, reprogramming factor; ROCKi, r-associated protein kinase 1 inhibitor; rtTA, reverse tetracycline-controlled transactivator; SMC4, small molecule cocktail 4.
measuring the percentage of cells expressing MYHC, and included seeding cells at low density and using FBS and collagen. To prime cells for differentiation, iPSCs were seeded in growth media without basic FGF but with ROCK inhibitor (Thiazovivin) to promote survival and attachment. Under these optimized conditions, MyoD expression induced quick and dramatic changes in cell morphology of FTD2, C2 iPSCs, from rounded into spindle-like cells and eventually elongated myotubes (Fig. 1A, 1B). Elongated multinucleated spindle-like cells that were positive to MYHC were visible by day 3 following Dox addition (supplemental online Fig. 1D). Interestingly, we observed a correlation between the expression level of MyoD and that of MYHC, suggesting that a minimum level of MyoD expression is needed to achieve successful myogenic conversion (supplemental online Fig. 1E). Maturation of multinucleated myotubes was promoted by switching from the induction media (15% FBS) to the differentiation media (5% HS). These myotubes displayed a striated pattern of MYHC staining (Fig. 1C, 1D). To explore the extent of myocyte fusion and myofiber development, we measured the fusion index (percentage of nuclei in myotubes relative to total number of nuclei). Determining the fusion index for the iPSC-derived myoblasts indicated efficient myocyte fusion into multinucleated myotubes (33.9% ± 6.6%) (Fig. 1G). Additionally, ectopic expression of MyoD induced expression of the myogenic regulatory protein Myogenin. The differentiation efficiency was determined to be 49.5% ± 5.6% by calculating the percentage of Myogenin+ cells relative to the total number of cells 6 days post-Dox addition (Fig. 1E, 1F). Our MyoD-mediated approach was not cell-line specific as it was also effective in inducing myogenic differentiation of another iPSC line derived from a healthy donor (FTD2, C1; supplemental online Fig. 2). Further characterization of the MyoD-induced myogenic differentiation was provided by flow cytometry analyses: by day 6 after the addition of Dox, the majority of FTD2, C1 iPSCs had already assumed the myogenic identity, as reflected by expression of the skeletal myoblast markers NCAM (60%) and CD44 (52%) and the late myogenesis marker MYHC (21%) (supplemental online Fig. 2). FTD2, C2 iPSCs expressed even higher levels of the MYHC (56%) by day 6. Collectively, these results validate the use of MyoD as an efficient method to mediate the direct myogenic differentiation of human iPSCs without the need for an intermediate differentiation stage or flow cytometry-based sorting procedure.

Generation and Direct Myogenic Differentiation of iPSC Lines From Muscular Dystrophy Backgrounds

We applied the same MyoD-mediated approach to generate myoblasts from iPSCs generated from patients with muscular dystrophy. Muscular dystrophies are a group of genetic disorders characterized by progressive skeletal muscle degeneration. The Duchenne (DMD) and Becker (BMD) types of muscular dystrophies are caused by mutations in the DYSTROPHIN gene that codes for the muscle structural protein Dystrophin (DMD). We derived 16 iPSC lines from fibroblasts of 4 patients with DMD or BMD (supplemental online Table 1). Generation of the disease-specific iPSC lines proceeded routinely, and characterization of iPSCs revealed no major differences compared with iPSCs generated from healthy donors (Fig. 2; supplemental online Fig. 1A). The patient-specific iPSC lines expressed pluripotency markers to comparable levels and maintained stable genomes (Fig. 2A–2D). The disease-specific iPSC lines displayed potential to differentiate into cell types representative of the three germ layers as assayed in vitro (Fig. 2E) and in vivo (Fig. 2F). To test myogenic differentiation potential, the DMD-specific iPSC line GM05112-M5.1 was infected with the lentiviral vector containing Dox-inducible MyoD, seeded at variable cell densities on collagen-coated plates and cultured in the presence of Dox. As seen with healthy donor iPSCs, MyoD expression induced immediate morphological changes leading to formation of elongated spindle-like cells at 1×10^6 and 2×10^6 but not the higher 3×10^6 cells per cm^2 cell densities (Fig. 3A), suggesting that high cell density inhibits MyoD-mediated myogenic differentiation of iPSCs. Expression of the skeletal muscle marker NCAM was detected in response to MyoD expression, whereas the differentiation media without Dox failed to induce the expression of NCAM or MyoD (Fig. 3B). Flow cytometry analysis revealed that the ectopic expression of MyoD induced rapid myogenic specification, resulting in the expression of the myoblast markers NCAM (49%), CD44 (64%), and CD29 (70%) within 36 hours of Dox addition (Fig. 3C). Efficient myogenic differentiation of the DMD-specific iPSCs proceeded only under optimized conditions (seeding at low-cell density on collagen in FBS-containing media), and MYHC staining was barely detectable in the presence of Dox under alternative culture systems such as Matrigel and SR-containing media (Fig. 3D). MyoD expression induced Myogenin expression to levels similar to those seen in iPSCs from healthy donors (Figs. 1E, 1F, 3E, 3G). Staining the dystrophy-specific iPSC-derived myotubes for MYHC revealed a clear striated pattern (Fig. 3F). Additionally, measuring the fusion index for myoblasts derived from dystrophic iPSCs revealed efficient fusion (38.7% ± 8.5%) into multinucleated myotubes, suggesting that the Dystrophin dysregulation does not impact the efficiency of cell fusion under the conditions used (Fig. 3H).

MyoD-induced myogenic differentiation could be tracked using stage-specific markers. Pluripotency was quickly suppressed, as judged by the loss of expression of the pluripotency master regulator NANOG by day 3 following Dox addition (Fig. 4A). Exogenous expression of MYOD1 peaked at day 4 of differentiation. In contrast, endogenous MYOD1 continued to rise. The mesoderm marker T (Brachyury) was expressed transiently and was not detected after the switch to the differentiation media on day 4 (Fig. 4B). The early myogenesis markers PAX3 and PAX7 peaked at day 3 following the addition of Dox and declined after the addition of the differentiation media (Fig. 4B). However, PAX3 and PAX7 maintained a minimum level of expression at all times. There was a progressive increase in the expression of mature skeletal muscle markers (MYH1, MYOG, DESMIN, TNNC1, RYR1, and DMD) following the induction of MyoD expression (Fig. 4C, 4D). The parental fibroblasts of iPSC line GM05112-M5.1 were derived from a patient with a deletion in exon 45 of the DMD gene. Ectopic expression of MyoD in GM05112-M5.1 iPSCs led to increased expression level of DMD, but, as expected, the DMD transcripts were truncated (Fig. 4D). Wnt signaling plays an essential role during embryonic myogenesis and regulation of homeostasis of skeletal muscle cells in the adult [13–15]. Wnt5a and Wnt7a are upregulated in the early phase of muscle regeneration, whereas Wnt3a acts in later stages to regulate differentiation [16, 17]. As expected, the expression of WNT5A and WNT7A was induced by the ectopic expression of MyoD, whereas the expression of WNT3A, which is expressed in undifferentiated iPSCs, decreased in the early stage of differentiation before increasing again (Fig. 4E). In addition, there was selective expression of members of the Frizzled family of Wnt...
Figure 2. Generation of DMD- and BMD-specific iPSC lines. (A): Real-time reverse transcription polymerase chain reaction expression analyses of pluripotency genes OCT4 and SOX2 and reprogramming transgene (WPRE viral element) in indicated induced pluripotent stem cell (iPSC) lines, H1 human embryonic stem cells, and uninfected and/or infected (with reprogramming virus, day 4) fibroblasts. (B): Flow cytometry analysis of indicated undifferentiated iPSC lines for expression of SSEA4 and TRA-1-81. (C): Karyotype analysis of GM05112-M5.1 iPSCs at passage 16 showing normal genome. (D): Immunofluorescence staining of GM05169-M20.3 iPSCs for pluripotency markers Nanog, Oct4, TRA-1-60, and TRA-1-81. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. (E): In vitro trilineage differentiation potential of GM05112-M5.1 iPSCs. Immunofluorescence staining for markers of neural stem cells (Nestin), α-SMA, and definitive endoderm (Sox17) is shown in red. Nuclei were stained with DAPI (blue). Scale bar = 100 μm. (F): In vivo trilineage differentiation potential of GM05169-M20.3 iPSCs. Histological sections of teratoma derived from GM05169-M20.3 iPSCs. Arrows point to areas of interest: neuroectoderm (left panel), endoderm (glands; middle), mesoderm (muscle; right). Scale bar = 200 μm. Abbreviations: BMD, Becker muscular dystrophy; DAPI, 4',6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; Nanog, Nanog homeobox; Oct4, POU class 5 homeobox 1 (POU5F1/Oct4); SMA, α-smooth muscle Actin; Sox2, SRY (sex determining region Y)-box 2; Sox17, SRY (sex determining region Y)-box 17; SSEA4, stage-specific embryonic antigen-4; TRA, tumor-related antigen.
Figure 3. MyoD-induced myogenic differentiation of Duchenne muscular dystrophy (DMD)-specific iPSCs (GM05112-M5.1). (A): Phase images of DMD-specific GM05112-M5.1 iPSCs on indicated days following Dox treatment. Day 0 refers to iPSCs 1 day after seeding in seeding media and before adding Dox. Numbers on top represent seeding cell densities (cells per cm²). Scale bar = 1,000 μm. (B): Immunofluorescence staining for NCAM (red) and MyoD (green) 8 days following incubation of iPSCs with or without Dox. Nuclei were stained with DAPI (blue). Scale bar = 200 μm. (C): Flow cytometry analysis of NCAM (CD56), CD44, and CD29 expression in MyoD-infected GM05112-M5.1 iPSCs cultured without Dox (purple) or with Dox for 36 hours (blue) and human primary myoblasts (red). Control (black line) is unstained pooled cells. Numbers on the right reflect percentage of indicated cells relative to total cell population. (D): Immunofluorescence staining for MYHC (green) and nuclei (blue). Cells were seeded at indicated densities and cultured for 5 days in media containing 15% FBS and Dox or 5% SR and Dox before switching to differentiation media (5% HS) for 3 more days. Scale bar = 200 μm. (E): Immunofluorescence staining for MYHC (green), Myogenin (red), and nuclei (blue). Cells were cultured with (left) or without Dox (right). Scale bar = 200 μm. (F): Left panel shows iPSC-derived multinucleated myotube stained for MYHC (green), MyoD (red), and nuclei (blue). Right panel shows enclosed region of interest displaying striated pattern of staining. Scale bar = 50 μm. (G): Efficiency of myogenic differentiation with or without Dox treatment. Average of triplicate samples (three wells; \( n \geq 100 \) each from at least three fields) and standard deviations are shown from a representative experiment. Abbreviations: FBS, fetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole; Dox, doxycycline; iPSCs, induced pluripotent stem cells; MYHC, myosin-heavy chain; MyoD, myogenic differentiation 1; NCAM, neural cell adhesion molecule; SR, serum replacement.
Figure 4. MyoD mediates direct and rapid induction of the myogenic program in dystrophic iPSCs. (A–F): Gene expression analysis by real-time reverse transcription polymerase chain reaction of untreated (day 0) and Dox-treated MyoD-infected GM05112-M5.1 iPSCs (days 3, 4, 5, 6, and 8). (A): Expression of pluripotency marker NANOG, total MYOD1, and endogenous MYOD1. (B): Expression of mesoderm marker T (Brachyury) and skeletal muscle specification genes PAX3 and PAX7. (C): Expression of skeletal muscle markers MYH1, MYOG, DES, TNNC, and RYR1. (D): Expression of DMD as detected using TaqMan probes specific to exons 63 and 64 or exons 44 and 45. Note that parental fibroblasts of GM05112-M5.1 iPSCs have a deletion of exon 45 of the DMD gene. (E, F): Expression of components of the Wnt signaling pathways: indicated Wnt genes (E) and Wnt receptors Frizzled (FZD) genes (F). (G–I): Genome-wide gene expression analysis (Affymetrix Human Genome U133 Plus 2.0) of MyoD-infected GM05112-M5.1 iPSCs on days 0 (iPSCs-3), 3, and 8 post-Dox treatment, human primary myoblasts (undifferentiated and as differentiated myotubes), and undifferentiated iPSCs from healthy donors (iPSCs-1 and iPSCs-2). (G): The Pearson correlation coefficients for all possible sample pairs were visualized in a correlation matrix as a heat map. (H): Data for the top 100 genes (unselected; see supplemental online Table 2) with highest expression levels in primary myotubes were extracted for all the samples and visualized as a heat map. (I): Genes upregulated (>fourfold) in the Dox-treated iPSCs (day 8) relative to the undifferentiated GM05112-M5.1 iPSCs were subjected to Ingenuity Pathway Analysis to reveal the most significant associated functions. Abbreviations: DES, Desmin; DMD, dystrophin; Dox, doxycycline; Endo-MYOD1, endogenous MYOD1; iPSCs, induced pluripotent stem cell; MYH1, myosin, heavy chain 1, skeletal muscle, adult; MYOD1, myogenic differentiation 1; MYOG, Myogenin; Nanog, Nanog homeobox; Pax3, paired box 7; Pax7, paired box 7; RYR1, Ryanodine receptor 1, skeletal; TNNC1, Troponin C type 1; WNT, wingless-type MMTV integration site family, member.
receptors, with FZD2 (Wnt5A receptor [18]) and FZDS (Wnt5a and Wnt7a receptor [19, 20]) increasing and FZD9 decreasing.

We next characterized and compared the iPSC-derived myoblasts with human primary myoblasts using genome-wide expression analysis. We performed analysis and cross-comparison on seven samples: MyoD-infected DMD-derived GM05112-M5.1 iPSCs (days 0 [iPSCs-3], 3, and 8 post-Dox addition), human primary myoblasts (undifferentiated and as differentiated myotubes), and undifferentiated iPSCs derived from healthy donors and dystrophic iPSCs (iPSCs-1 and iPSCs-2). Examination of the Pearson correlation coefficients revealed that after 8 days of Dox addition, the DMD-specific GM05112-M5.1 iPSCs most closely resembled primary myotubes and were divergent from the undifferentiated iPSCs (Fig. 4G; supplemental online Fig. 3A, 3B). We next focused on the expression of skeletal muscle genes. We ranked all the gene expression values for the primary myotube sample and determined the top 100 genes (unselected) with the highest expression levels. The gene list contained well-established skeletal muscle genes, such as MEF2C, MYH3, MYH8, ENO3, TNNT3, TNNT1, TTN, and NCAM1 (supplemental online Table 2). The expression levels of the 100 genes were compared across all samples and visualized as a heat map (Fig. 4H). The vast majority of the genes in the list were expressed in the Dox-treated iPSCs by day 3, and their expression increased further by day 8, whereas the same genes were generally not expressed in undifferentiated iPSCs. We performed Ingenuity Pathway Analysis (IPA), which maps gene expression to specific pathways and functions. Analysis of the most upregulated genes (>fourfold; n = 1,662) in the day 8 Dox-treated iPSCs (relative to undifferentiated parental iPSCs) revealed that among the most significant associated functions were regulation of development, function, and associated disorders of skeletal muscle (Fig. 4I; supplemental online Fig. 3C). The IPA analysis of the upregulated genes predicted their most significant upstream regulators, which included MyoD and components of the transforming growth factor β and Wnt signaling pathways (supplemental online Fig. 3D). Other signaling pathways that were also significantly associated with the upregulated genes were PI3K/AKT, Notch, IGF-1, and FGF signaling pathways, all of which are involved in regulating muscle development and function [21] (supplemental online Fig. 3E). The MyoD-mediated myogenic differentiation potential was further confirmed in two more iPSC lines specific to patients with DMD and BMD (supplemental online Fig. 4). Collectively, our results indicate that MyoD induction is capable of directing the differentiation of iPSCs from healthy donors and dystrophic iPSCs into cells that closely resemble primary muscle cells and therefore may represent an efficient and robust method for modeling diseases of muscle.

**DISCUSSION**

Significant progress has been made in understanding the genetic and cellular causes of many neuromuscular diseases. However, there are no therapeutics currently available that have been specifically developed and approved for the treatment of the most common forms of muscular dystrophy. This is partially because of the challenges associated with developing accurate and robust cellular models of disease for research and drug discovery. Derivation of muscle progenitor cells from patient biopsy samples is challenging, as very small numbers of muscle satellite stem cells can be obtained [21]. Furthermore, the expansion of satellite cells in vitro is limited because of spontaneous differentiation and reduced regenerative capacity [26]. Derivation of satellite cells or their derivative myoblasts from patients with muscular dystrophies can be even more challenging [1–3]. For example, Blau et al. [1] reported a defect in the proliferative capacity of satellite cells derived from DMD patients and a 95% decrease in the yield of myoblasts from the diseased muscle biopsies compared with normal. Therefore, there is a need for alternative sources for healthy and dystrophic myoblasts to facilitate research in developmental biology, drug discovery, and potentially for the generation of large numbers of cells required for cell therapies.

Human iPSCs represent an attractive source for the generation of skeletal muscle cells, as they have unlimited self-renewal capacity and the potential to differentiate into any cell type given the availability of robust differentiation technology and protocols [4]. However, the differentiation of human iPSCs into skeletal muscle cells is challenging, and only a small number of studies have attempted to address this problem. In an early work, Barberi et al. [5] generated skeletal myoblasts from human iPSCs through a multistage differentiation process (>6 weeks) that included generation of mesenchymal precursors followed by two rounds of cell enrichment using FACS. In a perhaps more promising but still time-consuming approach (>4 weeks), Darabi et al. [6] derived myoblasts by differentiating iPSCs into EBs, followed by overexpression of exogenous Pax7-green fluorescence protein (GFP) and purification of GFP+ cells by FACS. MyoD is a myogenic regulatory factor that was shown more than 2 decades ago to induce myogenesis in multiple somatic cell types [8, 9]. MyoD functions downstream of Pax7 (and the related Pax3) in mediating skeletal myogenesis [21] and thus could potentially induce a faster and more efficient way to generate muscle cells from iPSCs.
myogenic differentiation of human iPSCs compared with the use of Pax7 or Pax3. Although an indirect path, the ectopic expression of MyoD in mesodermal derivatives of iPSCs (mesoangioblasts) led to efficient generation of myoblasts in a multistage process that took 3 weeks [7]. A more direct approach for the myogenic differentiation of iPSCs was performed recently by introducing exogenous MyoD into undifferentiated iPSCs but surprisingly led to little observed myogenesis except when a component of the SWI/SNF chromatin-remodeling complex (BAF60C) was ectopically coexpressed with MyoD [27]. Another recent attempt to directly induce myogenic differentiation in human embryonic stem cells (ESCs) by the forced expression of MyoD using adenoviral system reported very low direct myogenic conversion (<1%) based on desmin expression, potentially because of the

Figure 5. IGF-1 and Wnt7a induce hypertrophy in DMD- and Becker muscular dystrophy (BMD)-specific myotubes. Human primary myotubes and dystrophic iPSC-derived myotubes (day 8 post-doxycline addition) were treated with either formulation control, 100 ng/ml Wnt7a, or 100 ng/ml IGF-1 for 2 days. Cells were stained with myosin-heavy chain and 4',6-diamidino-2-phenylindole, and images were analyzed by Axiovision software for measurement of fiber diameter (n = 100 fibers per treatment). (A): Representative images of primary myotubes (lower panels) and DMD-specific GM05112-M5.1 iPSC-derived myotubes treated as indicated. Scale bar = 200 μm. (B–E): Fiber diameter measurements for human primary myotubes (B) and DMD-specific GM05112-M5.1 (C), DMD-specific GM05169-M20.3 (D), and BMD-specific GM05081-C3 (E) iPSC-derived myotubes. Bars show median values with interquartile ranges. ****, p < .0001. Abbreviations: DMD, Duchenne muscular dystrophy; IGF-1, insulin-like growth factor 1; iPSC, induced pluripotent stem cell; Wnt7a, wingless-type MMTV integration site family, member 7A.
observed low infection rate and elevated cell death [28]. It was therefore unclear whether the inability to directly convert human ESCs/iPSCs into myoblasts through the ectopic expression of MyoD is because of inherent cellular barriers or technical complications.

In this study, we described an optimized, simple, and efficient monolayer method based on the ectopic expression of MyoD for the generation of myoblasts from normal and dystrophic iPSCs. The forced expression of MyoD was sufficient to quickly reprogram iPSCs into myoblasts that differentiated into myocytes capable of in vitro cell fusion into multinucleated myotubes. The MyoD-mediated induction of myogenic differentiation of iPSCs was dramatically faster than traditional methods of myogenic differentiation that rely on differentiation into mesodermal precursors or EBs and/or cell sorting of differentiated cells (<1 week compared with 3–6 weeks) [5–7]. iPSCs were cultured on Matrigel (feeder cell-free) in small molecule 4 (SMC4)-supplemented media enabling single-cell passage, enhanced survival, and efficient infection [11, 12]. MyoD induction was sufficient to direct myogenic differentiation of iPSCs cultured on collagen without the need for coculture with mouse stromal cells (e.g., OP9). These improvements could potentially enable the use of our differentiation scheme in high-throughput applications such as those needed in drug screening, optimization of differentiation, and transgenic manipulation and cloning. Optimizing cell culture, transgene delivery, and differentiation conditions was needed for efficient induction of myogenesis in iPSCs. For example, the use of suboptimal conditions that promoted the undifferentiated state such as the use of high cell densities along with the use of Matrigel (instead of collagen) and serum replacement (instead of FBS) led to low rate of myogenic conversion. In addition, the level of MyoD expression correlated with the level of MYHC expression. Such functional consequences of heterogeneity in levels of gene expression have been observed in a previous study: subpopulations of satellite cells expressing high levels of Pax7 were found to exhibit less metabolic activity and to appear less mature compared with satellite cells expressing lower levels of Pax7 [29]. It is therefore possible that differing levels of MyoD expression might exert variable effects on the ability to mediate myogenesis.

As observed with healthy donor iPSCs, induction of exogenous MyoD expression led to rapid (36-hour) and efficient (50%–70%) commitment toward the myogenic identity, as determined by expression of myogenic markers (Myogenin, MYHC, NCAM, CD44, and CD29). The MyoD-mediated differentiation proceeded in expected phases: suppression of pluripotency (NANOG), followed by induction of mesodermal markers (Bra-chury), early muscle markers (PAX3/7), and finally mature muscle markers (e.g., MYHC, MYOG). Switching from the induction media to the differentiation media led to suppression of the muscle progenitor markers (PAX3/7) and further increases in the expression levels of late muscle markers such as MYHC, MYOG, DES, TNNC1, RYR1, and DMD. DMD, however, was expressed in its truncated form, reflecting the disease-specific background of the cell lines. Interestingly, we were unable to detect the expression of the myogenic transcription factor MYF5 during the course of differentiation. This observation is in agreement with findings reported in other studies [28, 30]. MyF5 is an early marker of the myogenic lineage and is known to be inhibited by MyoD during myogenesis [31]. Cell fusion of mature myocytes was visible as early as 3 days from Dox addition and increased further upon the addition of differentiation media. Global gene expression of iPSCs over time following Dox addition confirmed the conversion of iPSCs into cells that closely resembled primary muscle cells and diverged greatly from their parental undifferentiated iPSCs. In addition, we have shown that the dystrophic iPSC-derived myoblasts have the potential to functionally respond to hypertrophy-inducing factors Wnt7a and IGF-1, which are being under investigation as potential treatments for DMD in preclinical and clinical studies, respectively. Treatment of myotubes derived from three different iPSC lines (from three patients) with Wnt7a or IGF-1 induced significant hypertrophy effects similar to those observed on primary myotubes, highlighting the utility of our myogenic differentiation approach for use in drug validation and screening.

CONCLUSION

Collectively, our results indicate that there are no intrinsic barriers specific to undifferentiated human iPSCs preventing direct MyoD-induced myogenesis, as has been suggested recently [27]. Our findings are in agreement with the observed MyoD-mediated effects in other cell types. Our results are also consistent with the findings of a recent report that was published while the present manuscript was being prepared [30]. Tanaka et al. [30] used a drug-inducible piggyBac expression system to ectopically express MyoD in undifferentiated iPSCs derived from patients with Miyoshi Myopathy (a congenital myopathy caused by mutations in DYSFERLIN) leading to direct, rapid, and efficient myogenesis.

One goal of regenerative medicine is the correction of genetic disease through cellular replacement therapy. To facilitate this, safer methods for the generation of human iPSCs and for the delivery of MyoD would need to be devised. Recent studies have demonstrated the feasibility of using episonal expression systems, mRNAs, or proteins in lieu of integrating lentiviral systems for the generation of transgene-free iPSCs [32–34]. Similar approaches might also be used for safer MyoD delivery and myogenic differentiation of iPSCs [34]. However, our current platform for the myogenic differentiation of human iPSCs is rapid, efficient, feeder/stroma-free, and amenable to high-throughput applications and thus could facilitate the more immediate applications of drug discovery and disease modeling.

AUTHOR CONTRIBUTIONS

R.A.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; M.B. and B.V.: collection and/or assembly of data, data analysis and interpretation; T.T.L., M.R., D.R., T.L., and K.L.: collection and/or assembly of data; P.F.: conception and design, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

R.A. has compensated employment with Fate Therapeutics, Inc., is an inventor on a patent application, and has stock ownership. M.B. has compensated employment. B.V. has compensated employment and stock options. T.T.L. has compensated employment with Fate Therapeutics, Inc. B.V. and M.R. have compensated employment and stock options with Fate Therapeutics, Inc. T.L. has compensated employment and stock options. K.L. has compensated employment and stock options. P.F. has compensated employment with Fate Therapeutics, Inc., has uncompensated intellectual property rights, and compensated stock options.
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