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Core Transcription Factors Promote Induction of PAX3-Positive Skeletal Muscle Stem Cells

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SUMMARY

The use of adult skeletal muscle stem cells (MuSCs) for cell therapy has been attempted for decades, but still encounters considerable difficulties. MuSCs derived from human induced pluripotent stem cells (hiPSCs) are promising candidates for stem cell therapy to treat Duchenne muscular dystrophy (DMD). Here we report that four transcription factors, HEYL, KLF4, MYOD, and PAX3, selected by comprehensive screening of different MuSC populations, enhance the derivation of PAX3-positive myogenic progenitors from fibroblasts and hiPSCs, using medium that promotes the formation of presomitic mesoderm. These induced PAX3-positive cells contribute efficiently to the repair of DMD-damaged myofibers and also reconstitute the MuSC population. These studies demonstrate how a combination of core transcription factors can fine-tune the derivation of MuSCs capable of contributing to the repair of adult skeletal muscle.

INTRODUCTION

The muscular dystrophies are a group of inherited skeletal muscle disorders characterized clinically by progressive muscle weakness and wasting. Duchenne muscular dystrophy (DMD) is among the most common and severe forms of these muscle diseases. DMD is caused by mutations or deletions in the Dmd gene, which lead to loss of muscle fiber integrity and continuous muscle damage. This damage leads to the rapid wasting of skeletal muscles, and there is as yet no cure, although a number of promising approaches are being developed to retard the progression of DMD symptoms (Guiraud et al., 2015). Cell replacement therapy uses extrinsic myogenic cells that express functional dystrophin protein to replace the abnormally affected skeletal muscle tissue of individuals with DMD (Negroni et al., 2016).

Skeletal muscle tissue has its own intrinsic repair and maintenance system, which depends on adult muscle stem cells (MuSCs). MuSCs are closely associated with the muscle fiber, hence their description as satellite cells, and are normally quiescent, but begin to proliferate in response to muscle injury or during intense exercise. They enter the myogenic differentiation program, fuse with damaged myofibers or form de novo fibers, and also reconstitute the quiescent MuSC population (Collins et al., 2005). Mouse MuSCs that have no Dmd mutation, when engrafted into the damaged muscle of DMD mice, contribute to the regeneration of DMD myofibers, which are now positive for functional dystrophin protein (Cerletti et al., 2008).

Skeletal muscle regeneration is regulated by families of transcription factors also essential for skeletal muscle formation in the embryo. PAX3, a paired box transcription factor, is expressed in the paraxial mesoderm of newly forming somites and then in the dorsal compartment of somites, the dermomyotome, which will give rise to myogenic progenitor cells. Pax3, and its sister gene, Pax7, also expressed later in all muscle progenitor cells, lie upstream of the MYOD family of basic-helix-loop-helix transcription factors that play an essential role in muscle formation. The MYOD family is specific to skeletal muscle, whereas PAX3 and PAX7 are also expressed in some cells of the central nervous system, and PAX3 plays an important role in the development of neural crest cells (Buckingham and Relaix, 2015). In the skeletal muscle context, the population of PAX3/PAX7-positive progenitors is essential for myogenic growth during development and is also regarded as the source of adult MuSCs (Gros et al., 2005). Recent studies have revealed that core transcription factors, expressed in adult stem cells of a number of different tissues, can induce and maintain a specific cell lineage directly when introduced into pluripotent stem cells (PSCs) and into other cell types (Morris, 2016). A classic example of such tissue-specific conversion is provided by the MYOD family, which when expressed in non-muscle cells activates the endogenous MyoD family genes and leads to the establishment of the gene regulatory network required for the subsequent formation of skeletal muscle (Weintraub et al., 1989).
In the study reported here, we established a defined system to induce myogenic stem cells from fibroblasts, identified by expression of a Pax3-GFP reporter. This is based on a combination of transcription factors which, together with appropriate mesodermal culture medium, are more effective than any single factor in generating PAX3-positive myogenic progenitors that when engrafted into dystrophic mouse muscle efficiently regenerate dystrophin-positive fibers and reconstitute the PAX7-positive stem cell compartment. These results provide new insight into the core transcription factors that underlie the conversion of non-muscle cells to myogenic progenitors of potential therapeutic interest.

RESULTS

MYOD Induces Myogenic Cell Conversion, but Not PAX3-Positive Muscle Stem Cells

We have developed Pax3\(^{GFP/+};\) MyoD-Cre; Rosa26\(^{CAG-LSL-tdTomato/+}\) (Pax3-GFP; MYOD-tdTomato) compound mice to visualize GFP-positive MuSCs (green in Figure 1A) and MYOD-primed myogenic cells (red in Figure 1A). Mouse embryonic fibroblasts isolated from this mouse line (Figures S1 and S2A) were transfected with a Pax3- and/or MyoD-expression pMX vector. MyoD- or Pax3+MyoD-infected cells are gradually converted into myogenic cells, which express the endogenous MyoD gene, shown as tdTomato-positive cells (Figure 1B), but not Pax3-GFP-positive cells corresponding to myogenic progenitor/muscle stem cells (right panels in Figure 1B). We could detect MyoD-primed, tdTomato-positive cells from day 3 after MyoD transfection, and the proportion of these tdTomato-positive cells increased for 2 weeks after the transfection, whereas Pax3-GFP-positive cells were rarely detected (Figure 1C). Expression of Pax3 and MyoD was not enough to induce PAX3-positive cells from fibroblasts.

Transcription Factors that Induce Pax3-Positive Myogenic Cells

Next, we investigated the expression of potential regulatory genes in Pax3-GFP-positive cells isolated from skeletal muscle of fetal, postnatal, and adult mice (Figure S1A). We used these three sources of cells to identify genes that are common to the MuSC identity, irrespective of other features of the cells at different developmental stages. Genes encoding transcription factors that are highly expressed in all three conditions, compared with other cell types, were selected (Figures S1B and S1C; Tables S1 and S2). We first investigated whether expression of a combination of these transcription factors could induce Pax3-GFP-positive cells derived from mouse embryonic fibroblasts of Pax3-GFP; MYOD-tdTomato embryos. Pax3-GFP-expressing cells were generated most efficiently by a mixture of eight transcription factors (+8F), Egr2, Hey1, Klf4, Mef2c, MyoD, Pax3, Pax7, and Pitx2, which were highly expressed in all three PAX3-positive muscle progenitor/stem cell populations (Figures 2A, 2B, and S3B). We attempted to determine which of the eight transcription factors were essential for the induction of PAX3-positive MuSCs. Pax3, Hey1, Klf4, and MyoD had the ability to induce Pax3-GFP cells effectively (Figure S2C). However, MYOD protein could not be detected in adult quiescent MuSCs as previously reported (Montarras et al., 2005). We therefore controlled the expression level of MYOD with doxycycline (DOX). Pax3, Hey1, Klf4 (3F), and constant MYOD (+DOX) could also induce Pax3-GFP as seen for 8F; however, 3F and transient MYOD expression for 72 h (+DOX) effectively enhanced the population of GFP-expressing cells (Figure 2C). These induced GFP-positive cells, selected by fluorescence-activated cell sorting (FACS), were detected as Pax7-positive cells (Figure 2D). These GFP-positive cells also expressed endogenous Pax7, Calcitonin receptor, Sprout1, and Syndecan4 transcripts, markers of muscle satellite cells (Pukada et al., 2007; Shea et al., 2010), and did not show elevated levels of transcripts for Sox1 or Sox10, markers of neural or neural crest cells (Figure 2E). To compare induced GFP-positive cells with endogenous Pax3-GFP MuSCs, we expanded both populations on cell-culture dishes for 4 days. The numbers of growing cells were similar, although induced GFP-positive cells had a tendency to form aggregates (lower panel in Figure 2F) and less tendency to spontaneously form muscle fibers (Figure 2F), with maintenance of mononucleated progenitors reflected by a higher level of Pax7 transcripts (Figure 2G). Both GFP-positive cell populations underwent myogenic differentiation when cultured under low serum conditions, as shown by staining for skeletal muscle myosin heavy chain (MyHC, Figure 2H). Differentiated MyHC-positive muscle cells from induced GFP cells were elongated and formed fewer, thinner fibers (Figure 2I). In these cultures, compared with differentiated muscle satellite cells, transcripts of Pax7 were higher indicating more maintenance of muscle progenitors, with similar levels of transcripts for embryonic MyHC (Myh3) and lower levels of transcripts of Myh7 and Myh1 encoding slow and fast adult MyHCs, respectively (Figure 2J). These results suggest that the induced GFP cells have a less mature skeletal muscle phenotype under these culture conditions. In conclusion, the four factors (4F) have the competence to convert mouse embryonic fibroblasts to PAX3- and PAX7-positive cells that have myogenic differentiation potential as indicated by the activation of genes for skeletal muscle-specific proteins such as Myogenin (Myog) and skeletal muscle MyHC. This was also the case for human dermal fibroblasts (Figure S3).
Mesodermal Differentiation with PAX3\textsuperscript{GFP/+} Human Induced PSCs

To identify PAX3-positive human MuSCs, we generated PAX3\textsuperscript{GFP/+} (PAX3-GFP) knockin human induced PSCs (hiPSCs) using a BAC construct with a GFP insertion in the ATG start codon of the human PAX3 gene (Figure S4A). Intrinsic GFP expression in cells derived from these hiPSCs could be detected in teratomas or neural precursors after SFEBq induction (GFP expressions in Figure 3A; Watanabe et al., 2005). After cell sorting with induced cells, GFP-positive cells were successfully immunostained with an anti-PAX3 antibody, while GFP-negative cells did not show the transcription of PAX3 (Figure 3B). This hiPSC PAX3\textsuperscript{GFP/+} knockin cell line was further genetically modified by the introduction of a DOX-inducible MYOD cassette using the piggyBac transposon system (Tanaka et al., 2013). To induce differentiated myogenic cells derived from these hiPSCs, we administered DOX continuously for at least a week (Figure S4B). This resulted in myogenic cell differentiation but GFP-positive cells were only rarely detected, in keeping with the result seen with mouse fibroblasts (Figure 1C and +3F++DOX in Figure 2C). However, transiently DOX-treated hiPSCs gave rise to significantly more GFP-positive cells than continuous or no DOX administration (DOX for 72 h in Figure 3C). To investigate gene expression in these GFP-positive cells we analyzed myogenic, neural, and neural crest markers, since Pax3 is expressed in the corresponding cell types of developing embryos. GFP-positive cells

Figure 1. MYOD and PAX3 Are Not Sufficient to Induce Myogenic Stem Cells, but Result in Differentiated Cells
(A) Schematic representation of skeletal muscle development in mouse embryogenesis. Pax3\textsuperscript{GFP/+} (Pax3-GFP) indicates dermomyotome (DM; green), and MyoD-Cre;Rosa26\textsuperscript{CAG-LSL-tdTomato/+} (MyoD-tdTomato) primed populations were initially labeled in myotome (M; Red). NT, neural tube; DRG, dorsal root ganglia; N, notochord; DML, dorsomedial lip; VLL, ventrolateral lip. (B) Mouse Pax3, MyoD, and Pax3+MyoD infected mouse embryonic fibroblasts (MEFs) derived from Pax3-GFP;MyoD-tdTomato embryos after 7 days. Scale bars, 50 μm. (C) FACS analyses with MEFs following the time course after MyoD infection, from day 3 to day 14.
Figure 2. The Combination of Four Transcription Factors Induces PAX3-Expressing Myogenic Cells from Mouse Fibroblasts

(A and B) Detectable GFP expression in MEFs (arrowheads) infected with eight transcription factors (+8F) after 14 days.

(C) FACS analyses of GFP-expressing cells with mouse Pax3, HeyL, and Klf4 (+3F) and persistent MYOD (++DOX) or transient MYOD for 72 h (+DOX).

(D) Immunofluorescence for GFP (labeled with Alexa 488, green) and Pax7 (labeled with Alexa 647, red) with GFP-positive cells induced by 3F and transient MYOD acceleration (+3F+DOX).

(E) Expression levels of Pax7, Calcr, Spry1, Sdc4, Sox1, and Sox10 transcripts levels in induced Pax3-GFP-positive (gray) or -negative (white) cells with 3F+DOX. n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. **p < 0.01, *p < 0.05.

(F) Morphological features of cultured GFP-positive satellite cells (GFP+ MuSCs; upper panel) and three transcription factors with transient DOX treatment (+3F+DOX GFP+ cells; lower panel) for 4 days.

(G) Myogenic transcripts of Pax7, endogenous MyoD (MyoD UTR), and Myog relative to Rpl13a transcripts in GFP+ cells cultured for 4 days. n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. *p < 0.05.

(H) Differentiated myogenic cells (DAPI, blue; MyHC, Alexa 647, red) from induced GFP-positive cells with 3F+DOX (right panels) compared with mouse Pax3-GFP satellite cells (GFP+ MuSCs; left panels) on 2% horse serum for 7 days.

(I and J) Quantification of the ratio of DAPI-positive mono (N = 1) or multiple nuclei (N = 2, 3 or N > 3) present in single MyHC-positive myofibers of (H). n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. *p < 0.05.

(J) Transcriptional levels of myogenic markers Pax7, MyoD, Myog, Myh3, Myh7, and Myh1, differentiated for 7 days from GFP+ cells. n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. *p < 0.05.

Error bars indicate ±SEM. Scale bars, 50 μm.
Figure 3. Mesodermal Differentiation from \textit{PAX3}GFP/+ Derived hiPSCs

(A) GFP expression in induced teratoma (left panels) and neural precursors by SFEBq induction (right panels) derived from \textit{PAX3}GFP/+ hiPSCs. Scale bars, 200 μm.

(B) Immunostaining with Pax3 antibody in GFP-sorted cells (upper panel) and the ratio of \textit{PAX3} transcript relative to \textit{RPL13A} in GFP-sorted cells (lower panel). Red, \textit{PAX3}; blue, DAPI. Scale bar, 100 μm.

(C) FACS profiles of \textit{Pax3GFP/+; Tet-On MYOD} hiPSCs with or without continuous doxycycline (DOX) treatment (left panel, no DOX; middle panel, continuous DOX) and transient treatment for 72 h and cultured for a total of 7 days (right panel, DOX for 72 h).

(D) Relative transcription levels of dermomyotome (\textit{DMRT2}), myogenic (\textit{MYF5}), and neural (\textit{SOX1}, \textit{SOX10}) progenitors in induced GFP-positive cells treated with doxycycline for 72 h and cultured for a total of 7 days. \textit{n} = 3 independent replicates; \textit{p} values are determined by \textit{t} test from a two-tailed distribution. *\textit{p} < 0.05.

(E) Schematic representation of the induction to mesodermal cells from hiPSCs.

(F) Morphological features and GFP expression of mesodermal differentiated cells derived from \textit{PAX3}GFP/+; \textit{Tet-On MYOD} hiPSCs.

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derived from DOX-treated hiPSCs acquired myogenic potential, as shown by the presence of MYF5 transcripts; however, neural transcripts of SOX1 or SOX10 were also detected (Figure 3D). This suggests that neural Pax3-positive cells may also arise even in the MuSC culture condition with transient MYOD expression (Figure 54C). We therefore attempted to exclude neural induction from hiPSCs by using modified cell-culture conditions to favor mesodermal, not ectodermal, lineages. Several groups have reported culture conditions that promote induction of somitic cells in the mesodermal lineage from human PSCs, and we adopted a method to induce paraxial mesodermal cells by manipulation of transforming growth factor β, bone morphogenetic protein, and Wnt signaling (Nakajima et al., 2018, Figure 3E). Pax3-GFP-expressing cells were obtained in induced aggregates of cells, reminiscent of mouse somites (Figure 3F), showing partial expression of DLL1, which marks the paraxial mesodermal lineage, during somite differentiation (green in Figure 3G). We evaluated induced DLL1+ or DLL1+Pax3 cells for expression of transcripts for MESOGENIN (MSGN), TBX6, and PDGFRα, which are presomitic mesodermal markers, or DMRT2, which is a dermomyotome marker of early somites (Sato et al., 2010). Successful mesodermal induction was demonstrated by high levels of MSGN, TBX6, or PDGFRα transcripts in total DLL1+ cells, and high DMRT2 expression in DLL1+GFP+ double-positive cells (Figure 3H). We next analyzed neural or non-neural transcripts in Pax3-GFP cells under different differentiation conditions. As shown in Figure 3I, on treatment with 5 μM CHIR99021, which promotes neural crest lineages derived from hiPSCs, including hiPSCs transiently treated with DOX for 72 h, the level of SOX1 or SOX10 transcripts was highly upregulated; however, GFP-positive cells treated with the mesodermal induction procedure for 1 week did not express neural crest markers but did express transcripts for DMRT2, also seen for a longer period of 2 weeks in culture (Figure 3I).

**Additional Expression of Four Transcription Factors in Mesodermal Cells Induces Pax3-Positive Muscle Stem Cells**

Using induced mesodermal cells labeled with DLL1, derived from hiPSCs, we attempted to determine which of the eight transcription factors was essential for the induction of Pax3-positive MuSCs (Figures 4A and S2). The combination of Pax3, HEYL, KLF4, and inducible transient MYOD (+3F+DOX) had the ability to induce Pax3-GFP (mCherry-positive/negative) cells effectively, in contrast to single transient or continuous MYOD (+DOX or +3F+DOX; Figures 4B and 4C). To evaluate whether these induced GFP cells have the characteristics of MuSCs, we checked cell-surface antigens, transcripts in these cells, and myogenic differentiation activity. Both CD56 and CD82, markers of myogenic stem cells and myocytes (Alexander et al., 2016), were co-expressed on 60% of 4F-induced Pax3-GFP cells (Figure 4D). These GFP-sorted cells showed mainly Pax7-positive staining; however, Pax7-negative cells were detected as MYO-G-positive differentiated muscle cells (Figure 4E). When GFP-positive or -negative populations of CD56-positive myogenic cells were seeded under myogenic differentiation conditions with 2% horse serum, GFP-positive cells labeled the differentiation marker MYOG or MYHC, although only a few myofibers were observed (Figures 4F–4H) in comparison with GFP-negative cells of the CD56+ population. This is consistent with downregulation of Pax3, and subsequently the GFP reporter on muscle differentiation and muscle fiber formation; the continuing presence of exogenous Pax3 transcripts in this population of myogenic cells may explain why the number of myofibers is low (Figure 4I; Relax et al., 2006). Comparison of transcript levels of DMRT2, NFIX that marks myogenic progenitors at fetal stages (Mesina et al., 2010), or NAPIL5 that marks adult MuSCs (Palafacchina et al., 2010) shows that +3F+DOX leads to myogenic progenitor/stem cells of fetal and adult types (Figure 4J). Furthermore, the levels of Pax7 or CalcR transcripts specifically expressed in adult quiescent MuSCs were high in induced GFP cells derived from mesodermal lineages compared with DOX-induced GFP cells (Figure 4K). In the absence of exogenous Pax3, the three factors HEYL, KLF4, and MYOD also induced Pax3-GFP cells from mesodermal cells; however Pax7, CalcR, or NAPIL5 transcripts in GFP+ cells were all decreased (Figure 5A), and the cells showed less myogenic differentiation (Figures 5B–5D). Comparison of the results obtained with induction by the four factors identified here with previously published protocols for inducing MuSCs, using growth factors (Chal et al., 2015) or with induced Pax7 (Darabi et al., 2012), showed that the protocol we describe leads to more mature MuSCs expressing adult satellite cell

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**Notes:**

(G) Cultured cell populations on mesodermal induction for 7 days (left panel) and subpopulation of ectodermal GFP (GFP+DLL1+) and mesodermal GFP (green box, GFP+DLL1+) derived from presomitic mesoderm (GFP+DLL1+) labeled with the hDLL1 antibody (right panel).

(H) Transcription analyses of each type of sorted cells with presomitic mesodermal markers (MSGN, TBX6, PDGFRα) and the dermomyotome marker DMRT2. n = 3 independent replicates; p values are determined by Dunnett’s multiple-comparisons test. *p < 0.05, **p < 0.01.

(I) Transcription analyses with Pax3-GFP-sorted cells on each differentiated condition. n = 3 independent replicates; p values are determined by Dunnett’s multiple-comparisons test. *p < 0.05, **p < 0.01.

Error bars indicate ±SEM.
**A** day7
mesodermal induction

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

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markers (Figure S5). We conclude that cells from mesodermal cells with the four transcription factors have the characteristics of skeletal MuSCs and show myogenic potential when cultured in vitro.

Transplanted PAX3-GFP Cells in the DMD Mouse Model Contribute to Skeletal Muscle Regeneration

Quiescent adult MuSCs are highly potent for skeletal muscle regeneration and form muscle fibers when they are transplanted into damaged muscle. We have previously reported that embryonic muscle precursors that are PAX3-GFP positive do not contribute effectively to regenerating muscle fibers in Dmd<sup>−/−</sup> mice (Sakai et al., 2013). We now tested the in vivo myogenic capacity of PAX3-GFP-positive cells obtained from hiPSCs by the three factors and transient MYOD procedure. Dmd<sup>−/−</sup>;NSG (DMD) mice were engrafted with GFP<sup>+</sup> cells, which were directly collected after sorting to evaluate their MuSC regenerative capacity (Figure 6A). These GFP-positive cells effectively contributed to regenerated muscle fibers after their engraftment into DMD mice, as detected by human Dystrophin (DYS) expression in regenerating muscle fibers (mean = 282 DYS<sup>+</sup> fibers/section of the tibialis anterior (TA) muscle, 9.4% positives in total myofibers of the TA muscle, red in Figures 6B and 6C). When cultured for 4 days ex vivo, GFP-negative cells induced by the four factors (+cultured GFP<sup>+</sup> or +GFP<sup>+</sup>, +3F+DOX), or GFP-positive cells induced by the four factors (GFP<sup>+</sup>, +3F+DOX) or after DOX alone (+GFP<sup>+</sup>, +DOX), these cells showed significantly reduced regenerative efficiency when the same number of cells was injected (0.43%, 0.60%, or 0.31%, respectively, Figure 6C). To investigate the capacity of PAX3-GFP-positive cells to renew the satellite cell compartment, we analyzed numbers of human-derived PAX7<sup>+</sup> cells underneath the basal lamina of mouse skeletal muscle fibers (arrowheads in Figure 6D). GFP-positive cells obtained after treatment with four factors occupied a satellite cell position on regenerated fibers, thus indicating renewal of the satellite cell compartment (mean = 27 PAX7<sup>+</sup>hLMNA<sup>+</sup> cells/section, Figures 6D and 6E). In contrast, GFP-negative cells or other induced GFP cells, including MYOD-expressing cells induced by DOX alone, did not contribute to reconstitution of the satellite cell compartment (Figure 6E). Transplanted cells, which were induced by four factors, were detected in the position of muscle satellite cells and also in the interstitial area. Whereas cells in the satellite position did not show MYOD-positive signals (green arrowheads in left panel of Figure 6F), those in the interstitium were MYOD positive, consistent with their status as activated myogenic cells that are still contributing to regeneration (green arrowheads in right panel of Figure 6F). To understand the relationship between regeneration and cell expansion of transplanted cells, we found Ki67-positive cells in the transplanted area and non-proliferating transplanted cells on reconstituted muscle fibers, which were DYS positive (red arrowheads in Figure 6G). Ki67-positive

Figure 4. Selected Four Transcription Factors Induce Human PAX3-Positive Muscle Stem Cells Derived from the Mesodermal Lineage

(A) Procedure for the induction of PAX3-GFP cells derived from mesodermal lineage.

(B) GFP or mCherry expression in transient MYOD-induced populations after expression of PAX3, HEYL, and KLF4 transcription factors (+3F), and doxycycline for 72 h (+DOX).

(C) The contrasting proportion of induced PAX3-GFP cells with transient single doxycycline treatment (+DOX) and three transcription factors and transient/continuous doxycycline (+3F+DOX/+3F+DOX).

(D) The FACS profile of CD56 and CD82 expression, human myogenic cell-surface markers, in induced cells. More than 60% of PAX3-GFP cells were merged with CD56<sup>+</sup>CD82<sup>+</sup> as double positives.

(E) Immunostaining with anti-PAX7 (left panel, labeled with Alexa 488, light blue) and anti-MYOGENIN (MYOG; right panel, labeled with Alexa 647, purple) antibodies in CD56<sup>+</sup>CD82<sup>+</sup> GFP<sup>+</sup> sorted cells. GFP signals were not detected after immunostaining.

(F and G) In vitro myogenic differentiation assay with induced PAX3-GFP-positive (CD56<sup>+</sup>GFP<sup>+</sup>) or -negative (CD56<sup>+</sup>GFP<sup>−</sup>) cells of CD56<sup>+</sup> positive cells in 2% of horse serum medium for 7 days. MYOG, purple; MYHC, light blue (labeled with Alexa 647); DAPI, blue.

(H) Percentage of differentiated myogenic cells presented as the ratio of MYOG or MYHC/DAPI fluorescence. n = 3 independent replicates; p values are determined by Dunnett’s multiple-comparisons test. *p < 0.01.

(I) Fetal (NFDX) and adult (NAP1L5) transcriptional analyses with PAX3-GFP-positive or -negative cells of CD56-positive cells induced by transient DOX treatment plus three transcription factors (+3F+DOX). Mesodermal GFP-expressing cells, marked by DMRT2 (dermomyotome), were used as control (GFP<sup>+</sup> mesoderm). n = 3 independent replicates; p values are determined by Dunnett’s multiple-comparisons test. *p < 0.01.

(J) Transcriptional analyses with GFP-positive cells induced by transient DOX treatment plus three transcription factors (+3F+DOX). GFP-expressing cells with or without DOX were used as control (GFP<sup>+</sup> – DOX/+DOX). n = 3 independent replicates; p values are determined by Dunnett’s multiple-comparisons test. *p < 0.01. Error bars indicate ±SEM. Scale bars, 50 μm.
cells were detected in the interstitial area, not in the DYS-expressing area (white arrowhead in Figure 6G). We conclude that induced-PAX3 positive cells, grown under mesodermal culture conditions and expressing the four transcription factors, contribute to the formation of new skeletal muscle fibers, thus recapitulating aspects of normal regeneration after cell transplantation in vivo.

**DISCUSSION**

Here we define a combination of myogenic reprogramming factors that can induce MuSCs, capable of regenerating adult skeletal muscle. This is in contrast to MYOD, which converts non-muscle cells to the myogenic lineage, with the formation of differentiating myoblasts. However, these cells, which do not express PAX3 or PAX7, do not have the regenerative potential of adult MuSCs, as illustrated by our analysis of engraftment with mesodermal cells expressing MYOD alone or the effect of culturing MuSCs, which leads to the activation of MYOD expression with downregulation of PAX3 or PAX7 and subsequent loss of their capacity to repair injured muscle after engraftment (Montarras et al., 2005). Genetic tracing experiments have shown that most MuSCs are derived from cells that had expressed myogenic regulatory factors, MYOD, MYF5, or MRF4, and indeed still transcribe the Myf5 gene; however the corresponding proteins are undetectable in adult MuSCs (Crist et al., 2012; Sato et al., 2014).

To test the effects of controlled MYOD activation on the induction of MuSCs, we used the Tet-On MYOD system. MYOD activation for over a week efficiently leads to

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**Figure 5. The Effect of Removing PAX3 from the Candidate Four Transcription Factors for Inducing PAX3-Expressing Muscle Stem Cells from hiPSCs**

(A) FACS profile for detecting PAX3-GFP cells with or without PAX3 from mesodermal cells.

(B) Transcriptional analyses with GFP-positive cells induced by transient DOX treatment plus three transcription factors (+3F+DOX) or two transcription factors (no PAX3, +2F+DOX). n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. *p < 0.01.

(C) Myogenic differentiation with induced PAX3-GFP-positive cells in 2% of horse serum medium for 7 days. MYOG, purple (labeled with Alexa 647); DAPI, blue.

(D) Percentage of differentiated myogenic cells presented as MYOG/DAPI ratio. n = 3 independent replicates; p values are determined by t test from a two-tailed distribution. *p < 0.01.

Error bars indicate ±SEM.
terminally differentiated skeletal muscle cells derived from hiPSCs (Figure S4B); however, transient induction of MYOD expression led to the presence of PAX3-expressing cells and the absence of differentiated cells marked by skeletal muscle MyHC. In these cultured cells, we detected not only myogenic but also neural cells in the presence of persistent MYOD activation under conditions of high fetal bovine serum (FBS), suitable for ex vivo expansion of myogenic cells (Figure S4C). Transient MYOD activation and exogenous factors in the serum appear not to prevent some non-skeletal muscle derivatives from iPSCs, although MYOD might be essential for the induction of MuSCs from mouse fibroblasts as previously reported (Bar-Nur et al., 2018). Non-myogenic neural derivatives were excluded in these experiments when hiPSCs were cultured under medium conditions that favor mesoderm. In these conditions, early somite markers were observed. In the experiments described here, we employed the same hiPSC line to generate two subclones, which have different copy numbers of MYOD transgenes after treatment with the piggyBac transposon system, and these were used for the induction of PAX3-positive cells. Results with other hiPSC
lines, together with a different gene integration system to control MYOD levels, will be checked in further investigations.

In our investigation to identify regulatory factors common to MuSCs during development and in the adult, we identified four factors that promote the formation of this cell type from mouse and also human fibroblasts, and from hiPSCs. Moreover we show that this induction method leads to more mature MuSCs compared with previously reported methods to induce PAX3-positive myogenic precursor cells from hiPSCs with several growth and chemical factors (Chal et al., 2015) or induced PAX7 expression in human embryonic stem cells/hiPSCs after the formation of embryoid bodies (Darabi et al., 2012). This is illustrated by high levels of transcripts of CALCR or NAPILS, which mark adult MuSCs under the conditions we describe with HEY, KLF4, PAX3, and transient MYOD and which we have not detected with the other reported induction methods.

MYOD, although unable to promote the formation of Pax3-GFP-positive MuSCs when overexpressed on its own, had a positive effect in combination with other factors. This is perhaps surprising but might reflect its role as a chromatin remodeler (de la Serna et al., 2005), facilitating the function of the other factors that more specifically render upstream myogenic factors accessible for transcription. Pax3 and Pax7 genes continue to be expressed in myoblasts and are only downregulated on muscle cell differentiation (Young and Wagers, 2010).

One of the four transcription factors, HEY1, is involved in Notch signaling. This signaling directly targets Hes1, Hey1, or HeyL, known to be highly expressed in quiescent MuSCs and essential for the generation of adult MuSCs, to suppress MyoD transcription and to regulate myogenic differentiation (Fukada et al., 2011). It has been reported that interstitial PAX7-positive myogenic stem cells have the ability to traverse the basal lamina and locate in the interstitial region, and that this process is dependent on Notch signaling (Bröhl et al., 2012). Therefore, transplanted cells affected by Notch signaling may be detected as activated myogenic cells in the interstitium, although the reason why and how Pax7+ MuSCs come to be located outside the basal lamina are poorly understood. Another of the factors, KLF4, a member of the family of Krüppel-like factors, is known as a reprogramming factor that can induce PSCs (Takahashi and Yamanaka, 2006). This factor was chosen for the MuSC transcriptome screening because the KLF family is essential for the maintenance of MuSCs (Hayashi et al., 2016).

Exogenous PAX3 and/or PAX7 expression in PSCs has been proposed to enable efficient propagation of myogenic progenitors and has been used to produce myogenic stem cells for engraftment (Darabi et al., 2012; Kim et al., 2017). We have found that the three factors HEYL, KLF4, and MYOD, without exogenous PAX3 also induced PAX3-GFP cells from mesodermal cells; however, PAX7, CALCR, or NAPILS transcripts in GFP+ cells without exogenous PAX3 were all decreased and showed less myogenic differentiation in vitro. Furthermore, additional expression of PAX3/PAX7 is probably important for the long-term maintenance of MuSCs (Buckingham and Relaix, 2015), and the activation of transient PAX3 promotes ex vivo expansion of mouse PAX7-positive MuSCs (Filareto et al., 2015). Manipulation of culture medium by itself, without the introduction of expression vectors, is being used to promote the formation of myogenic cells for cell therapy (Chal et al., 2015), although the addition of the four factors that we describe increased the production of MuSCs and also conferred more adult MuSC-like properties as discussed. However, in culture these PAX3-GFP cells did not all differentiate, probably because of high levels of PAX3 in some cells that prevent the normal downregulation of the protein required for the efficient onset of myogenic differentiation. Furthermore, the PAX3-GFP-expressing cells are not probably forming mature muscle on in vitro differentiation, as indicated by the MyHC isofoms expressed, some of which require innervation. Pax3 expression marks all myogenic progenitors in the early embryo, and continues to be transcribed in a subset of adult MuSCs (Relaix et al., 2005, 2006) but not in the hind limbs, including the TA muscles. In our experiments, GFP signals were not detected in mouse TA muscles after transplantation with induced PAX3-expressing cells, suggesting that Pax3 expression continues to be downregulated in mouse limbs (Boutet et al., 2012) and thus will not interfere with in vivo myogenic differentiation of transplanted cells.

Obtaining mature muscle fibers in culture presents another challenge. The important point for any therapeutic application to muscular disorders is that candidate muscle stem cells should be capable of behaving like intact adult MuSCs in an adult skeletal muscle environment, as we show using the induced cells derived from fibroblasts and hiPSCs described here. Further investigation would be required to understand the cellular and molecular mechanisms of these transcription factors to induce MuSCs, and in order for them to be used as valuable sources for stem cell therapies.

**EXPERIMENTAL PROCEDURES**

**Mice**

All animal experiments were approved by the Ethics Committee of Animal Experimentation of Kyoto Prefectural University of Medicine (permission number M26-237). All injections with needles were performed under anesthesia, and all efforts were made to minimize suffering. Pax3-GFP+, MyoD-CreIZ, and
Rosa26^CAG-LSL-Tdtomato^/+ mice were used for obtaining skeletal muscle cells, and female Dmd^−/− crossed with male NSG mice (Sato et al., 2014, Charles River) were used for the transplanted donors. Male Dmd^−/−;NSG mice were used for all experiments at the indicated ages.

Eight-week-old Dmd^−/−;NSG host mice were used for grafting of freshly isolated or cultured cells derived from hiPSCs (5.0 × 10^5 cells per 20 μL of PBS with 2% horse serum [Sigma]), and 10 μM Y-27632 (Nacalai Tesque) into TA muscle fibers. Mice were anesthetized with diethyl ether prior to engraftment. TA muscle was removed 4 weeks after transplantation, fixed, and stained as described below. Three independent mice for the transplantation and ten sections of each transplanted mouse were analyzed.

cDNA Constructs
For overexpression in mouse fibroblasts, pMXs-Gateway vector (Addgene #18656) was used. All coding sequences of selected 38 mouse genes were amplified by RT-PCR with KOD FX Neo DNA polymerase (Toyobo) and single strand cDNA synthesized by SuperScript 3 Reverse Transcriptase (Invitrogen) from mouse Pax3-GFP adult MuSCs. These cDNAs were subsequently subcloned into the pENTR1 and pMXs vectors. For human iPSCs, piggyBac-Gateway vector was used (Addgene #20960). All human cDNA constructs including coding sequences were obtained from the human proteome expression resource HuPEx library (Goshima et al., 2008). Human cDNAs were transferred into the pLVX vector (Clontech) to overexpress in human fibroblasts and the pPiggyBac-Gateway vector with the LR clonase (Invitrogen) to overexpress in human iPSCs.

Cell Sorting
For live cell sorting and culture, skeletal muscle of limbs and diaphragms of embryonic-day-16.5 embryos, diaphragms of 1-week-old mice at a juvenile stage, and 12-week-old mice at an adult stage were dissected into small pieces and treated with 0.2% of collagenase ( Worthington) in DMEM (Nacalai Tesque) at 37°C for 30 min to a few hours until the tissues disappeared. Dissociated cell suspensions were centrifuged and resuspended with 1% BSA (Sigma) in PBS stained with SYTOX Green or Red Dead Cell Stain (Sigma) in PBS for 30 min to 3 h until the tissues disappeared. Dissociated cells, and female Dmd^−/−;C0/Y;NSG mice were used for the transplanted donors.

Electroporated by NEPA21 (Nepagene), mixed with 1.0 μg of plasmid DNAs, as described in Tanaka et al. (2013), and seeded onto Gelrite-coated dishes in DMEM/F12 medium supplemented with 20% FBS and 10 ng/mL basic FGF (MuSC medium) for 24 h. At day 8, culture medium was changed to MuSC medium supplemented with 1 ng/mL doxycycline (DOX; Tocris) for 3 days. After DOX treatment, culture medium was changed to MuSC medium for 2 weeks. The observation of cultured cells was performed by BZ-X710 microscopy (Keyence).

Microarray Analyses
The datasets for gene expression in mouse Pax3-GFP-expressing cells (embryonic day 16.5, 1 week old, 12 weeks old) and other stem cells were obtained from the NCBI GEO database (GEO: GSE11274 and GSE15155 [Ko et al., 2009; Pallafacchina et al., 2010]). All of the obtained Affymetrix CEL files were imported into GeneSpring GX (Agilent Technologies) for the calculation of expression levels and principal component analysis. Expression values were calculated with the GC-RMA summarization algorithm as implemented in GeneSpring GX software.

qRT-PCR Analyses
Total RNAs from sorted or cultured cells were extracted using an RNeasy Micro Kit (Qiagen). For qPCR analyses, synthesized cDNAs were prepared using SuperScript VILO MasterMix (Invitrogen). All qRT-PCR reactions were carried out in triplicate using Thunder Bird SYBR qPCR Mix (Toyobo) and Thermal Cycler Dice Realtime System (Takara), and normalized to mRNA expression level of mouse or human ribosomal protein L13A as a control. Primer sequences (5’ to 3’) are listed in Table S3.

Immunofluorescence
Cells and skeletal muscle tissues were fixed with 4% paraformaldehyde in PBS for 15 min at 4°C, prior to embedding in Frozen Section Compound (Leica Microsystems) for cryosections. Fixed samples were incubated with 0.1% Triton X-100 in PBS for 5 min, BlockingOne (Nakacai Tesque) for 30 min, and anti-GFP (Molecular Probes A6459; diluted 1:500), anti-PAX3 (DSHB AB528426; diluted 1:100), anti-PAX7 (DSHB AB528428; diluted 1:100), anti-myogenin (DSHB AB528429; diluted 1:100), anti-MyoD (Abcam ab64159; diluted 1:500), anti-laminin (Enzo Life Sciences ALX-804-190; diluted 1:500), anti-dystrophin (Abcam ab15277; diluted 1:200), anti-MyHC (R&D MAB4470; ME20, diluted 1:200), anti-FSP1 (Abcam ab124805; diluted 1:200), anti-laminin A/C (Abcam ab8984; diluted 1:200), and anti-Ki67 (Abcam ab15580; diluted 1:500) antibodies in 5% blocking One overnight at 4°C. After three washes with 0.1% Tween 20 in PBS, cells were incubated with Alexa-conjugated anti-mouse immunoglobulin G1 (IgG1), mouse IgG2b, rabbit IgG, or rat IgG antibodies (Molecular Probes; diluted

Cell Culture
Isolated embryonic fibroblasts of Pax3-GFP;MyoD-Tdtomato mouse, or human primary dermal fibroblasts (Lonza) were expanded onto coated dishes with 0.1% gelatin, and 10% FBS (Gibco) in DMEM. Two independent human iPSC lines (#11-8 and #11-19 Pax3^GFP/Tet-MYOD) were cultured on iMatrix (Nippi)-coated dishes in StemFit AK02 medium (Ajinomoto). Cells were passaged as single cells. To induce PAX3-positive cells, we carried out the initial procedure on single cells plated on Gelrex-coated (Gibco) 6-well plates (5 × 10^5 cells/well) in mTeSR medium (STEMCELL Technologies) supplemented with 10 μM Y-27632 for 3 days. At day 3, culture medium was changed to mesodermal differentiation medium (50% Iscove’s modified Dulbecco’s medium and 50% F12 supplemented with 1% BSA, 1% CD lipid concentrate [Gibco], 1% insulin-transferrin-selenium [GIBCO], 20 μM 1-thioglycerol, 10 μM SB431542 [StemGent], 10 μM CHIR99021 [StemGent], 2 μM DMM1 (Tocris), and 20 ng/mL basic fibroblast growth factor [FGF; Wako]) for 4 days. At day 7, cells were dissociated with TrypLE select (Gibco) and mesodermal PAX3-GFP positives were selected by the cell sorter as DLL1-positive cells, electroporated by NEPA21 (Nepagene), mixed with 1.0 × 10^6 cells and 5 μg of plasmid DNAs, as described in Tanaka et al. (2013), and seeded onto Gelrite-coated dishes in DMEM/F12 medium supplemented with 20% FBS and 10 ng/mL basic FGF (MuSC medium) for 24 h. At day 8, culture medium was changed to MuSC medium supplemented with 1 ng/mL doxycycline (DOX; Tocris) for 3 days. After DOX treatment, culture medium was changed to MuSC medium for 2 weeks. The observation of cultured cells was performed by BZ-X710 microscopy (Keyence).
1:500). Cells were washed and mounted in ProLong Diamond antifade reagent with DAPI (Molecular Probes). Images were collected and processed by BZ-X710 microscopy.

**Statistics**

We report the statistical data including results of at least three biological replicates. Statistical analyses were performed with StatPlus software (AnalystSoft) to determine significant differences from a two-tailed distribution using a paired or unpaired Student’s t test. The statistical significance for the comparison of multiple sample sets was determined with Dunnett’s multiple comparisons following ANOVA. p values are indicated on each figure as <0.05 (*) and <0.01 (**). All error bars represent mean ± SD.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.stemcr.2019.06.006.

**AUTHOR CONTRIBUTIONS**

T.S. conceived and designed the experiments. T.S., K.H., H.S., T.Y., and N.G. performed the experiments and analyzed data. T.S., H.S., M.U., and C.S. contributed reagents, material, and analysis tools. T.S. and H.S. wrote the paper. T.S. edited the manuscript.

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