Stem Cells International

Skeletal Muscle Cells Generated from Pluripotent Stem Cells

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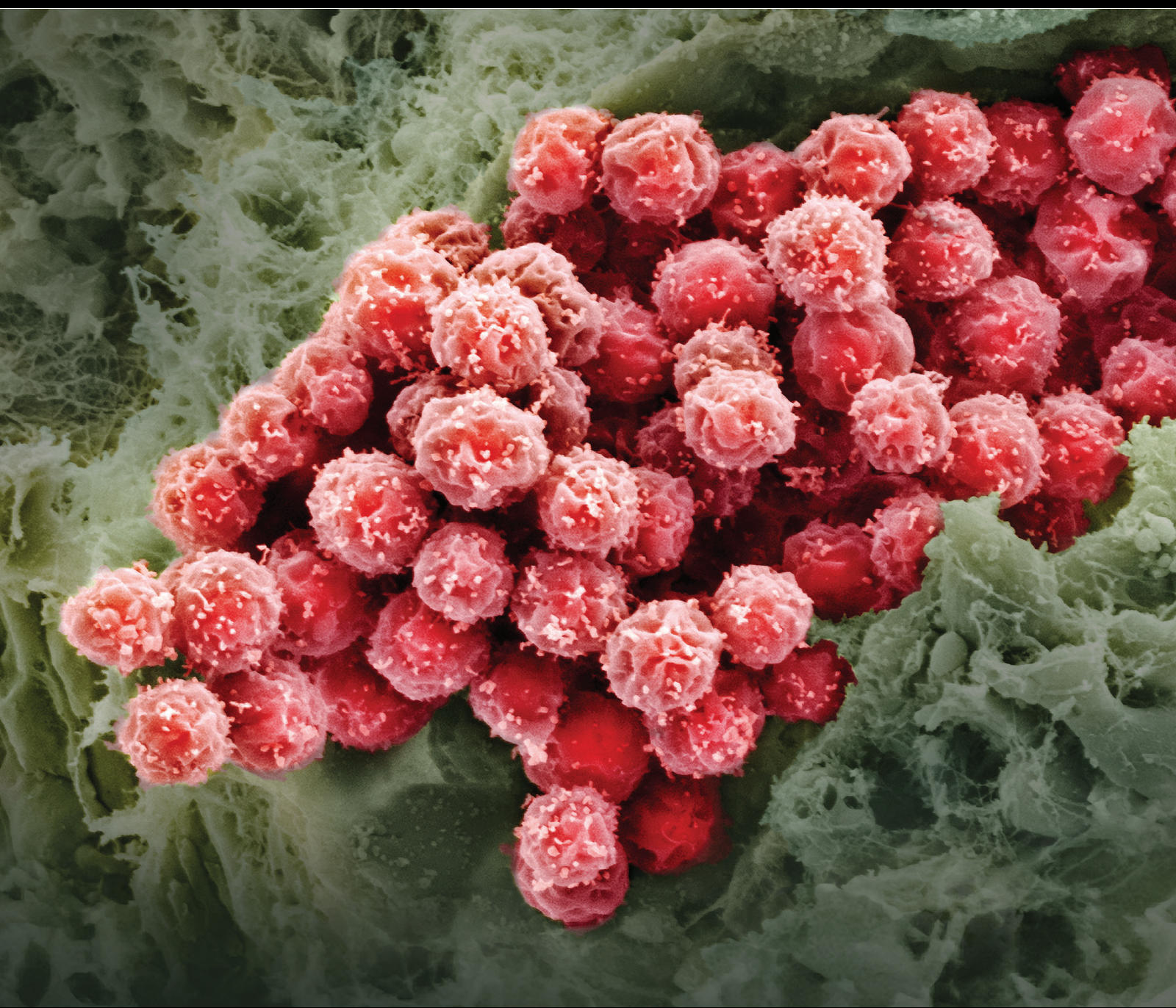

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Editorial Board

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Editorial

Skeletal Muscle Cells Generated from Pluripotent Stem Cells

Yuko Miyagoe-Suzuki,[1] Atsushi Asakura,[2] and Masatoshi Suzuki[3]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan*
[2]*Stem Cell Institute, Paul and Sheila Wellstone Muscular Dystrophy Center, Department of Neurology, University of Minnesota Medical School, Minneapolis, MN 55455, USA*
[3]*Department of Comparative Biosciences, Stem Cell and Regenerative Medicine Center, University of Wisconsin, Madison, WI 53706, USA*

Correspondence should be addressed to Yuko Miyagoe-Suzuki; miyagoe@ncnp.go.jp, Atsushi Asakura; asakura@umn.edu, and Masatoshi Suzuki; masatoshi.suzuki@wisc.edu

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Pluripotent stem cells, which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer a promising cell source for cell-based therapy to target many degenerative diseases including Duchenne muscular dystrophy (DMD) as well as modeling disease conditions *in vitro*. The proliferation and differentiation properties of ESCs/iPSCs can contribute to preparing a large yield of skeletal muscle stem cells (myogenic progenitors), which is necessary for cell-based therapy. Particularly, iPSC technology allows us to create patient-derived stem cells, which can recapitulate pathophysiological conditions *in vitro*. These *in vitro* disease models are expected to work as a unique platform for drug screening and allow comprehensive studies of disease mechanisms. In addition, patient-derived iPSCs are an especially ideal cell source to obtain an unlimited number of myogenic cells that escape immune rejection after engraftment. In the last decade, a number of culture methods have been published almost annually from different groups for myogenic differentiation from human ESCs/iPSCs. Currently, however, derivation of skeletal muscle stem cells/progenitors with high regenerative potential from human ESCs/iPSCs is still challenging. Further, *in vitro*-generated human ESC/iPSC-derived myofibers are morphologically and functionally less mature than postnatal myofibers.

This special issue was proposed to introduce both comprehensive review articles and an original cutting-edge research article to describe novel insights into skeletal muscle differentiation of ESCs/iPSCs. The topics cover the current status of research progress on ESC/iPSC-based disease modelling and ESC/iPSC-based cell therapy, the technical barriers for the successful induction of skeletal muscle suitable for regenerative medicine and ESC/iPSC-based drug development, and technical tips for *in vitro* disease modeling and ESC/iPSC-based regenerative medicine.

When considering cell-based therapies targeting muscle diseases including DMD, a significant number of myogenic cells with high differentiation quality would be essential. The research article by Y. Ando et al. introduces their current efforts to generate mesenchymal stromal cells for future myoblast therapy from a working cell bank of human ESCs. The authors identified that ESC-derived $CD105^+$ cells possess extensive *in vitro* proliferation capability and exhibit efficient myogenic differentiation capacity with genetic stability. Therefore, ESC-derived $CD105^+$ cells may be an alternative cell source for myogenic cells in cell-based therapy for patients with genetic muscular disorders.

The review article authored by P. Gee et al. overviews recent advances and applications of the CRISPR/Cas 9 system in the field of stem cell research and how iPSCs and the CRISPR technologies can be applied to gene therapy for DMD. The CRISPR/Cas 9 technology allows us to make precise and targeted editing to the genome of living cells. For DMD, an attractive therapeutic approach is to restore the expression of the dystrophy gene using the CRISPR/Cas

9 system. The authors summarized the current approaches using various CRISPR/Cas 9 strategies to target DMD mutation *in vitro* (patient-derived myoblast and iPSCs) and *in vivo* (DMD mouse model).

The review article by Y. Kodaka et al. summarized the recent protocols of efficient myogenic differentiation using EC/iPSCs. Current approaches of skeletal muscle cell induction of ESCs/iPSCs utilize techniques including overexpression of myogenic transcription factors such as MyoD or Pax3 using small molecules to induce mesodermal cells followed by myogenic progenitor cells. In addition, the authors noticed that epigenetic myogenic memory exists in muscle cell-derived iPSCs, which causes increased myogenic differentiation capacity compared with fibroblast-derived iPSCs. Therefore, muscle cell-derived iPSCs may be utilized for the efficient myogenic differentiation of iPSCs.

Skewed X chromosome inactivation (XCI) is a cause of a severe dystrophic phenotype in female carriers of DMD. Therefore, XCI status in human iPSCs is important for disease modeling of manifesting female DMD carriers. In this special issue, Y. Miyagoe-Suzuki et al. reported frequent reactivation of inactive X chromosomes during iPSC reprogramming. Consequently, many human iPSC clones showed biallelic expression of the androgen receptor (AR) gene and loss of X-inactivation-specific transcript and trimethylhistone H3 (Lys27) signals on X chromosomes. Particularly, a wild-type dystrophin allele was expressed in multinucleated myotubes differentiated from a manifesting carrier of DMD-iPSCs with a XaXa pattern. Therefore, myotubes differentiated from manifesting female carrier of DMD-iPSCs with two active X chromosomes restored dystrophin expression due to acquirement of nonskewed XCI.

The other review article authored by T. Akiyama et al. presents the roles of epigenetic and transcriptional manipulation for skeletal muscle differentiation from human pluripotent stem cells. Enforced expression of specific myogenic factors such as PAX7 and MYOD1 has been known to promote skeletal muscle differentiation. Current work demonstrates that direct differentiation of human pluripotent stem cells hardly occurs with the ectopic expression of these transcriptional factors due to chromatin features unique to human pluripotent stem cells, which hinder the access of transcription factors to genes involved in muscle differentiation. Recent studies have demonstrated that epigenetic manipulation can enhance myogenic differentiation from human ESCs/iPSCs.

Human iPSCs are a useful tool to investigate the molecular mechanisms of myogenesis in human. K. Higashioka et al. generated MYOGENIN-mutated human iPS cells using CRISPR/Cas9 technology. MYOGENIN is known to function as an essential myogenic transcription factor during the terminal differentiation stage. MYOGENIN gene-knockout mice display deficiency of differentiated skeletal myofibers, while there are residual myofibers in the mutant mice possibly due to functional compensation by MYOD1 and/or MRF4. Interestingly, in the paper, the authors found that human iPSCs can differentiate into skeletal muscle without MYOGENIN activity *in vitro*, indicating similar compensation mechanisms by MYOD1 and/or MRF4 for myogenic differentiation of human iPSCs.

The editors together with the involved authors have discussed the current status of skeletal muscle differentiation using ESCs/iPSCs, the epigenetic alteration occurring during the myogenic reprogramming process, and the related research to expand our knowledge of skeletal muscle differentiation using ESCs/iPSCs. We hope that this special issue can provide valuable information that will further improve protocols for skeletal muscle cell induction from ESCs/iPSCs and that will facilitate the application of ESCs/iPSCs for the future in regenerative medicine of muscle diseases.

Yuko Miyagoe-Suzuki
Atsushi Asakura
Masatoshi Suzuki

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Research Article

Can Human Embryonic Stem Cell-Derived Stromal Cells Serve a Starting Material for Myoblasts?

Yu Ando,[1] Marie Saito,[1] Masakazu Machida,[1] Chikako Yoshida-Noro,[2] Hidenori Akutsu,[1] Masataka Takahashi,[1] Masashi Toyoda,[3] and Akihiro Umezawa[1]

[1]*Center for Regenerative Medicine, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan*
[2]*Department of Applied Molecular Chemistry, College of Industrial Technology, Nihon University, Tokyo, Japan*
[3]*Research Team for Geriatric Medicine (Vascular Medicine), Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan*

Correspondence should be addressed to Akihiro Umezawa; umezawa@1985.jukuin.keio.ac.jp

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A large number of myocytes are necessary to treat intractable muscular disorders such as Duchenne muscular dystrophy with cell-based therapies. However, starting materials for cellular therapy products such as myoblasts, marrow stromal cells, menstrual blood-derived cells, and placenta-derived cells have a limited lifespan and cease to proliferate in vitro. From the viewpoints of manufacturing and quality control, cells with a long lifespan are more suitable as a starting material. In this study, we generated stromal cells for future myoblast therapy from a working cell bank of human embryonic stem cells (ESCs). The ESC-derived $CD105^+$ cells with extensive in vitro proliferation capability exhibited myogenesis and genetic stability in vitro. These results imply that ESC-derived $CD105^+$ cells are another cell source for myoblasts in cell-based therapy for patients with genetic muscular disorders. Since ESCs are immortal, mesenchymal stromal cells generated from ESCs can be manufactured at a large scale in one lot for pharmaceutical purposes.

1. Introduction

Duchenne muscular dystrophy is an intractable genetic disorder, and effective therapies have not yet been developed. Novel approaches to treat Duchenne muscular dystrophy include small molecules, gene therapy, and biologics such as cytokines and cell-based therapy [1, 2]. Among these advanced therapeutic approaches, regenerative therapies have been focused due to the recent advances of pluripotent stem cells with different types of reprogramming technologies [3, 4]. In vitro expansion of quality-controlled stem cells and transplantation into patients with degenerative diseases in an allogeneic manner can be one of the ideal therapeutic scenarios. Somatic cells such as myoblasts, marrow stromal cells, menstrual blood-derived cells, and placenta (amnion, cholate plate, umbilical cord)-derived cells have been introduced as starting materials for cellular therapy products [5–9]. However, these somatic cells have a limited lifespan and cease to proliferate in vitro, and thus, sufficient numbers of cells cannot be prepared to treat muscles of a whole body in cell-based therapies. From this viewpoint, cells with a long lifespan are more suitable for starting materials.

Human pluripotent stem cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are immortal and can therefore be a good source of large number of cellular therapy products with one lot for genetic muscular disorders [1]. In addition to immortality, ESCs and iPSCs exhibit pluripotency, that is, capability to differentiate theoretically into almost all types of cells including myoblasts and their progenitor cells [10]. As therapeutic cellular products, myoblasts and mesenchymal stromal cells are considered the most suitable. In this study, we generated mesenchymal stromal cells from ESCs for the production of

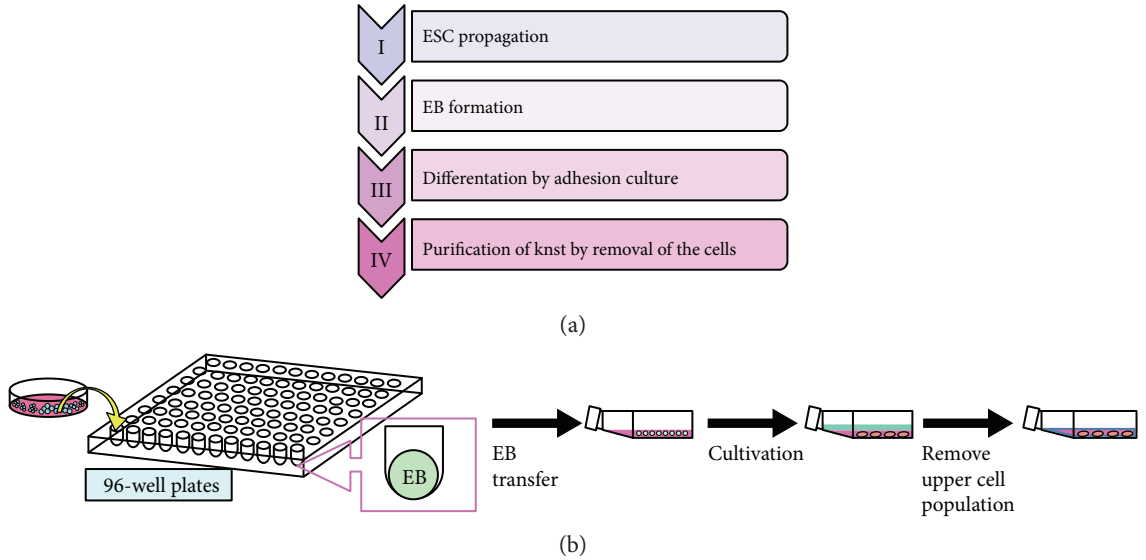


Figure 1: Generation of mesenchymal stromal cells from sees2. (a) Step-by-step manufacturing process. (b) Scheme for generation of mesenchymal stromal cells from sees2.

cellular therapy products to treat patients with genetic muscular disorders [11, 12]. We developed a novel protocol to manufacture mesenchymal stromal cells from ESCs with certified materials that had been analyzed for viruses.

2. Results

2.1. Generation of Mesenchymal Stromal Cells. To generate mesenchymal stromal cells from human ESCs, we propagated sees2 cells on mouse embryonic fibroblasts (MEFs) and formed embryoid bodies (EBs) for 4 days on a feeder layer of freshly plated gamma-irradiated mouse embryonic fibroblasts (Figure 1). The EBs were then transferred to the collagen-coated flasks and cultivated for 60 to 70 days. The upper adherent cell layer was detached to obtain a resource of mesenchymal stromal cells.

2.2. Propagation of Mesenchymal Stromal Cells. We repeated generation of mesenchymal stromal cells from sees2 cells in 4 different independent experiments (#3, #14, #23, and #25) and investigated proliferation rate of the mesenchymal stromal cells (#3, #14, #23, and #25) for over 50 days (Figure 2(a)). The mesenchymal stromal cells rapidly proliferated in culture and propagated continuously, however stopped replicating, became broad and flat, and exhibited SA-β-galactosidase activity as indicated by blue staining of their cytoplasm at passage 11 (Figures 2(b) and 2(c)). The enlargement of the cell size was passage-dependent.

2.3. Flow Cytometric and Karyotypic Analysis. Flow cytometric analysis revealed that the mesenchymal stromal cells #2 and #3 were positive for CD90, CD105, and HLA-ABC and negative for HLA-DR (Figure 3(a)). The expression level and pattern of these markers remained unchanged after 3 or 4 passages (12 or 16 population doublings, resp.). Karyotypic analyses of the mesenchymal stromal cells #2 and #3 were performed at passages 3 and 2, respectively (Figure 3(b)). They were found to be diploid and not to exhibit any significant abnormalities. The chromosome number of both #2 and #3 was 46 without exception.

2.4. Global Outlook by Hierarchical Clustering and Principal Component Analysis (PCA). To investigate myogenic potential, mesenchymal stromal cells were analyzed, depending on gene expression levels. Hierarchical clustering analysis based on all probes, mesenchyme-associated genes, and stem cell-associated genes revealed that the mesenchymal stromal cells were categorized into the same group in a passage-dependent manner (Figures 4(a), 4(b), and 4(c)). Likewise, hierarchical clustering analysis and PCA on the expression pattern of the myogenic and cardiomyogenic genes also show passage-dependent categorization (Figures 4(d) and 4(e), Supplemental Table 1 available online at https://doi.org/10.1155/2017/7541734). After the induction, the mesenchymal stromal cells started to form multinucleated myotubes (Figure 4(f)).

3. Discussion

For the development of cell-based therapeutic strategies to genetic myogenic disorders, immortal cells as a raw material are required to gain sufficient number of cells, and detailed studies are therefore essential with regard to the characteristics of differentiated mesenchymal stromal cells. This present study demonstrated the detailed alterations of the mesenchymal stromal cells during expansion from P0 to P11 in monolayer culture. The fate of mesenchymal stromal cells generated from ESCs depended on passage number or population doubling levels in culture. In our previous study, we showed that human marrow stromal cells and umbilical cord blood-derived cells reach senescence, exhibit large, flat morphology at late passages, and have different characteristics, depending on passage number and population doublings [13, 14]. Myogenic ability of ESC-derived

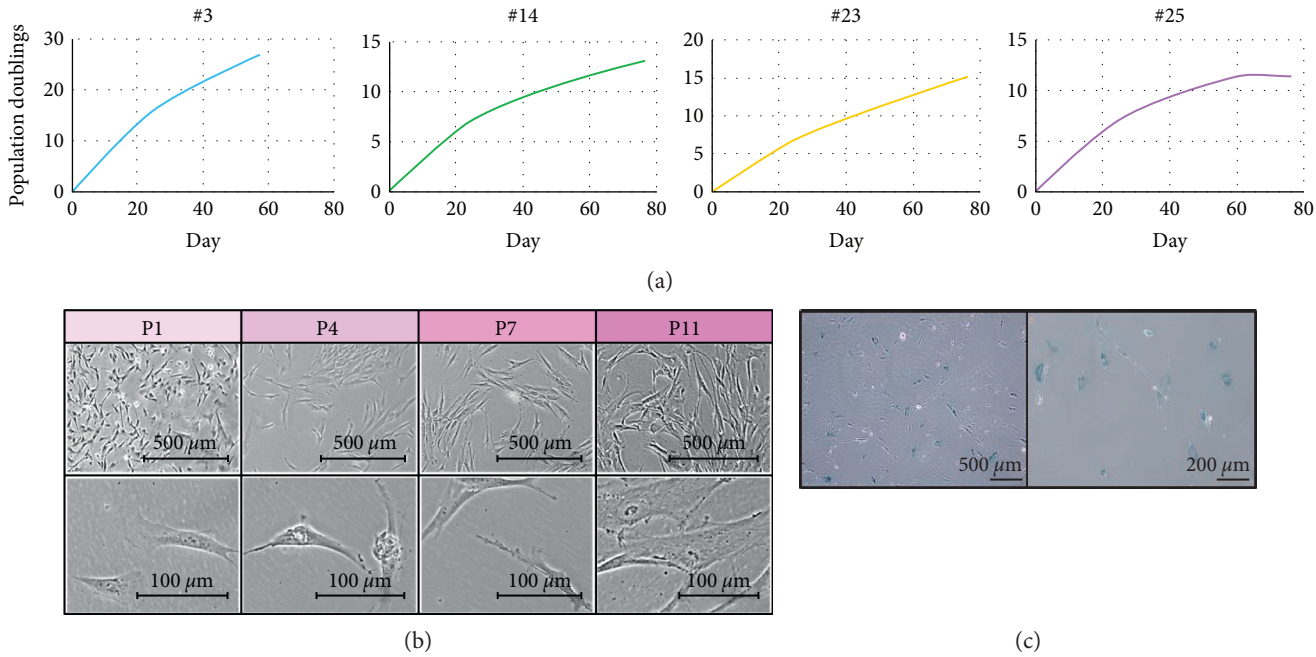
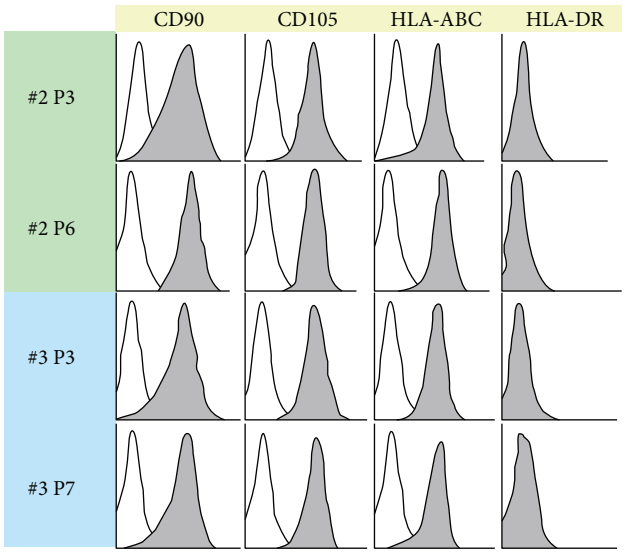


Figure 2: Characterization of mesenchymal stromal cells. (a) Growth curve of mesenchymal stromal cells (knst#3, #14, #23, #25). (b) Phase contrast photomicrography of mesenchymal stromal cells (knst#2: passages 1, 4, 7, and 11). (c) Senescence-associated beta-galactosidase stain (knst#3, passage 11).



Cell	Mode	Karyotype
#2 P3	46 (50)	46, XX (20)
#3 P2	46 (50)	46, XX (20)

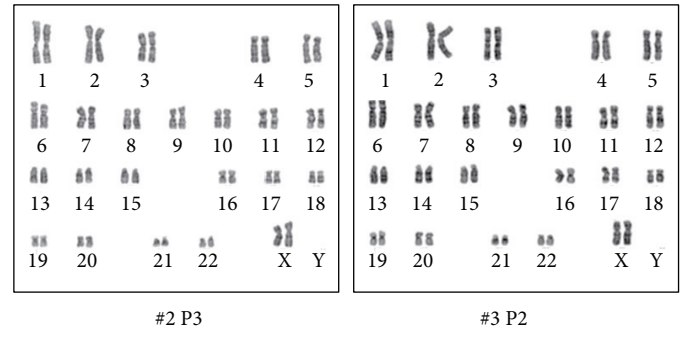


Figure 3: Flow cytometric analysis and karyotypic analysis. (a) Flow cytometric analysis of knst#2 (passages 3 and 6) and knst#3 (passages 3 and 7). (b) Karyotypic analysis of knst#2 (passage 3) and knst#3 (passage 2).

mesenchymal stromal cells is possibly associated with surface markers, morphology, cytokines, and differentiation capacity. c-kit, CD34, and CD140 serve as good markers to distinguish murine mesenchymal cells with multipotency, that is, mesenchymal stem cells [15]. CD29+, CD44+, CD59+, and CD90+ cells from menstrual blood are capable of differentiating into myoblasts/myocytes and conferring human dystrophin expression in the murine model for Duchenne muscular dystrophy [6]. In this study, we generated high-purity mesenchymal stromal cells for future myoblast therapy from a working cell bank of ESCs. The ESC-derived CD105+ cells with in vitro extensive proliferation capability exhibited myogenesis and genetic stability in vitro, implying that ESC-derived CD105+ cells are another cell sources for myoblasts in cell-based therapy to patients with genetic muscular

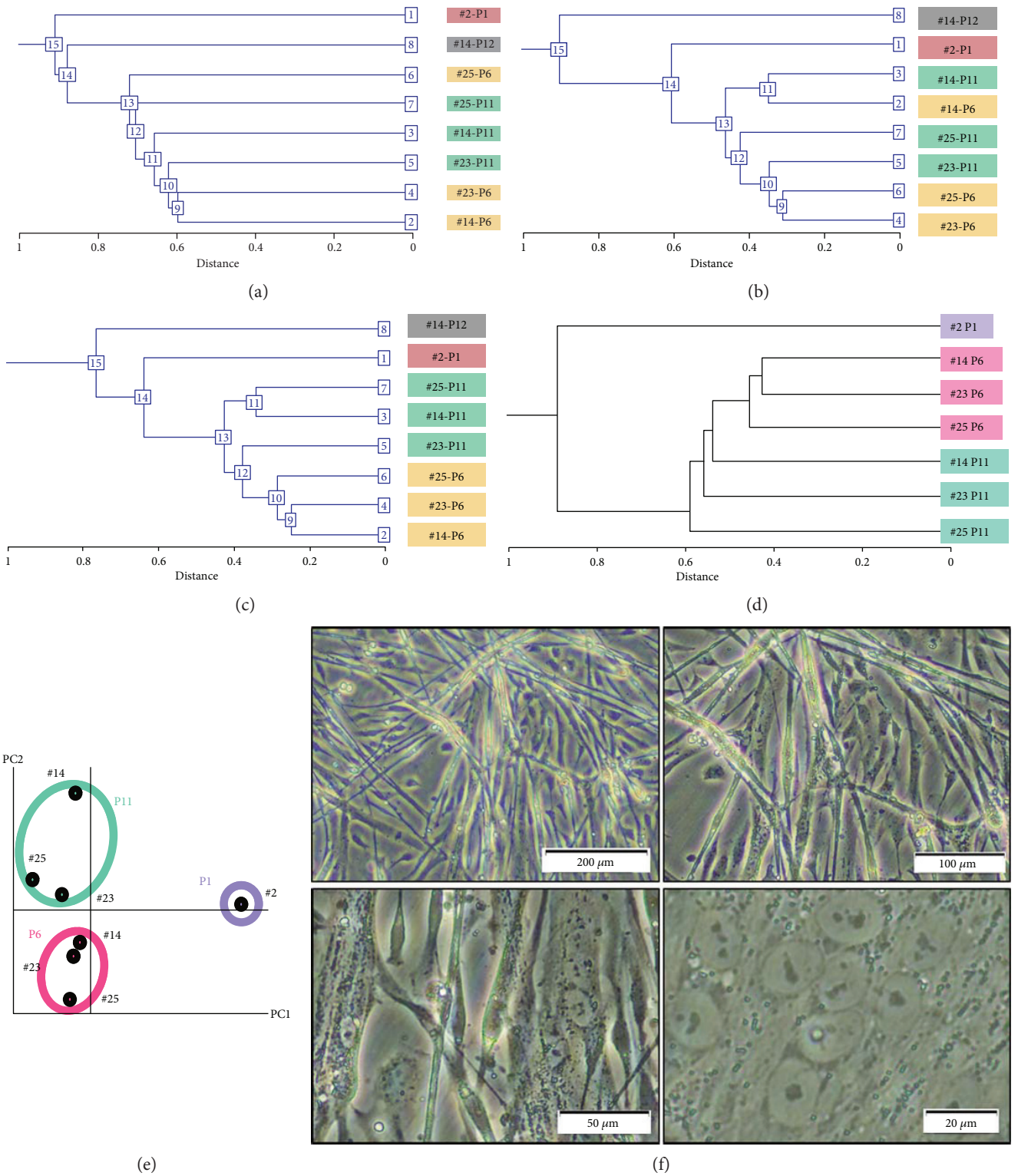


Figure 4: Global gene expression analysis of ESC-derived mesenchymal stromal cells. (a) Hierarchical clustering analysis based on the expression of all genes (58,201 probes on an Agilent SurePrint G3 Human GE v3 8x60K Microarray). (b) Hierarchical clustering analysis based on expression levels of the mesenchyme-associated genes. (c) Hierarchical clustering analysis based on expression levels of the stem cell-associated genes. (d) Hierarchical clustering analysis based on expression levels of the muscle-associated genes. (e) Principal component analysis of the muscle-associated genes. (f) Phase-contrast photomicrographs of knst myogenesis.

disorders. Since ESCs are immortal, mesenchymal stromal cells generated from ESCs can be manufactured in a large scale with one lot in pharmaceutical purpose.

Mesenchymal stromal cells derived from ESCs have been examined from the viewpoints of differentiation propensity, surface markers, proliferation, and morphology [10, 16–20]. They exhibit multipotency, that is, adipogenic, osteogenic, and chondrogenic differentiation in vitro [10]. They also show myogenic differentiation in vitro like mesenchymal stem cells derived from the bone marrow, menstrual blood, and placenta [6, 8, 13, 21–23]. These mesenchymal stromal cells can be used for therapeutic agents or delivery vehicles to patients with graft-versus-host disease, ischemic heart disease, and lysosomal storage disorders. With the robust scalable manufacturing process described in this study, ESC-derived CD105+ cells serve as a starting material of these possible cellular therapy agents. ESC-derived CD105+ cells were mortal while the original ESCs (sees2) were immortal. The cells are, therefore, nontumorigenic because they reach senescence or stop dividing after a limited number of replications. This limited cell lifespan could be an advantage from the viewpoint of tumorigenicity, but a disadvantage for scalable manufacturing of cell therapy products. iPSC-derived mesenchymal stromal cells exhibit almost the same phenotypes in differentiation propensity, surface markers, proliferation, and morphology as ESC-derived CD105+ cells [19, 24]. Taken together, there is no great distinction in quality attributes of mesenchymal stromal cells derived from ESCs, iPSCs, and various tissues.

Implantation of myoblasts induced from ESC-derived mesenchymal stromal cells into patients with genetic muscular disorders is indeed an ideal strategy, from the viewpoint of industry-based, sustainable supply of large quantities of affordable, quality-controlled cells. It is unlikely that it is possible to prepare unaffected somatic cells in sufficient quantity, necessitating the use of stem cells from suitable, cost-effective allogeneic sources, such as ESCs and iPSCs. The cellular therapy products manufactured from ESCs and iPSCs can cover whole-body muscle because of their immortality. In addition, the proliferation capability and genetic stability of the ESC-derived mesenchymal stromal cells open up significant new possibilities in regenerative medicine. ESCs can be a promising cellular source for cell-based therapy to treat Duchenne muscular dystrophy, a lethal human disease for which no effective treatment currently exists [11, 12].

4. Materials and Methods

4.1. Ethical Statement. Human cells in this study were performed in full compliance with the Ethical Guidelines for Clinical Studies. The cultivation of hESC lines were performed in full compliance with "the Guidelines for Derivation and Distribution of Human Embryonic Stem Cells (Notification of the Ministry of Education, Culture, Sports, Science, and Technology in Japan (MEXT))" and "the Guidelines for Utilization of Human Embryonic Stem Cells (Notification of MEXT)." The experimental procedures were approved by the Institutional Review Board (IRB) at the National Center for Child Health and Development. Animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the National Research Institute for Child Health and Development. All experiments with mice were subject to the 3 R consideration (refine, reduce, and replace), and all efforts were made to minimize animal suffering and to reduce the number of animals used.

4.2. hESC Culture. sees2 and sees5 were routinely cultured onto a feeder layer of freshly plated gamma-irradiated mouse embryonic fibroblasts (MEFs), isolated from ICR embryos at 12.5 gestations, in the hESC culture media. The hESC media consisted of Knockout™-Dulbecco's modified Eagle's medium (KO-DMEM) (Life Technologies, CA, USA; number 10829-018) supplemented with 20% 35 kGy irradiated Knockout™-Serum Replacement (KO-SR; number 10828-028), 2 mM Glutamax-I (number 35050-079), 0.1 mM nonessential amino acids (NEAA; number 11140-076), 50 U/ml penicillin-50 μg/ml streptomycin (Pen-Strep) (number 15070-063), and recombinant human full-length bFGF (Kaken Pharmaceutical Co. Ltd.) at 50 ng/ml. Cells were expanded using enzymatic passaging by recombinant trypsin (Roche Diagnostics, Indianapolis, USA).

4.3. Manufacturing Procedure. To generate EBs, sees2 and sees5 (5×10^3/well) were dissociated into single cells with 0.5 mM EDTA (Life Technologies) after exposure to the rock inhibitor (Y-27632: A11105-01, Wako, Japan) and cultivated in 96-well plates (Thermo Fisher Scientific) in the EB medium (76% Knockout DMEM, 20% 35 kGy irradiated Xeno-free Knockout Serum Replacement (XF-KSR, Life Technologies, CA, USA), 2 mM GlutaMAX-I, 0.1 mM NEAA, Pen-Strep, and 50 μg/ml l-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA)) for 4 days. The EBs were transferred to T25 flasks coated with NMP collagen PS (Nippon Meat Packers Inc.) and cultivated in the XF32 medium (85% Knockout DMEM, 15% 35 kGy-irradiated XF-KSR, 2 mM GlutaMAX-I, 0.1 mM NEAA, Pen-Strep, 50 μg/ml l-ascorbic acid 2-phosphate, 10 ng/ml heregulin-1β (recombinant human NRG-beta 1/HRG-beta 1 EGF domain; Wako, Japan), 200 ng/ml recombinant human IGF-1 (LONG R3-IGF-1; Sigma-Aldrich), and 20 ng/ml human bFGF (Kaken Pharmaceutical Co. Ltd.)) for 60 to 70 days. The flasks were gently shaken to detach the cells. The detached cells were aggregated and could thus be easily removed by a pipette. The remaining adherent cells in the flasks were used for a resource of mesenchymal stromal cells. The adherent cells were then propagated in α-MEM medium supplemented with 10% FBS (Gibco or HyClone) and 1% Pen-Strep for further in vitro analysis.

4.4. Karyotypic Analysis. Karyotypic analysis was contracted out to Nihon Gene Research Laboratories Inc. (Sendai, Japan). Metaphase spreads were prepared from cells treated with 100 ng/ml of colcemid (Karyo Max, Gibco Co. BRL) for 6 h. The cells were fixed with methanol:glacial acetic acid (2 : 5) three times and placed onto glass slides (Nihon Gene Research Laboratories Inc.). Chromosome spreads were Giemsa banded and photographed. A minimum of 10

metaphase spreads were analyzed for each sample and karyotyped using a chromosome imaging analyzer system (Applied Spectral Imaging, Carlsbad, CA).

4.5. Gene Chip Analysis. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions. RNA quantity and quality were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was amplified and labeled with cyanine 3 (Cy3) using an Agilent Low Input Quick Amp Labeling Kit, one-color (Agilent Technologies) following the manufacturer's instructions. Briefly, total RNA was reversed transcribed to double-strand cDNA using a poly dT-T7 promoter primer. Primer, template RNA, and quality control transcripts of known concentration and quality were first denatured at 65°C for 10 min and incubated for 2 hours at 40°C with 5X first-strand buffer, 0.1 M DTT, 10 mM dNTP mix, and AffinityScript RNase Block Mix. The AffinityScript enzyme was inactivated at 70°C for 15 min. cDNA products were then used as templates for in vitro transcription to generate fluorescent cRNA. cDNA products were mixed with a transcription master mix in the presence of T7 RNA polymerase and Cy3-labeled CTP and incubated at 40°C for 2 hours. Labeled cRNAs were purified using QIAGEN's RNeasy mini spin columns and eluted in 30 μl of nuclease-free water. After amplification and labeling, cRNA quantity and cyanine incorporation were determined using a Nanodrop ND-1000 spectrophotometer and an Agilent Bioanalyzer. For each hybridization, 0.60 μg of Cy3-labeled cRNA were fragmented and hybridized at 65°C for 17 hours to an Agilent SurePrint G3 Human GE v3 8x60K Microarray. After washing, microarrays were scanned using an Agilent DNA microarray scanner. Intensity values of each scanned feature were quantified using Agilent feature extraction software version 11.5.1.1, which performs background subtractions. We only used features which were flagged as no errors (detected flags) and excluded features which were not positive, not significant, not uniform, not above background, saturated, and population outliers (not detected and compromised flags). Normalization was performed using Agilent GeneSpring software version 13.0 (per chip:normalization to 75 percentile shift). There are total of 58,201 probes on an Agilent SurePrint G3 Human GE v3 8x60K Microarray without control probes. Hierarchical clustering analysis and Principal Component Analysis were performed using NIA Array Analysis (https://lgsun.grc.nia.nih.gov/ANOVA/).

Conflicts of Interest

The authors declare no financial conflicts of interest.

Authors' Contributions

Akihiro Umezawa designed the experiments. Yu Ando, Marie Saito, Masataka Takahashi, and Masakazu Machida performed the experiments. Akihiro Umezawa, Yu Ando, and Marie Saito analyzed the data. Masashi Toyoda contributed the reagents, materials, and analysis tools. Hidenori Akutsu, Chikako Yoshida-Noro, Akihiro Umezawa, Masashi Toyoda, and Yu Ando discussed the data and manuscript. Akihiro Umezawa and Marie Saito wrote this manuscript.

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Review Article

Cellular Reprogramming, Genome Editing, and Alternative CRISPR Cas9 Technologies for Precise Gene Therapy of Duchenne Muscular Dystrophy

Peter Gee,[1,2] Huaigeng Xu,[1] and Akitsu Hotta[1,2]

[1]*Center for iPS Cell Research and Application (CiRA), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[2]*Institute for Integrated Cell Material Sciences (iCeMS), Kyoto University, Yoshida Ushinomiya-cho, Sakyo-ku, Kyoto 606-8507, Japan*

Correspondence should be addressed to Akitsu Hotta; akitsu.hotta@cira.kyoto-u.ac.jp

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In the past decade, the development of two innovative technologies, namely, induced pluripotent stem cells (iPSCs) and the CRISPR Cas9 system, has enabled researchers to model diseases derived from patient cells and precisely edit DNA sequences of interest, respectively. In particular, Duchenne muscular dystrophy (DMD) has been an exemplary monogenic disease model for combining these technologies to demonstrate that genome editing can correct genetic mutations in DMD patient-derived iPSCs. DMD is an X-linked genetic disorder caused by mutations that disrupt the open reading frame of the *dystrophin* gene, which plays a critical role in stabilizing muscle cells during contraction and relaxation. The CRISPR Cas9 system has been shown to be capable of targeting the *dystrophin* gene and rescuing its expression in in vitro patient-derived iPSCs and in vivo DMD mouse models. In this review, we highlight recent advances made using the CRISPR Cas9 system to correct genetic mutations and discuss how emerging CRISPR technologies and iPSCs in a combined platform can play a role in bringing a therapy for DMD closer to the clinic.

1. Induced Pluripotent Stem Cells for Disease Modeling

Induced pluripotent stem cells (iPSCs), first established by Takahashi et al. in 2006, are invaluable tools for disease modeling that could one day provide a source of healthy autologous cells needed for regenerative medicine applications [1, 2]. Induced by reprogramming somatic cells, such as skin or blood, with four key transcription factors (OCT3/4, SOX2, KL4, and c-MYC), human pluripotent stem cells with similar features to embryonic stem cells can be obtained. These cells possess several advantageous features including unlimited self renewal in culture and the ability to be differentiated into endoderm, ectoderm, and mesoderm, including pancreatic β-cells, cardiomyocytes, neurons, and myocytes [2]. Moreover, iPSCs are particularly useful for studying the underlying mechanism of genetic disorders because they can recapitulate patient genotypes and cellular phenotypes upon differentiation, thus, can be used for screening chemical libraries to identify novel therapies in disease relevant cells [3]. In fact, initiatives to establish a large number of patient-derived iPSCs in the USA, EU, UK [4], and Japan [5] are ongoing, demonstrating the importance of iPSCs for disease modeling and new drug development.

While patient-derived iPSCs have been established for many diseases (e.g., FTLD-Tau [6], type I diabetes [7], Down syndrome, Parkinson's disease [8], etc.), we focus on the utility of those from Duchenne muscular dystrophy (DMD) patients [8–11]. Many DMD iPSC lines have been established containing different types of mutations in the *dystrophin* gene ranging from nonsense mutations to whole exon deletions or duplications (Table 1).

Table 1: Reported DMD-iPSC lines and genotypes.

Patient information (sex/age/cell type)	iPSC reprogramming method	Mutation description	Ref.
Male/6 YR/fibroblast (Coriell ID: GM04981)	Multiple lentiviral vectors	ΔExons 45–53	[8]
Male/28 YR/fibroblast (Coriell ID: GM05089)	Multiple lentiviral vectors	ΔExons 3–5	[8]
Male/9 YR/fibroblast (Coriell ID: GM05169)	Multiple retroviral vectors	ΔExons 4–43	[11]
Male/3 YR/fibroblast	Multiple retroviral vectors	ΔExon 44	[10]
Male/9 YR/fibroblast	Multiple retroviral vectors	ΔExons 46-47	[10]
Male/9 YR/fibroblast (Coriell ID: GM05169)	Multiple sendai virus vectors	ΔExons 4–43	[9]
Male/10 YR/fibroblast (Coriell ID: GM03783)	Multiple sendai virus vectors	ΔExons 3–17	[9]
Male/23 YR/fibroblast (Coriell ID: GM04327)	Multiple sendai virus vectors	Exons 5–7 duplication	[9]
Male/18 YR/fibroblast (Coriell ID: GM05127)	Multiple sendai virus vectors	DNA 5533 G→T (protein E→X)	[9]
Male/11 YR/fibroblast (Coriell ID: GM03781)	Multiple sendai virus vectors	ΔExons 3–17	[9]
Male/NA/fibroblast	Polycistronic lentivirus vector	ΔExons 46–51	[12]
Male/NA/fibroblast	Polycistronic lentivirus vector	ΔExons 46-47	[12]
Male/NA/fibroblast	Polycistronic lentivirus vector	Exon 50 duplication	[12]
Male/3 YR/fibroblast	Multiple episomal vectors	ΔExon 44	[13]
Male/31 YR/T lymphocytes	Multiple sendai virus vectors	ΔExons 48–54	[14]
Male/13 YR/T lymphocytes	Multiple sendai virus vectors	ΔExons 46-47	[14]
Male/18 YR/fibroblast	Multiple lentiviral vectors	ΔExons 48–50	[15]
Male/14 YR/fibroblast	Multiple lentiviral vectors	ΔExons 47–50	[15]
Male/13 YR/fibroblast	Multiple lentiviral vectors	DNA 3217 G→C (protein E→X)	[15]
Male/10 YR/fibroblast	Multiple lentiviral vectors	ΔExons 45–52	[15]
Male/10 YR/fibroblast	Multiple lentiviral vectors	DNA 10171 C→T (protein R→X)	[15]
Male/8 YR/fibroblast	Multiple lentiviral vectors	DNA 4918-4919 ΔTG	[15]
Male/20 YR/fibroblast	Multiple lentiviral vectors	DNA 7437 G→A (protein W→X)	[15]

NA: not available; YR: years old.

2. What Is Duchenne Muscular Dystrophy and What Treatments Are Currently Available?

DMD is an X-linked genetic muscle wasting disease that occurs in approximately 1 in 3500 males and affects mainly skeletal and cardiac muscles [16]. DMD patients are born apparently normal, but start to display symptoms of delayed muscle development during childhood, lose their walking ability after 10 years of age, become dependent on aspirators in their 20s, and generally do not survive past the age of late 30s, eventually succumbing to cardiac or respiratory failure [17]. The main cause of the disease is attributed to mutations in one of the largest protein-coding genes in the human genome called dystrophin that spans 79 exons (2.2 megabases) and stabilizes muscle cells by anchoring the cytoskeleton to the extracellular matrix with other proteins, in a complex known as the dystrophin-associated glycoprotein complex (DAGC) [18]. Dp427m is the main muscle isoform of dystrophin protein, which has an mRNA length of 14 kb and protein molecular weight of 427 kDa. Importantly, the N- and C-terminal regions of the protein are critical for functional anchoring of dystrophin to actin and DAGC while the central rod domain, consisting of 24 triple helix rod domains and 4 hinge regions, connects both ends and appears to be expendable to a certain extent [19]. The majority of genetic mutations in the *dystrophin* gene found in DMD patients are large deletions that disrupt the ORF of the dystrophin protein, which are mainly found in frontal exons 2–20 or a deletion hot spot exons 45–55, accounting for over 60% of mutations in DMD patients [20]. On the other hand, in-frame mutations in the *dystrophin* gene are known to cause a milder disease phenotype, named Becker muscular dystrophy (BMD) [21]. BMD patients show a broad spectrum of disease phenotypes, but in general, retain their ability to walk and live longer than DMD patients. In BMD patients, in-frame deletions in the *dystrophin* gene lead to the translation of truncated but functional dystrophin protein. In fact, BMD patients who lack more than half of the dystrophin protein coding sequence in the rod domain have been documented [21, 22]. The existence of these BMD patients offers evidence that expression of a truncated dystrophin protein serves as a basis to treat DMD patients with smaller dystrophin protein variants.

Skeletal muscle cells missing functional dystrophin protein are susceptible to membrane permeability and leakage of Ca^{2+} ions after muscle contraction and relaxation, ultimately resulting in cell death. Over time, muscle cells are replaced by fat and scar tissue. Typical therapies for DMD include corticosteroid treatments that can delay a wheelchair-bound state; however, caution is needed for adverse effects that include behavioral changes, fractures, cataracts, weight gain, and cushingoid appearance [23].

Moreover, current therapeutic options to treat the root cause of the disease itself are limited.

To this end, several therapies have been explored in clinical trials to treat the cause of DMD. In one clinical trial, gene therapy was used to supply minidystrophin [24], a 60% truncated form of dystrophin cDNA lacking a large portion of the rod domain and a C-terminal domain [25], delivered through a viral vector. Unfortunately, viral-mediated delivery of microdystrophin into patients failed to establish sustained protein expression, possibly due to an immune response [24]. Another therapy used antisense oligonucleotides (AONs) that are able to induce exon skipping at the pre-mRNA level for rescuing truncated dystrophin protein expression. Recently, the FDA conditionally approved Eteplirsen (Exondy51) [26], an AON that skips exon 51, but its half-life in blood is only 3 hours, hence a weekly injection is required. It is still unclear whether AONs will be able to provide long-term benefits in preventing muscular dystrophy in patients.

3. Considerations for Allogeneic or Autologous iPS Cell Therapy to Treat DMD Going Forward

As DMD patients suffer from severe muscle atrophy, cell transplantation therapy would be a rational approach. Obtaining functional myoblast progenitor cells from autologous or human leukocyte antigen- (HLA-) matched allogenic iPSCs has advantages for expansion and potential clinical use over other methods such as direct isolation of primary myoblasts and myoblasts derived from reprogrammed fibroblasts. Primary myoblasts are generally immortalized by oncogenic factors including the SV40 large T antigen and telomerase reverse transcriptase (*TERT*), making their use for transplantation unreasonable. Reprogrammed myoblasts from fibroblasts by *MYOD1* [27] also suffer from the same problem as primary myoblasts because they require immortalization for long-term survival and expansion [28]. On the other hand, iPSCs can be maintained indefinitely and then converted into myoblast progenitors [29]. This is particularly important because clinical trials where allogeneic myoblasts were transplanted into DMD patients revealed that low cell survival, poor cell migration, and potential immune clearance are issues, which means a high cell number is needed [30]. To further improve cell transplantation, identification of an appropriate cell type with better survival and engraftment should be determined. For preparation of large cell numbers, the selected differentiation protocol from iPSCs needs to be robust and scalable.

To minimize immunogenic reactions, autologous human iPSCs could be edited ex vivo and then transplanted back into the patient. The autologous approach could work similarly to a mouse study in which fibroblasts from a severe DMD mouse model (*mdx*) that lacks both *utrophin* and *dystrophin* genes were reprogrammed into iPSCs ex vivo and transduced with a *Sleeping Beauty* transposon to express microdystrophin cDNA [31]. After differentiation into myogenic progenitor cells, the cells were transplanted back into the *mdx* mice by engraftment or systemic delivery [31]. Both led to dystrophin protein expressing skeletal muscle cells, improved muscle strength, and, importantly, the establishment of satellite muscle cells for a continual supply of corrected skeletal muscle cells. In the human context, iPSCs could be established and then differentiated into myoblast progenitor cells or muscle stem cells for transplantation back into the patient. As DMD iPSCs carry the same genetic mutation as the original patient, functional dystrophin protein must be restored before the transplantation. The classical approach would be to transduce cDNA by a vector, such as a human artificial chromosome [11] or *Sleeping Beauty* transposon vector [31]. More recently, genome editing approaches have been evolving to correct the *Dystrophin* mutation(s).

4. How Does Genome Editing Work?

Genome editing can be used to facilitate DNA repair after a double stranded DNA break (DSB) is induced by a programmable nuclease [32]. Thereafter, two predominant DNA repair pathways are induced. One involves homologous recombination (HR), which requires the presence of a DNA template with homology regions overlapping each side of the cleaved DNA to be precisely inserted into the DSB site [33]. The other DNA repair pathway is the predominant one, called NHEJ (nonhomologous end joining), and leads to insertions or deletions (indels) being introduced to patch up the DSB site. NHEJ is more frequent and has been estimated to occur within 30 minutes as opposed to HR, which takes as long as 7 hours [34]. Both of these approaches have been utilized in combination with DNA nucleases for genome editing purposes.

5. CRISPR Cas9 Nucleases

Nucleases available for gene editing such as meganucleases, TALENs (transcription activator-like effector nucleases), and ZFNs (zinc-finger nucleases) rely on engineering the DNA binding domain for recognizing specific DNA sequences (reviewed in [35, 36]). In contrast, CRISPR (clustered regularly interspaced short palindromic repeats) Cas9 (CRISPR associated protein) uses complementary guide RNA, hence it is highly versatile and has become the preferred nuclease of choice in the genome editing field, enabling scientists to quickly establish disease models, create gene knockouts for studying cellular phenotypes, and model gene correction for monogenic diseases.

CRISPR-Cas9 was first identified as an adaptive immune system in bacteria against invading bacteriophages and later harnessed into a tool for DNA editing [37]. It was quickly adapted for use in mammalian cells and has also been proven to function in many organisms. The Type II CRISPR system consists of a nuclease called Cas9 and a single guide RNA molecule (sgRNA), which is a fusion of two RNA components, transactivating RNA (tracrRNA) and CRISPR RNA. Cas9 complexes with sgRNA and is guided to a targeted DNA sequence by a programmable 20 bp sequence that lies

at the most 5′-terminal portion of the sgRNA. Importantly, the 20 bp targeting sequence must reside next to a defined protospacer adjacent motif (PAM) that varies in sequence and length depending on the Cas9 nuclease being used for DNA cleavage. In this respect, there are a variety of Cas9 nucleases to choose from depending on one's targeting needs.

CRISPR Cas9 from *Streptococcus pyogenes* (SpCas9) is the most commonly utilized for DNA editing because it is extensively characterized in respect to its structure and activity [37–40]. SpCas9 cDNA is approximately 4.1 kb long and translates into a 1368 amino acid protein [37]. Owing to the fact that its PAM sequence (NGG) is relatively common in the human genome, it offers flexibility for designing sgRNA against DNA target sequences. The Cas9 cDNA from *Staphylococcus aureus* (SaCas9) is smaller than that from SpCas9 by nearly 1000 bp and encodes a 1053 amino acid protein. SaCas9 has a "NGGRRT" PAM requirement; therefore, its targetable density is lower than that of SpCas9 [41].

In combination with iPSCs, genetic mutations in patient-derived iPSCs have been successfully corrected by CRISPR-Cas9 system for several diseases, such as β-thalassemia [42], Niemann-Pick disease Type C [43], hemophilia A [44], and DMD [12, 13, 45]. Both Sp- and SaCas9 have been used successfully to correct dystrophin gene mutations in *mdx* mice and patient-derived myoblasts and iPSCs.

6. How Can CRISPR Cas9 Be Applied to DMD?

There are mainly four approaches that have been demonstrated to restore the open reading frame of dystrophin transcripts by genomic editing with CRISPR Cas9: (i) exon skipping by splicing acceptor disruption, (ii) exon deletion, (iii) NHEJ mediated frame shift, and (iv) exogenous exon knock-in [46] (Figure 1). The use of each approach can be catered to the type of DMD mutation to be targeted (Table 2).

7. Exon Skipping

Similar to the AON strategy mentioned above, CRISPR-Cas9-mediated mutagenesis of splicing acceptor (SA) sites could induce exon skipping to permanently restore the *DMD* ORF. As an example, a DMD patient lacking exons 48–50 (Δ48–50 DMD) fails to express the protein when exon 47 is followed by exon 51. However, if exon 47 is followed by exon 52, then the ORF can be restored (Figure 1). Ousterout et al. demonstrated that this could be accomplished by removing a splicing acceptor (SA) in front of exon 51 by a pair of ZFNs in immortalized myoblasts from a DMD patient [47]. Similarly, another group reported the SA disruption of exon 51 in myoblasts derived from Δ48–50 and Δ45–52 DMD patients by CRISPR SpCas9 to restore dystrophin protein expression with a combination of TALEN and CRISPR SpCas9 strategies [48, 49]. Our group applied this approach to disrupt the SA of exon 45 in iPSCs derived from a Δ44 DMD patient using CRISPR SpCas9 and TALENs to skip exon 45 and successfully restore dystrophin protein expression after iPSCs were differentiated into myoblasts [13].

8. Exon Deletion

An alternative for exon skipping is to excise one or more targeted exons. Using CRISPR SpCas9 with Δ48–50 DMD patient-derived myoblasts, Ousterout et al. and colleagues deleted exon 51 by targeting the flanking introns 50 and 51 [50]. The result was a similar phenotype to that observed with SA disruption. The authors also employed a larger exon deletion strategy where they designed sgRNAs against introns 44 and 55 to remove a 336 kb genomic region of the *dystrophin* gene. Adeno-associated virus (AAV) delivery of either multiplexed TALENs or CRISPR Cas9 into Δ48–50 and Δ45–52 DMD patient myoblasts could also remove exons 45–55. Furthermore, Young et al. used three DMD patient-derived iPSC lines (Δ46–51, Δ46–47, and duplicated exon 50) to demonstrate that two sgRNAs could remove exons 45–55 and a larger portion of the intronic region [12]. Up to a 725 kbp region of genomic DNA was removed using this approach, and dystrophin-positive fibers could be observed in differentiated skeletal muscle cells in vitro as well as in vivo in transplanted *mdx* mice [12]. Although the adverse effects by the large genomic deletion need to be determined, these results are important from a cost perspective because they indicate that up to 60% of DMD patients could be converted to a BMD genotype by the multiexon deletion approach [50].

Exon deletion has also been useful in vivo in *mdx* mice, which have a premature stop codon in exon 23 of the *dystrophin* gene. Deletion of mouse exon 23 by CRISPR Cas9 can restore the ORF, similar to the approaches conducted in human cells. Three groups reported the delivery of either SpCas9 or SaCas9 and sgRNAs targeting upstream and downstream of exon 23 by AAV into *mdx* mice in vivo. All three of these groups performed localized and systematic delivery of Cas9 and sgRNAs by intramuscular or intraperitoneal injection and not only successfully recovered dystrophin expression but improved muscle function as well [51–53].

9. Frame Shifting

The NHEJ pathway can be utilized to induce insertions and deletions for resetting an ORF containing a premature stop codon and restoring dystrophin protein expression [54]. Theoretically, there is a one third chance of this event occurring, meaning that the approach is not highly efficient. Nonetheless, we applied this strategy to a region harboring a premature stop codon in exon 45 in iPSCs derived from a Δ44 DMD patient and successfully restored dystrophin protein expression [13]. Frame shift restoration of dystrophin by inducing indels in exon 51 and exon 53 of myoblasts derived from Δ48–50 and Δ45–52 DMD patients, respectively, was also shown to be effective [49].

10. Exon Knock-In

Exon knock-in with a DNA donor template by HR after nuclease-induced DNA cleavage offers the ability to restore full-length dystrophin protein expression. In Δ44 DMD

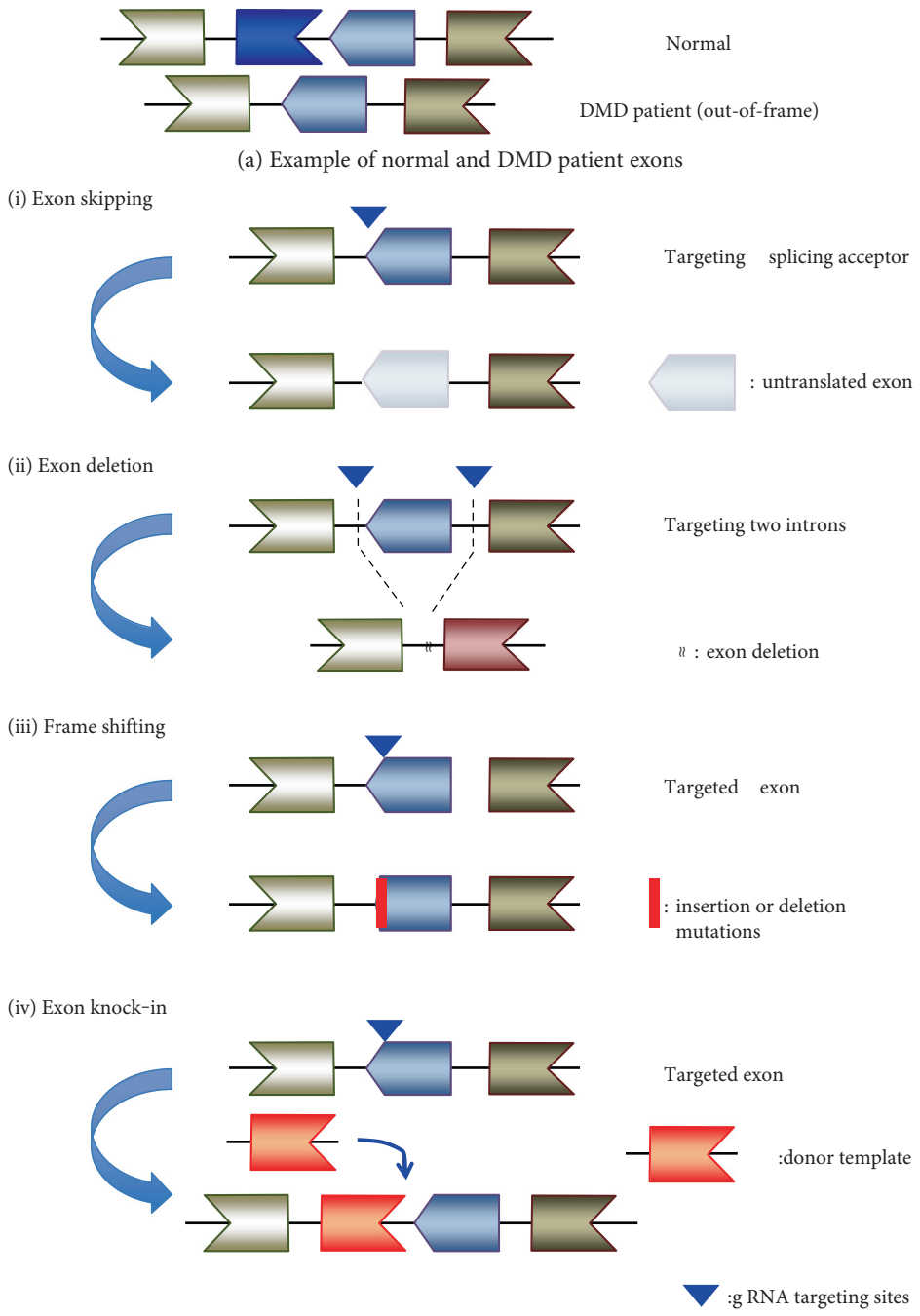

Figure 1: *Dystrophin* gene targeting strategies by CRISPR Cas9. (a) Examples of normal and patient *dystrophin* gene exons. Individual exons are represented by beige, dark blue, light blue, and brown. In healthy patient genomes, the exons are in frame and will lead to the expression of a full protein. In DMD patient genomes, the deletion of the dark blue exon leads to a frame shift, disrupting the ORF and causing a premature stop codon. (b) Four main strategies of genome editing to correct the ORF of the *dystrophin* gene: (i) for exon skipping, sgRNA is designed to target a splicing acceptor. This disruption would mask the exon as an intron, which would not be included in the final mRNA product; (ii) exon deletion involves the complete deletion of a single or multiple exons from the genome. Exon(s) within the range of two targeting sgRNAs would be excised. Mono-exon deletions could be designed for each *dystrophin gene* mutation type. For a multiexon deletion strategy, exons 45–55 (or exons 44–54) are deleted and could be applied to up to 60% of DMD patients, although this results in the production of a much smaller dystrophin protein as seen in Becker muscular dystrophy; (iii) another approach to avoid premature stop codons and recover the ORF is by inserting or deleting bases and making frame shifts instead of an exon deletion. NHEJ-mediated insertions or deletions may induce frame shifts and recover the ORF; (iv) *dystrophin* gene deletion mutations involving one or multiple exons could be rescued by a knock-in strategy of the deleted exon(s) to completely restore full length dystrophin protein expression. In this strategy, a donor template should be delivered in addition to Cas9 and sgRNA.

Table 2: Summary of papers utilizing various CRISPR-Cas9 strategies to target DMD mutations in patient-derived cells.

Strategy	Target cell	DMD type	Genome editing target	Deletion size	Ref.
Mono or multiexon deletion	Myoblast	ΔEx48–50	Ex51 Ex45–55	336 kbp	[50]
Multiexon deletion	iPSC	ΔEx46–51 ΔEx46-47 Dup ex50	Ex45–55	530 kbp 670 kbp 725 kbp	[12]
Exon deletion	Myoblast	ΔEx48–50 ΔEx45–52	Ex51 Ex44–54 Ex53		[49]
Exon deletion	*mdx* mice (in vivo)	Nonsense mut in Ex23	Ex23	~0.5 kbp	[52]
Exon deletion	*mdx* mice (in vivo)	Nonsense mut in Ex23	Ex23	~1.2 kbp	[51]
Exon deletion	*mdx* mice (in vivo)	Nonsense mut in Ex23	Ex23	~0.3 kbp	[53]
Exon skipping	iPSC	ΔEx44	Ex45	18 bp	[13]
Exon skipping	Myoblast	ΔEx48–50 ΔEx45–52	Ex51 Ex53		[49]
Frame shifting	iPSC	ΔEx44	Ex45	2 bp insertion	[13]
Frame shifting	Myoblast	ΔEx48–50 ΔEx45–52	Ex51 Ex53		[49]
Frame shifting and exon deletion	Myoblast	ΔEx51–53	Ex50 Ex54	>160 kbp	[57]
Exon knock-in	iPSC	ΔEx44	Ex45		[13]
Exon knock-in and exon deletion	*mdx* mice (in vivo)	Nonsense mut in Ex53	Ex52-53 Ex53		[55]

patient-derived iPSCs, we succeeded in knocking in exon 44 at the 5′ end of exon 45 to obtain full length dystrophin protein in differentiated myoblasts [13]. While this strategy is appealing because there would be a complete restoration of native dystrophin protein, the frequency of the HR pathway is low and antibiotic selection is normally required. HR does not take place in G1-arrested cells such as mature myofibers, so it would be much less efficient in vivo. Indeed, Bengtsson et al. used two AAV vectors to deliver SpCas9, multiplexed sgRNAs, and a donor template in an *mdx* mouse harboring a nonsense mutation in exon 53 and found that HR was successful in 0.18% of total genomes compared with editing occurring in 2.3% of total genomes. In other words, NHEJ accounted for 92% of the edited genomes [55]. In this context, a target specific integration approach, such as HITI (homology-independent targeted integration) [56], might be applicable to insert the missing exon(s) at the appropriate locus. In addition, there is a limitation to the length of the HR template DNA that can be used depending on the delivery approach being used, meaning that it would be challenging to apply to large deletion mutations for restoring the original genomic DNA form of dystrophin.

11. The Expanding CRISPR Toolbox for Alternative Options to Treat DMD Patients

Depending on the targeted *dystrophin* gene sequence, other type II CRISPR Cas9 nucleases which have been reported such as those from *Streptococcus thermophilus* [58], *Neisseria meningitis* [59], or type V CRISPR Cpf1 may be appropriate for use. Cpf1 is approximately the same size as SpCas9 but does not have a tracrRNA component and consists of only crRNA. It has a PAM that is more applicable for T rich regions (i.e. "TTTN") at the 5′ side of the targeted sequence. Cpf1 has distinct cleavage patterns compared with SpCas9 because it induces a staggered DSB as opposed to a blunt DSB. Insertion mutations and single base deletions were rarely observed with Cpf1, but out-of-frame mutation frequencies were comparable with SpCas9 [60].

Recently, new nucleases have been identified which may also be applicable for editing the *dystrophin* gene in the future. *Natronobacterium gregoryi* Argonaute (NgAgo) was reported to induce DSB guided by phosphorylated single-stranded DNA without the requirement of a PAM sequence, despite the fact that mammalian Argonaute normally processes RNA guided by an RNA (siRNA) template [61]. Although the reproducibility of the published results is still being debated [62], the potential to use other programmable nuclease systems would greatly expand the targeting range of candidate *DMD* mutations. Furthermore, through metagenomics of DNA extracted from bacteria and archaea that cannot be typically cultivated in the laboratory, new Cas9 nucleases, named CasX and CasY, have been recognized as smaller than SpCas9 and have unique sequence recognition for PAMs [63]. Continued exploration of these untapped sources for novel Cas9 nucleases may yield additional powerful tools for genome editing.

12. Alternatives to Genome Cleavage

Alternative approaches to treat DMD patients that do not rely on DNA cleavage could involve the utilization of catalytically dead Cas9 (dCas9) proteins fused with effector

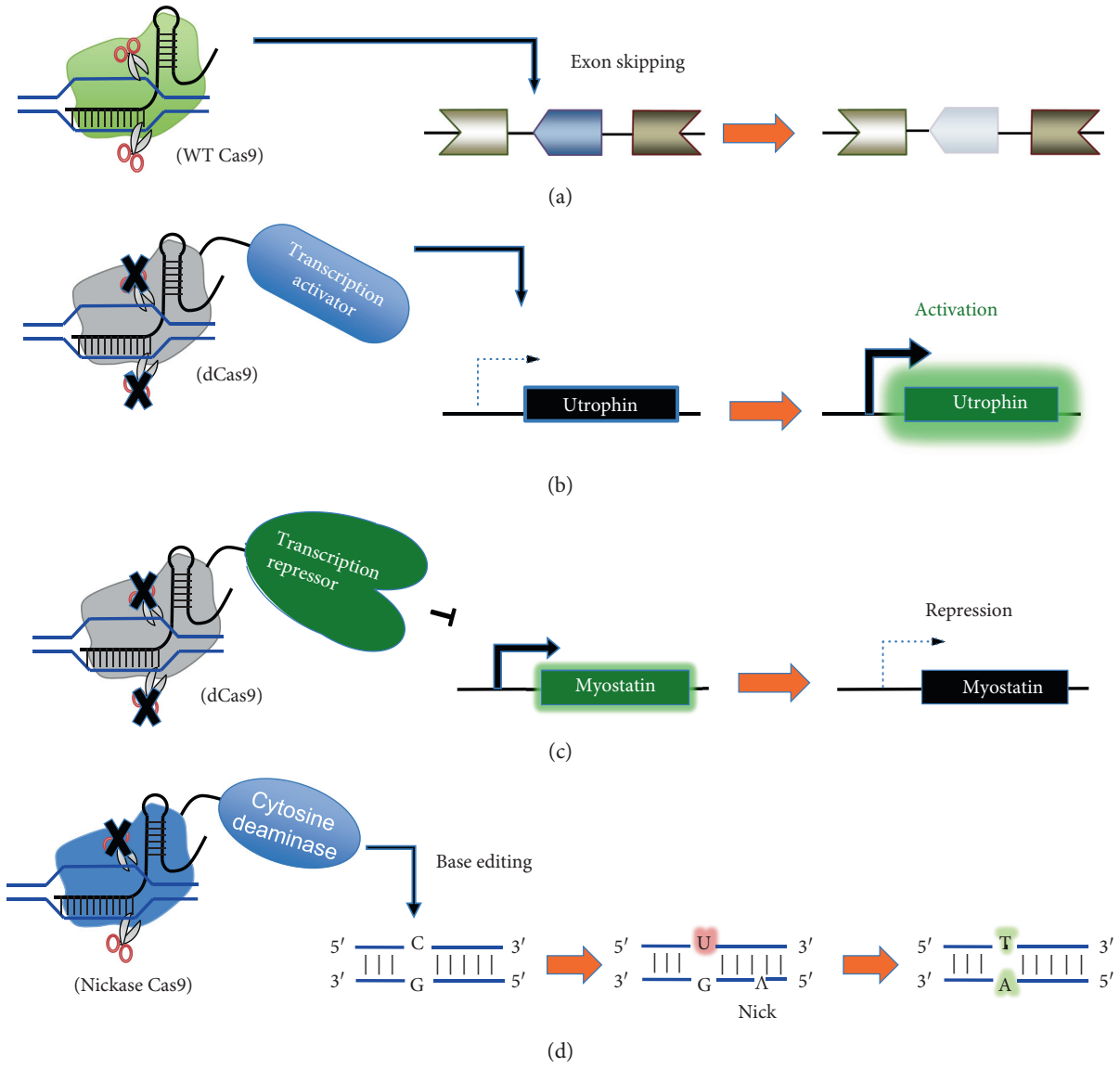

FIGURE 2: DSB and non-DSB mediated therapeutic approaches to potentially treat DMD. (a) WT Cas9 nuclease can be used to cleave DNA for exon skipping, frame shifting, or exon deletion as mentioned in Figure 1. (b) Catalytically inactive dCas9 fused with a transcription repressor such as KRAB can work as a sequence-dependent transcription repressor for a target gene such as *myostatin* to attenuate muscle wasting. (c) dCas9 fused with a transcription activator such as VP64 or p65 can work as a sequence-dependent transcription activator, in this case for activating *utrophin* expression to compensate for the absence of dystrophin. (d) Nickase Cas9 fused with a cytosine deaminase (i.e. APOBEC1 or AID homologue) can revert C to T by cytosine deamination. This can be used for correcting T → C mutations, or to disrupt premature stop codons or splicing acceptor sequences to induce exon skipping.

molecules for transcriptional activation, transcriptional inhibition, or specific base editing.

Utrophin (*UTRN*) is a paralog of dystrophin that is structurally similar and may be a candidate for transcriptional upregulation. Previous reports have shown that the overexpression of utrophin cDNA reduces the pathology of muscular dystrophy in *mdx* mice when overexpressed as a transgene [64]. Currently, phase I studies are being conducted with a drug molecule, called SMT 1100, to increase utrophin expression [65]. To transcriptionally activate a gene using dCas9, several groups have fused it with transcriptional activators such as VP64 (four tandem repeats of VP16) and p65 [66, 67]. Wojtal et al. recently reported that dCas9 fused with VP160 (ten tandem repeats of VP16) could boost utrophin protein expression in DMD patient myoblasts nearly 7-fold depending on the targeted promoter [68]. Thus, this technology is able to increase utrophin expression and bypass the risks associated with genomic cleavage of a functional Cas9 nuclease (Figure 2). In this context, stable activation of the utrophin gene is critical, hence a combination with demethylation of CpG or alike should also be considered.

Fusion of dCas9 with transcriptional repressor Kruppel-associated box domain (KRAB) has been shown to effectively inhibit the transcription of endogenous loci through the recruitment of heterochromatin [69]. One potential target

of dCas9-KRAB in DMD patients is *Myostatin (MSTN)*, which is a cytokine released specifically by skeletal muscles cells that causes muscle atrophy [70]. One study showing the in vivo delivery of SaCas9 nuclease into wild-type mice targeting *MSTN* was able to attenuate muscle loss [71]. In a similar manner, dCas9-KRAB could be used to suppress Myostatin expression (Figure 2); however, the effectiveness of dCas9-KRAB will depend on the chromatin status of the target DNA of the sgRNA, which was shown in cancer cells to cause a variation ranging between 60 and 80% transcriptional repression [69]. Again, stable alteration of the epigenetic state to maintain transcriptional suppression after the elimination of dCas9-KRAB will be essential to successfully incorporate this type of method.

Recently, the development of a nucleotide-specific base editor consisting of rat cytidine deaminase APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) [72] and AID ortholog PmCDA1 from sea lamprey [73] fused with a nickase version of Cas9 or dCas9, respectively, was shown to be able to induce C→T mutations. While it is estimated that 10% of DMD patients have point mutations [20], the base editing approach would be applicable to patients with T→C mutations that could be reverted back by Cas9-APOBEC1. This strategy may also be used to disrupt premature stop codons or splicing acceptor sites for inducing exon skipping (Figure 2). Although these new non-DSB techniques need to be optimized further, they may open new possibilities towards DMD therapy.

13. Considerations for Direct Use of CRISPR Cas9 to Treat DMD Patients In Vivo

AAV is a small (about 22 nm diameter) parvorvirus with a genome size of 4.7 kb and cannot replicate in the absence of a helper virus such as adenovirus or herpes simplex virus [74]. It has been engineered into a vector for transducing transgenes in vivo and has also been tested in gene therapy clinical trials for treating diseases such as hemophilia B and cystic fibrosis. For in vivo gene editing with the CRISPR Cas9 system, several groups have developed AAV vectors for the delivery of either Sp- or SaCas9 and sgRNA to treat DMD. These constructs were tested in *mdx* mice and could partially restore dystrophin expression either through local or systemic injection in skeletal muscle and cardiac cells [51–53]. However, when considering their use in human patients, AAV has several disadvantages. Up to 73% of adults have preexisting antibodies against different AAV serotypes from exposure to naturally occurring AAV infection during childhood [75], which is important because the anti-AAV antibodies could neutralize the viral particles before cell penetration and Cas9 transduction. The second disadvantage of AAV is that transgenes are constitutively expressed over a long period of time, especially in nondividing cells, which in the case of Cas9, may increase the probability of off-target cleavage. The third disadvantage is that the restrictive packaging capacity of the AAV capsid prevents both the Cas9 nuclease and sgRNA to be contained within the same vector. Indeed, for all three in vivo studies testing the effectiveness of the CRISPR Cas9 system, the nuclease and sgRNA were packaged into separate vectors [51–53]. Ideally, an all-in-one approach is desirable to increase in vivo delivery efficiency and to decrease the production cost.

The possibility of nonspecific DNA cleavage by CRISPR-Cas9 is a major concern in the field of gene editing [76] since it was reported by several groups that a high number of off-target events could occur depending on the uniqueness of the gene target sequence [77–79]. Off-target mutagenesis by ZFN nucleases has been associated with cellular toxicities [80]. Although numerous engineering approaches are being explored to minimize off-target cleavage by optimizing sgRNA structure and length or by adding regulatory elements to SpCas9 to limit nuclease activity, evaluation of the frequency of these events will be critical for therapeutic applications. Various evaluation methods exist, but the most sensitive only detects mutations at 0.1% frequency in in vitro cells, not in in vivo tissues [81]. Thus, the detection threshold may not be high enough to pick up rare off-target cleavage events, such as one in millions of cells. However, it should be noted that to date, no off target mutations for dystrophin sgRNAs have been reported in vitro or in vivo using CRISPR SpCas9 in pluripotent stem cells [12, 13, 51–53].

Another point of concern regarding the use of gene editing with CRISPR Cas9 in vivo is the immune response to the expressed Cas9 nuclease or corrected dystrophin protein. It is possible that newly expressed Cas9 nuclease and dystrophin may elicit an immune response. Indeed, it has been recently reported that Cas9 nuclease delivered into Ai9 or C57BL/6 mice by naked DNA electroporation or an AAV vector triggered an increase in the number of Cas9-reactive T cells [82]. Furthermore, the delivery of microdystrophin by AAV in a clinical trial failed to establish dystrophin protein expression but preexisting or de novo dystrophin protein reactive T cells were detected, suggesting that the transplanted cells were eliminated by immune clearance [24]. There is also evidence for an age-related increase in dystrophin reactive T-cells in DMD patients [83]. While these findings suggest that the immune system may be another obstacle to overcome in order to establish permanent treatment, limiting the expression of the Cas9 nuclease specifically to muscle cells utilizing a muscle-specific promoter may help to attenuate an immune response [55].

The way we envision the use of iPSCs going forward is to develop a screening platform to validate sgRNAs against the human *dystrophin* gene. Because each sgRNA has a variable activity and its own associated off target frequency, it is more relevant to test sgRNAs against the *dystrophin* gene in a human genome context, meaning that *mdx* mice are less informative for assessing specificity. To this end, patient-specific iPSCs could be used to test sgRNA activity and specificity directly in cells relevant to patients.

14. Conclusion

Much work has been done on editing strategies with CRISPR Cas9 to restore dystrophin expression in cells derived from DMD patients. Further discoveries of orthogonal CRISPR systems or other programmable nucleases will broaden our

ability to precisely target the *dystrophin* gene. Advances on non-DSB type base editors or transcriptional regulator-based epigenetic editors might open new therapeutic approaches. Specificity and off-target effects are the biggest concerns regarding the safety of any programmable sequence-specific editors. In this context, DMD patient-derived iPSCs not only provide a disease-relevant context for validating a novel therapeutic approach but could also serve as an abundant source for testing specificity in a human genome context. Although most of the technologies mentioned here are years away from being used in patients, they provide exciting options for DMD treatment in future.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Peter Gee, Huaigeng Xu, and Akitsu Hotta contributed to writing the manuscript.

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Review Article

Skeletal Muscle Cell Induction from Pluripotent Stem Cells

Yusaku Kodaka,[1,2,3] Gemachu Rabu,[1,2,3] and Atsushi Asakura[1,2,3]

[1]*Stem Cell Institute, University of Minnesota Medical School, Minneapolis, MN 55455, USA*
[2]*Paul and Sheila Wellstone Muscular Dystrophy Center, University of Minnesota Medical School, Minneapolis, MN 55455, USA*
[3]*Department of Neurology, University of Minnesota Medical School, Minneapolis, MN 55455, USA*

Correspondence should be addressed to Atsushi Asakura; asakura@umn.edu

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Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have the potential to differentiate into various types of cells including skeletal muscle cells. The approach of converting ESCs/iPSCs into skeletal muscle cells offers hope for patients afflicted with the skeletal muscle diseases such as the Duchenne muscular dystrophy (DMD). Patient-derived iPSCs are an especially ideal cell source to obtain an unlimited number of myogenic cells that escape immune rejection after engraftment. Currently, there are several approaches to induce differentiation of ESCs and iPSCs to skeletal muscle. A key to the generation of skeletal muscle cells from ESCs/iPSCs is the mimicking of embryonic mesodermal induction followed by myogenic induction. Thus, current approaches of skeletal muscle cell induction of ESCs/iPSCs utilize techniques including overexpression of myogenic transcription factors such as MyoD or Pax3, using small molecules to induce mesodermal cells followed by myogenic progenitor cells, and utilizing epigenetic myogenic memory existing in muscle cell-derived iPSCs. This review summarizes the current methods used in myogenic differentiation and highlights areas of recent improvement.

1. Introduction

Duchenne muscular dystrophy (DMD) is a genetic disease affecting approximately 1 in 3500 male live births [1]. It results in progressive degeneration of skeletal muscle causing complete paralysis, respiratory and cardiac complications, and ultimately death. Normal symptoms include the delay of motor milestones including the ability to sit and stand independently. DMD is caused by an absence of functional dystrophin protein and skeletal muscle stem cells, as well as the exhaustion of satellite cells following many rounds of muscle degeneration and regeneration [2]. The dystrophin gene is primarily responsible for connecting and maintaining the stability of the cytoskeleton of muscle fibers during contraction and relaxation. Despite the low frequency of occurrence, this disease is incurable and will cause debilitation of the muscle and eventual death in 20 to 30 year olds with recessive X-linked form of muscular dystrophy. Although there are no current treatments developed for DMD, there are several experimental therapies such as stem cell therapies.

Skeletal muscle is known to be a regenerative tissue in the body. This muscle regeneration is mediated by muscle satellite cells, a stem cell population for skeletal muscle [3, 4]. Although satellite cells exhibit some multipotential differentiation capabilities [5], their primary differentiation fate is skeletal muscle cells in normal muscle regeneration. Ex vivo expanded satellite cell-derived myoblasts can be integrated into muscle fibers following injection into damaged muscle, acting as a proof-of-concept of myoblast-mediated cell therapy for muscular dystrophies [6–9]. However, severe limitations exist in relation to human therapy. The number of available satellite cells or myoblasts from human biopsies is limited. In addition, the poor cell survival and low contribution of transplanted cells have hindered practical application in patients [6, 8, 9]. Human-induced pluripotent stem cells (hiPSCs) are adult cells that have been genetically reprogrammed to an embryonic stem cell- (ESC-) like state by being forced to express genes and factors important for maintaining the defining properties of ESCs. hiPSCs can be generated from a wide variety of somatic cells [10, 11]. They have the ability to self-renew and successfully turn

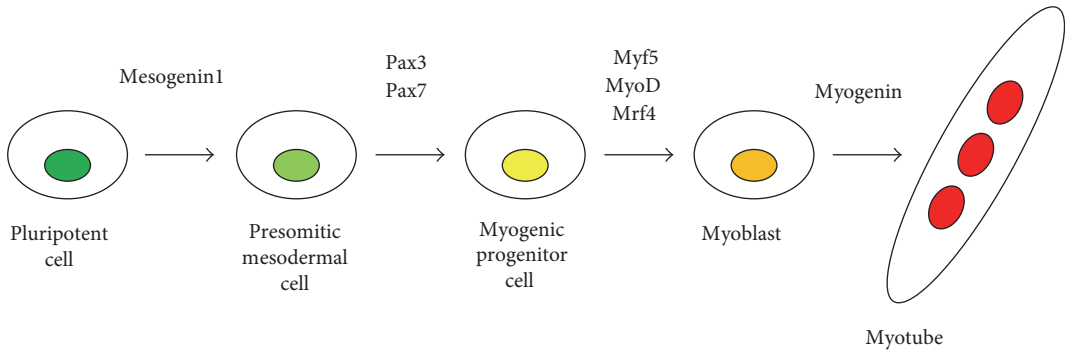

Figure 1: Hierarchal master transcription factor cascade for myogenesis. For myogenic differentiation during early embryogenesis, Mesogenin1 works as a master regulator for unsegmented presomitic mesoderm formation. Then, segmented somites are formed. Pax3 and Pax7 are activated in presomitic mesoderm, which generates somite-derived dermomyotome. Pax3 and Pax7 then work as master regulators for myogenic progenitor cell induction. Finally, MyoD and Myf5 are upregulated in the dorsomedial lip of dermomyotome and function as master regulators for myogenic specification to generate myoblasts. Eventually, myoblasts stop cell proliferation and express myogenin, which induces terminal differentiation of myoblasts to form multinucleated myotubes.

into any type of cells. With their ability to capture genetic diversity of DMD in an accessible culture system, hiPSCs represent an attractive source for generating myogenic cells for drug screening.

The ESC/iPSC differentiation follows the steps of embryonic development. The origin of skeletal muscle precursor cells comes from the mesodermal lineage, which give rise to skeletal muscle, cardiac muscle, bone, and blood cells. Mesoderm subsequently undergoes unsegmented presomitic mesoderm followed by segmented compartments termed somites from anterior to caudal direction. Dermomyotome is an epithelial cell layer making up the dorsal part of the somite underneath the ectoderm. Dermomyotome expresses Pax3 and Pax7 and gives rise to dermis, skeletal muscle cells, endothelial cells, and vascular smooth muscle [12]. Dermomyotome also serves as a tissue for secreted signaling molecules to the neural tube, notochord, and sclerotome [13, 14]. Upon signals from the neural tube and notochord, the dorsomedial lip of dermomyotome initiates and expresses skeletal muscle-specific transcription factors such as MyoD and Myf5 to differentiate into myogenic cells termed myoblasts. Myoblasts then migrate beneath the dermomyotome to form myotome. Eventually, these myoblasts fuse with each other to form embryonic muscle fibers. ESCs/iPSCs mimic these steps toward differentiation of skeletal muscle cells. Many studies utilize methods of overexpression of muscle-related transcription factors such as MyoD or Pax3 [15], or the addition of small molecules which activate or inhibit myogenic signaling during development. Several studies show that iPSCs retain a bias to form their cell type of origin due to an epigenetic memory [16–19], although other papers indicate that such epigenetic memory is erased during the reprogramming processes [20–22]. Therefore, this phenomenon is not completely understood at the moment. In light of these developments, we have recently established mouse myoblast-derived iPSCs capable of unlimited expansion [23]. Our data demonstrates that these iPSCs show higher myogenic differentiation potential compared to fibroblast-derived iPSCs. Thus, myogenic precursor cells generated from human myoblast-derived iPSCs expanded ex vivo should provide an attractive cell source for DMD therapy. However, since DMD is a systemic muscle disease, systemic delivery of myoblasts needs to be established for efficient cell-based therapy.

2. Myogenic Master Transcription Factors for Skeletal Muscle Development (Figure 1)

During developmental myogenesis, presomitic mesoderm is first formed by Mesogenin1 upregulation, which is a master regulator of presomitic mesoderm [24]. Then, the paired box transcription factor *Pax3* gene begins to be expressed from presomitic mesoderm to dermomyotome [25]. Following *Pax3* expression, *Pax7* is also expressed in the dermomyotome [26], and then *Myf5* and *MyoD*, skeletal muscle-specific transcription factor genes, begin to be expressed in the dorsomedial lip of the dermomyotome in order to give rise to myoblasts which migrate beneath the dermomyotome to form the myotome. Subsequently, *Mrf4* and *Myogenin*, other skeletal muscle-specific transcription factor genes, followed by skeletal muscle structural genes such as myosin heavy chain (MyHC), are expressed in the myotome for myogenic terminal differentiation (Figure 1) [27, 28]. *Pax3* directly and indirectly regulates *Myf5* expression in order to induce myotomal cells. Dorsal neural tube-derived Wnt proteins and floor plate cells in neural tube and notochord-derived sonic hedgehog (Shh) positively regulate myotome formation [13, 29]. Neural crest cells migrating from dorsal neural tubes are also involved in myotome formation: Migrating neural crest cells come across the dorsomedial lip of the dermomyotome, and neural crest cell-expressing Delta1 is transiently able to activate Notch1 in the dermomyotome, resulting in conversion of Pax3/7(+) myogenic progenitor cells into MyoD/Myf5(+) myotomal myoblasts [30, 31]. By contrast, bone morphogenetic proteins (BMPs) secreted from lateral plate mesoderm are a negative regulator for the myotome formation by maintaining Pax3/Pax7(+) myogenic progenitor cells [29, 32]. Pax3 also regulates cell migration of myogenic progenitor cells from ventrolateral lip of dermomyotome to the limb bud [33]. *Pax3* mutant

mice lack limb muscle but trunk muscle development is relatively normal [34]. *Pax3*/*Pax7* double knockout mice display failed generation of myogenic cells, suggesting that *Pax3* and *Pax7* are critical for proper embryonic myogenesis [35]. Therefore, both *Pax3* and *Pax7* are also considered master transcription factors for the specification of myogenic progenitor cells. Importantly, MyoD was identified as the first master transcription factor for myogenic specification since MyoD is directly able to reprogram nonmuscle cell type to myogenic lineage when overexpressed [36–38]. In addition, genetic ablation of MyoD family gene(s) via a homologous gene recombination technique causes severe myogenic developmental or regeneration defects [39–45]. Finally, genetic ablation of combinatory MyoD family genes demonstrates that $MyoD^{-/-}$:$Myf5^{-/-}$:$MRF4^{-/-}$ mice do not form any skeletal muscle during embryogenesis, indicating the essential roles in skeletal muscle development of MyoD family genes [28, 46]. It was proven that Pax3 also possesses myogenic specification capability since ectopic expression of Pax3 is sufficient to induce myogenic programs in both paraxial and lateral plate mesoderm as well as in the neural tube during chicken embryogenesis [47]. In addition, genetic ablation of Pax3 and Myf5 display complete defects of body skeletal muscle formation during mouse embryogenesis [48]. Finally, overexpression of Pax7 can convert CD45(+)Sca-1(+) hematopoietic cells into skeletal muscle cells [49]. From these notions, overexpression of myogenic master transcription factors such as MyoD or Pax3 has become the major strategy for myogenic induction in nonmuscle cells, including ES/iPSCs.

3. Overexpression Approaches of Myogenic Master Transcription Factors in ESCs/iPSCs

The overexpression of *MyoD* approach to induce myogenic cells from mESCs was first described by Dekel et al. in 1992. This has been a standard approach for the myogenic induction from pluripotent stem cells (Table 1). Ozasa et al. first utilized Tet-Off systems for *MyoD* overexpression in mESCs and showed desmin(+) and MyHC(+) myotubes in vitro [50]. Warren et al. transfected synthetic *MyoD* mRNA in to hiPSCs for 3 days, which resulted in myogenic differentiation (around 40%) with expression of myogenin and MyHC [51]. Tanaka et al. utilized a *PiggyBac* transposon system to overexpress *MyoD* in hiPSCs. The *PiggyBac* transposon system allows cDNAs to stably integrate into the genome for efficient gene expression. After integration, around 70 to 90% of myogenic cells were induced in hiPSC cultures within 5 days [52]. This study also utilized Miyoshi myopathy patient-derived hiPSCs for the MyoD-mediated myogenic differentiation. Miyoshi myopathy is a congenital distal myopathy caused by defective muscle membrane repair due to mutations in *dysferlin* gene. The patient-derived hiPSC-myogenic cells will be able to provide the opportunity for therapeutic drug screening. Abujarour et al. also established a model of patient-derived skeletal muscle cells which express NCAM, myogenin, and MyHC by doxycycline-inducible overexpression of *MyoD* in DMD patient-derived hiPSCs [53]. Interestingly, MyoD-induced iPSCs also showed suppression of pluripotent genes such as Nanog and a transient increase in the gene expression levels of *T (Brachyury T)*, *Pax3*, and *Pax7*, which belong to paraxial mesodermal/myogenic progenitor genes, upstream genes of myogenesis. It is possible that low levels of MyoD activity in hiPSCs may initially suppress their pluripotent state while failing to induce myogenic programs, which may result in transient paraxial mesodermal induction. Supporting this idea, BAF60C, a SWI/SNF component that is involved in chromatin remodeling and binds to MyoD, is required to induce full myogenic program in MyoD-overexpressing hESCs [54]. Overexpression of MyoD alone in hESC can only induce some paraxial mesodermal genes such as *Brachyury T*, *mesogenin*, and *Mesp1* but not myogenic genes. Co-overexpression of MyoD and BAF60C was now able to induce myogenic program but not paraxial mesodermal gene expression, indicating that there are different epigenetic landscapes between pluripotent ESCs/iPSCs and differentiating ESC/iPSCs in which MyoD is more accessible to DNA targets than those in pluripotent cells. The authors then argued that without specific chromatin modifiers, only committed cells give rise to myogenic cells by MyoD. These results strongly indicate that nuclear landscapes are important for cell homogeneity for the specific cell differentiation in ESC/iPSC cultures. Similar observations were seen in overexpression of *MyoD* in P19 embryonal carcinoma stem cells, which can induce paraxial mesodermal genes including Meox1, Pax3, Pax7, Six1, and Eya2 followed by muscle-specific genes. However, these MyoD-induced paraxial mesodermal genes were mediated by direct MyoD binding to their regulatory regions, which was proven by chromatin immunoprecipitation (ChIP) assays, indicating the novel role for MyoD in paraxial mesodermal cell induction [55].

hESCs/iPSCs have been differentiated into myofibers by overexpression of *MyoD*, and this method is considered an excellent in vitro model for human skeletal muscle diseases for muscle functional tests, therapeutic drug screening, and genetic corrections such as exon skipping and DNA editing. Shoji et al. have shown that DMD patient-derived iPSCs were used for myogenic differentiation via *PiggyBac*-mediated MyoD overexpression. These myogenic cells were treated with morpholinos for exon-skipping strategies for *dystrophin* gene correction and showed muscle functional improvement [56]. Li et al. have shown that patient-derived hiPSC gene correction by TALEN and CRISPR-Cas9 systems, and these genetically corrected hiPSCs were used for myogenic differentiation via overexpression of *MyoD* [57]. This work also revealed that the TALEN and CRISPR-Cas9-mediated exon 44 knock-in approach in the *dystrophin* gene has high efficiency in gene-editing methods for DMD patient-derived cells in which the exon 44 is missing in the genome.

Along this line of the strategy, Darabi et al. first performed overexpression of *Pax3* gene, which can be activated by treatment with doxycycline in mESCs, and showed efficient induction of MyoD/Myf5(+) skeletal myoblasts in EB cultures [15]. Upon removing doxycycline, these myogenic cells underwent MyHC(+) myotubes. However,

Table 1: Myogenic induction by overexpression of transgenes.

Authors	Year	Journals	Refs	Species	Cell types	Transgenes	Transgene systems	Remarks
Dekel et al.	1992	New Biol	[109]	Mouse	ESC	MyoD	Electroporation	EB culture
Rohwedel et al.	1995	Exp Cell res	[110]	Mouse	ESC	M-Twist	Transfection	EB culture
Prelle et al.	2000	Biochem Biophys Res Commun	[111]	Mouse	ESC	IGF2	Electroporation	EB culture
Myer et al.	2001	Dev Biol	[112]	Mouse	ESC	MyoD, myogenin	Electroporation	*Myogenin*$^{-/-}$ EB culture
Sumariwalla et al.	2001	Genesis	[113]	Mouse	ESC	MyoD, myogenin, MRF4	Electroporation	*Myogenin*$^{-/-}$ EB culture
Caron et al.	2005	Oncogene	[114]	Mouse	ESC	HMGA2/T	Transfection	EB culture
Kamochi	2006	Transplantation	[115]	Mouse	ESC	IGF2	Transfection	2D culture
Ozasa et al.	2007	Biochem Biophys Res Commun	[50]	Mouse	ESC	MyoD	Tet-Off system	2D culture
Darabi et al.	2008	Nat Med	[15]	Mouse	ESC	Pax3	Tet-ON system in integrated gene	EB culture, PDGFRα(+) Flk-1(−) cell sorting
Craft et al.	2008	Stem Cells	[116]	Mouse	ESC	Pax3, MyoD	Herpes simplex virus	EB culture
Warren et al.	2010	Cell Stem Cell	[51]	Human	iPSC	MyoD	mRNA transfection	EB culture
Meier-Stiegen et al.	2010	PLoS One	[117]	Mouse	ESC	Notch-IC-ERT	TMX-ERT system, electroporation	EB culture
Darabi et al.	2011	Stem Cells	[58]	Mouse	ESC	Pax7	Tet-ON system in integrated gene	EB culture, bFGF, PDGFRα(+) Flk-1(−) cell sorting
Iacovino et al.	2011	Stem Cells	[118]	Mouse, human	ESC	Myf5	Tet-ON system in integrated gene, Tet-ON-lentiviral vector	EB culture
Thoma et al.	2012	Cell Reprogram	[119]	Mouse	ESC	MyoD	TMX-induction system in transfection	EB culture
Goudenege et al.	2012	Mol Ther	[120]	Human	ESC	MyoD	Adenoviral vector	EB culture
Rao et al.	2012	Stem Cell Rev	[121]	Human	ESC	MyoD	Tet-On system in lentiviral vector	2D culture
Tedesco et al.	2012	Sci Transl Med	[80]	Human	iPSC	MyoD-ERT	TMX-ERT system, lentiviral vector	2D culture, induction of limb-girdle muscular dystrophy 2D, and DMD patient iPSC for mesoangioblast-like cells
Darabi et al.	2012	Cell Stem Cell	[59]	Human	ESC/ iPSC	Pax7	Tet-ON system in lentiviral vector	EB culture, Pax7(+) cell sorting
Tanaka et al.	2013	PLoS One	[52]	Human	iPSC	MyoD	PiggyBac transposon-Tet-ON system	2D culture, Miyoshi myopathy patient hiPSC
Albini et al.	2013	Cell Rep	[54]	Human	ESC	MyoD + Baf60c	Lentiviral vector	Myosphere culture
Abujarour et al.	2014	Stem Cells Transl Med	[53]	Human	iPSC	MyoD	Tet-Off system in lentiviral vector	2D culture, DMD patient-derived hiPSC
Yasuno et al.	2014	Biochem Biophys Res Commun	[122]	Human	iPSC	MyoD	PiggyBac transposon-Tet-ON system	EB culture, carnitine palmitoyltransferase II deficiency patient hiPSC
Albini et al.	2014	J Vis Exp	[123]	Human	ESC	MyoD + Baf60c	Lentiviral vector	Myosphere culture

Table 1: Continued.

Authors	Year	Journals	Refs	Species	Cell types	Transgenes	Transgene systems	Remarks
Li et al.	2015	Stem Cell Reports	[57]	Human	iPSC	MyoD	PiggyBac transposon-Tet-ON system	2D culture, DMD patient hiPSCs for gene correction by TALEN and CRISPR-Cas9
Maffioletti et al.	2015	Nat Protoc	[124]	Human	ESC/ iPSC	MyoD-ERT	TMX-ERT system, lentiviral vector	2D culture, induction of limb-girdle muscular dystrophy 2D, and DMD patient iPSC for mesoangioblast-like cells
Shoji et al.	2015	Sci Rep	[56]	Human	iPSC	MyoD	PiggyBac transposon-Tet-ON system	2D culture, DMD patient hiPSCs for exon skipping
Dixon et al.	2016	Proc Natl Acad Sci U S A.	[125]	Human	ESC	MyoD	GAG-binding motif for cell penetrating peptide	2D culture
Shoji et al.	2016	Methods Mol Biol	[126]	Human	iPSC	MyoD	PiggyBac transposon-Tet-ON system	2D culture
Akiyama et al.	2016	Development	[127]	Human	iPSC	MyoD + JMJD3	PiggyBac transposon-Tet-ON system	2D culture
Magli et al.	2016	Methods Mol Biol	[128]	Mouse	ESC	Pax3	Tet-ON system in integrated gene	EB culture, PDGFRα(+) Flk-1(−) cell sorting

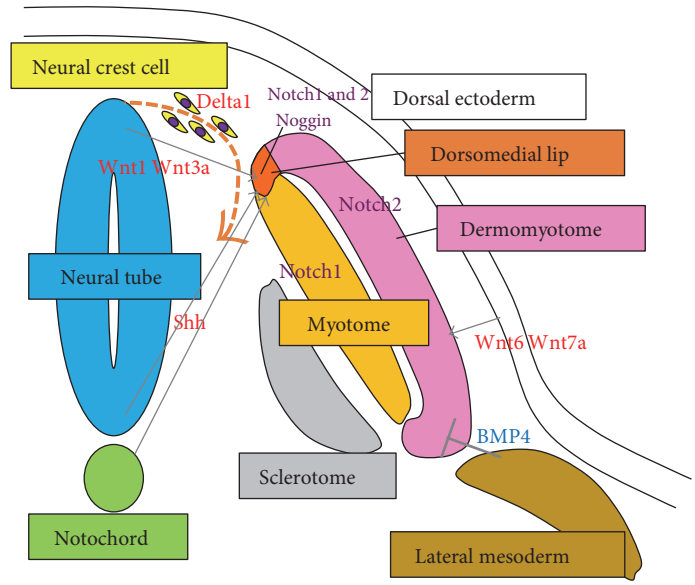

FIGURE 2: Positive and negative signals from surrounding tissues for embryonic myogenesis. Dermomyotome receives positive (Shh, Wnt1, Wnt3a, Wnt6, Wnt7a, Delta1, and Noggin) and negative (BMP4) signals from surrounding tissues (dorsal neutral tube, floor plate, notochord, dorsal ectoderm, and lateral mesoderm) to form myotomes. This occurs at the Notch1/2-positive dorsomedial lip of dermomyotome.

teratoma formation was observed after EB cell transplantation into cardiotoxin-injured regenerating skeletal muscle in *Rag2*$^{-/-}$:*γC*$^{-/-}$ immunodeficient mice [15]. This indicates that myogenic cell cultures induced by *Pax3* in mESCs still contain some undifferentiated cells which gave rise to teratomas. To overcome this problem, the same authors separated paraxial mesodermal cells from Pax3-induced EB cells by FACS using antibodies against cell surface markers as PDGFR*α*(+)Flk-1(−) cell populations. After cell sorting, isolated Pax3-induced paraxial mesodermal cells were successfully engrafted and contributed to regenerating muscle in *mdx:Rag2*$^{-/-}$:*γC*$^{-/-}$ DMD model immunodeficient mice without any teratoma formations. Darabi et al. also showed successful myogenic induction in mESCs and hES/iPSCs by overexpression of Pax7 [58, 59]. *Pax3* and *Pax7* are not only expressed in myogenic progenitor cells. They are also expressed in neural tube and neural crest cell-derived cells including a part of cardiac cell types in developmental stage, suggesting that further purification to skeletal muscle cell lineage is crucial for therapeutic applications for muscle diseases including DMD.

Taken together, overexpression of myogenic master transcription factors such as MyoD or Pax3/Pax7 is an excellent strategy for myogenic induction in hESCs and hiPSCs, which can be utilized for in vitro muscle disease models for their functional test and drug screening. However, for the safe stem cell therapy, it is essential to maintain the good cellular and genetic qualities of hESC/hiPSC-derived myogenic cells before transplantation. Therefore, random integration sites of overexpression vectors for myogenic master transcription factors and inappropriate expression control of these transgenes may diminish the safety of using these induced myogenic cells for therapeutic stem cell transplantation.

4. Supplement with Defined Factors for Myogenic Induction in ESCs/iPSCs

Stepwise induction protocols utilizing small molecules and growth factors have been established as alternative myogenic induction approaches and a more applicable method for therapeutic situations. As described above, during embryonic myogenesis, somites and dermomyotomes receive secreted signals such as Wnts, Notch ligands, Shh, FGF, BMP, and retinoic acid (RA) with morphogen gradients from surrounding tissues in order to induce the formation of myogenic cells (Figure 2). The canonical Wnt signaling pathway has been shown to play essential roles in the development of myogenesis. In mouse embryogenesis, Wnt1 and Wnt3a secreted from the dorsal neural tube can promote myogenic differentiation of dorsomedial dermomyotome via activation of Myf5 [31, 32, 60]. Wnt3a is able to stabilize *β*-catenin which associates with TCF/LEF transcription factors that bind to the enhancer region of Myf5 during myogenesis [61]. Other Wnt proteins, Wnt6 and Wnt7a, which emerge from the surface ectoderm, induce MyoD [62]. BMP functions as an inhibitor of myogenesis by suppression of some myogenic gene expressions. In the lateral mesoderm, BMP4 is able to increase Pax3 expression which delays Myf5 expression in order to maintain an undifferentiated myogenic progenitor state [63]. Therefore, Wnts and BMPs regulate myogenic development by antagonizing each other for myogenic transcription factor gene expression [64, 65]. Wnt also induces Noggin expression to antagonize BMP signals in the dorsomedial lip of the dermomyotome [66]. In this region, MyoD expression level is increased, which causes myotome formation. Notch signaling plays essential roles for cell-cell communication to specify the different cells in developmental stages. During myotome formation, Notch is expressed

Table 2: Myogenic induction without transgenes.

Authors	Year	Refs	Journals	Species	Cell types	Factors	Remarks
Zhuang et al.	1992	[129]	Proc Natl Acad Sci U S A	Mouse	ESC		$E2A^{-/-}$ EB culture
Dinsmore et al.	1996	[130]	Cell Transplant	Mouse	ESC	RA, DMSO	EB culture of androgenetic and parthenogenetic ESC
Rohwedel et al.	1998	[131]	Exp Cell Res	Mouse	ESC	LiCl	EB culture
Barberi et al.	2005	[82]	PLoS Med	Human	ESC	OP9 and C2C12 coculture	CD73(+) MSC sorting
Barberi et al.	2007	[83]	Nat Med	Human	ESC	OP9 coculture, insulin	CD73(+) MSC sorting, NCAM(+) cell sorting
Sakurai et al.	2008	[85]	Stem Cells	Mouse	ESC		2D culture, PDGFRα(+) Flk-1(−) cell sorting
Sasaki et al.	2008	[132]	Differentiation	Mouse	ESC	Spermine	EB culture
Chang et al.	2009	[86]	FASEB J	Mouse	ESC		EB culture, SM/C-2.6(+) cell sorting
Sakurai et al.	2009	[68]	Stem Cell Res	Mouse	ESC	LiCl, BMP4	2D culture, PDGFRα(+) E-cadherin(low) cell sorting
Mizuno et al.	2010	[87]	FASEB J	Mouse	iPSC		EB culture, SM/C-2.6(+) cell sorting
Teng et al.	2010	[133]	J Cell Biochem	Human	ESC	TGFβ inhibitor	$GNE^{-/-}$ EB culture
Awaya et al.	2012	[134]	PLoS One	Human	ESC/iPSC		EB culture
Sakurai et al.	2012	[135]	PLoS One	Mouse, human	ESC	LiCl, BMP4, Activin A	2D culture
Kuraitis et al.	2012	[136]	Eur Cell Mater	Mouse	ESC	sLeX-collagen matrices	EB culture
Xu et al.	2013	[70]	Cell	Human	iPSC	GSK3β inhibitor, bFGF, forskolin	EB culture
Leung et al.	2013	[137]	Biomacromolecules	Human	ESC	Chitosan-polycaprolactone (C-PCL) nanofibers + Wnt3a	2D culture
Borchin et al.	2013	[72]	Stem Cell Reports	Human	iPSC	GSK3β inhibitor	2D culture, c-met(+) cell sorting
Hosoyama et al.	2013	[75]	Stem Cells Transl Med	Human	ESC/iPSC	bFGF, EGF	EZ sphere culture
Hwang et al.	2014	[69]	Sci Rep	Mouse	ESC	Wnt3a	2D culture, PDGFRα(+) cell sorting
Shelton et al.	2014	[73]	Cell Reports	Mouse, human	ESC/iPSC	GSK3β inhibitor, BMP, VEGF, Inhibinβ, bFGF	EB culture
Chal et al.	2015	[76]	Nat Biotechnol	Mouse, human	ESC/iPSC	GSK3β inhibitor, BMP inhibitor	2D culture
Chal et al.	2016	[77]	Nat Protoc	Human	iPSC	GSK3β inhibitor, BMP inhibitor, bFGF, HGF, IGF1	2D culture
Caron et al.	2016	[74]	Stem Cell Transl Med	Human	ESC	GSK3β inhibitor, Ascorbic acid, Alk5 inhibitor, Dex, EGF, insulin	2D culture

in dermomyotome, and Notch1 and Notch2 are expressed in dorsomedial lip of dermomyotome. Delta1, a Notch ligand, is expressed in neural crest cells which transiently interact with myogenic progenitor cells in dorsomedial lip of dermomyotome via Notch1 and 2. This contact induces expression of the Myf5 or MyoD gene in the myogenic progenitor cells followed by myotome formation. The loss of function of Delta1 in the neural crest displays delaying skeletal muscle formation [67]. Knockdown of Notch genes or use of a dominant-negative form of mastermind, a Notch transcriptional coactivator, clearly shows dramatically decrease of Myf5 and MyHC(+) myogenic cells. Interestingly, induction of Notch intracellular domain (NICD), a constitutive active form of Notch, can promote myogenesis, while continuous expression of NICD prevents terminal differentiation. Taken together, transient and timely activation of Notch is crucial for myotome formation from dermomyotome [30].

Current studies for myogenic differentiation of ESCs/iPSCs have utilized supplementation with some growth factors and small molecules, which would mimic the myogenic development described above in combination with embryoid body (EB) aggregation and FACS separation of mesodermal cells (Table 2). To induce paraxial mesoderm cells from mESCs, Sakurai et al. utilized BMP4 in serum-free cultures [68]. Three days after treatment with BMP4, mESCs could be differentiated into primitive streak mesodermal-like cells,

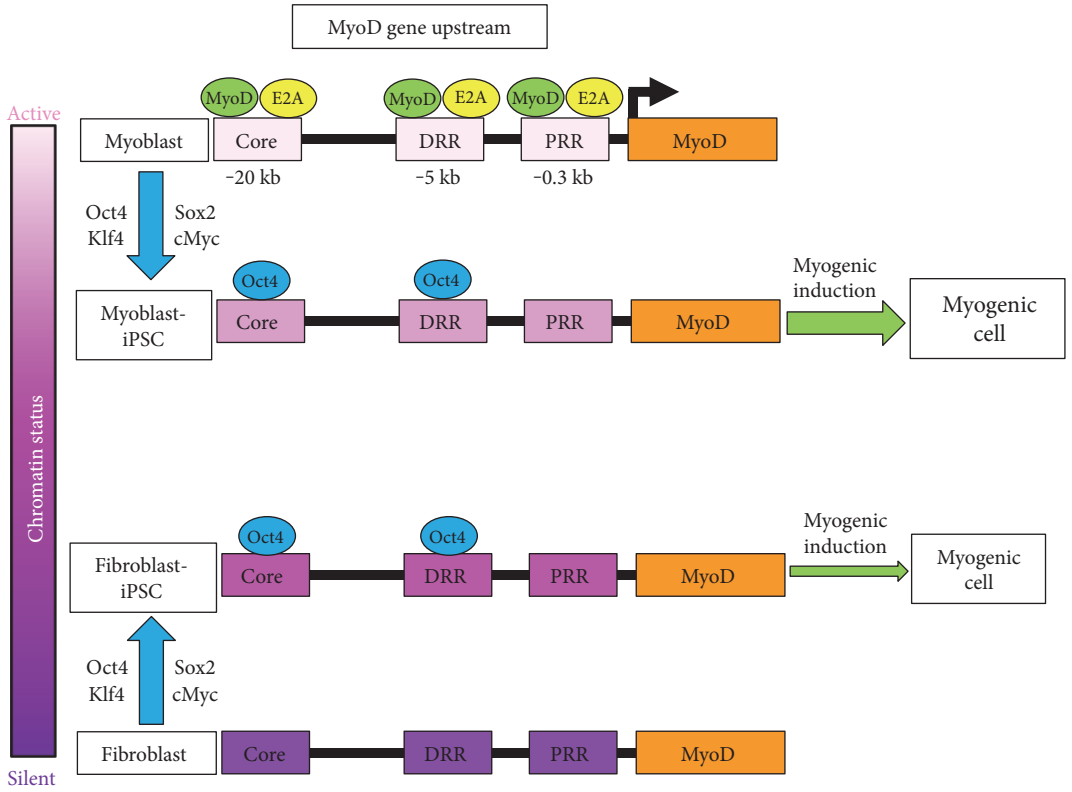

Figure 3: Schematic model for chromatin status of myoblast versus fibroblast-derived iPSCs for myogenic induction. In myoblasts, MyoD binds to the two MyoD enhancers (core and DRR) and promoter (PRR), and histone marks show the open chromatin state characteristic. During iPSC reprogramming via expression of Oct4, Sox3, Klf4, and cMyc, exogenous Oct4 binds to both MyoD enhancers which may lead to the bivalent state characteristic of pluripotent stem cells. In fibroblast, both MyoD enhancers and promoter show the closed chromatin state characteristic. During iPSC reprogramming, exogenous Oct4 binds to both MyoD enhancers which may lead to the bivalent state characteristic of pluripotent stem cells. However, myoblast-derived iPSCs may maintain the more open bivalent state characteristic, and thus, myogenic conversion efficiency is increased upon induction.

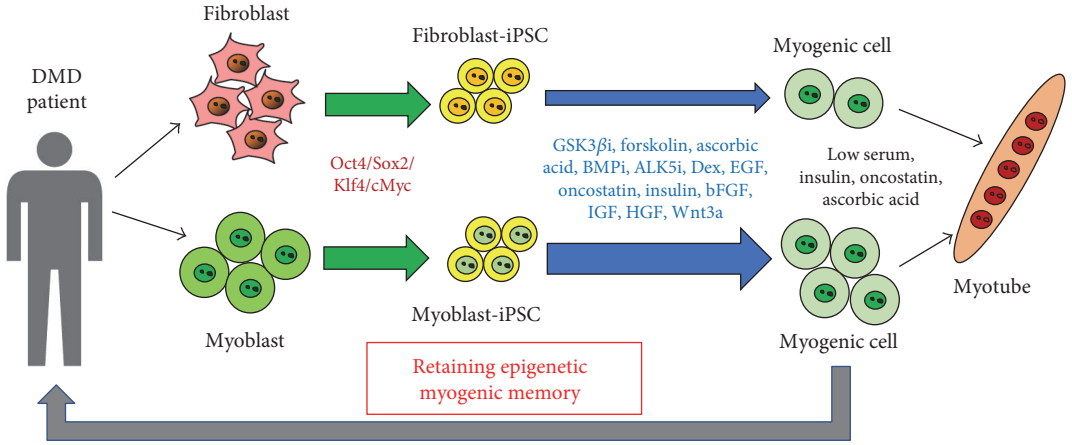

Figure 4: Myogenic cells induced from myoblast-derived iPSCs for DMD therapy. DMD patient-derived fibroblasts or myoblasts will be reprogramed into iPSCs by reprogramming factors (Oct4, Sox3, Klf4, and cMyc). These fibroblast- and myoblast-derived iPSCs will be induced to myogenic cells via combinatory small molecules and factors such as GSK3βi, forskolin, ascorbic acid, BMPi, ALK5i, Dex, EGF, oncostatin, insulin, bFGF, IGF, HGF, and Wnt3a. These iPSC-derived myogenic cells will be used for autologous cell therapy. Myoblast-derived iPSCs maintain epigenetic myogenic memory.

but the continuous treatment with BMP4 turned the ESCs into osteogenic cells. Therefore, they used LiCl after treatment with BMP4 to enhance Wnt signaling, which is able to induce myogenic differentiation. After treatment with LiCl, PDGFRα(+) E-cadherin(−) paraxial mesodermal cells were sorted by FACS. These sorted cells were cultured with

IGF, HGF, and FGF for two weeks in order to induce myogenic differentiation. Hwang et al. have shown that treatment with Wnt3a efficiently promotes skeletal muscle differentiation of hESCs [69]. hESCs were cultured to form EB for 9 days followed by differentiation of EBs for additional 7 days, and then PDGFRα(+) cells were sorted by FACS. These PDGFRα(+) cells were cultured with Wnt3a for additional 14 days. Consequently, these Wnt3a-treated cells display significantly increased myogenic transcription factors and structural proteins at both mRNA and protein levels. An interesting approach to identify key molecules that induce myogenic cells was reported by Xu et al. [70]. They utilized reporter systems in zebrafish embryos to display myogenic progenitor cell induction and myogenic differentiation in order to identify small compounds for myogenic induction. *Myf5-GFP* marks myogenic progenitor cells, while *myosin light polypeptide 2* (*mylz2*)-*mCherry* marks terminally differentiated muscle cells. They found that a mixed cocktail containing GSK3β inhibitor, bFGF, and forskolin has the potential to induce robust myogenic induction in hiPSCs. GSK3β inhibitors act as a canonical Wnt signaling activator via stabilizing β-catenin protein, which is crucial for inducing mesodermal cells. Forskolin activates adenylyl cyclase, which then stimulates cAMP signaling. cAMP response element-binding protein (CREB) is able to stimulate cell proliferation of primary myoblasts in vitro, suggesting that the forskolin-cAMP-CREB pathway may help myogenic cell expansion [71], However the precise mechanisms for CREB-mediated myogenic cell expansion remain unclear. The adenylyl cyclase signaling cascade leads to CREB activation [71]. During embryogenesis, phosphorylated CREB has been found at dorsal somite and dermomyotome. CREB gene knockout mice display significantly decreased Myf5 and MyoD expressions in myotomes. While activation of Wnt1 or Wnt7a promotes Pax3, Myf5, and MyoD expressions, inhibition of CREB eliminates these Wnt-mediated myogenic gene expressions without altering the Wnt canonical pathway, suggesting that CREB-induced myogenic activation may be mediated through noncanonical Wnt pathways. Several groups also utilized GSK3β inhibitors for inducing mesodermal cells from ESCs and iPSCs [72, 73]. These mesodermal cell-like cells were expanded by treatment with bFGF, and then ITS (insulin/transferrin/selenite) or N2 medium were used to induce myogenic differentiation. Finally, bFGF is a stimulator for myogenic cell proliferation. Caron et al. demonstrated that hESCs treated with GSK3β inhibitor, ascorbic acid, Alk5 inhibitor, dexamethasone, EGF, and insulin generated around 80% of Pax3(+) myogenic precursor cells in 10 days [74]. Treatment with SB431542, an inhibitor of Alk4, 5, and 7, PDGF, bFGF, oncostatin, and IGF was able to induce these Pax3(+) myogenic precursor cells into around 50–60% of MyoD(+) myoblasts in an additional 8 days. For the final step, treatment with insulin, necrosulfonamide, an inhibitor of necrosis, oncostatin, and ascorbic acid was able to induce these myoblasts into myotubes in an additional 8 days. Importantly, the same authors utilized ESCs from human facioscapulohumeral muscular dystrophy (FSHD) to demonstrate the myogenic characterization after myogenic induction by using the protocol described above. Hosoyama et al. have shown that hESCs/iPSCs with high concentrations of bFGF and EGF in combination with cell aggregation, termed EZ spheres, efficiently give rise to myogenic cells [75]. After 6-week culture, around 40–50% of cells expressed Pax7, MyoD, or myogenin. However, the authors also showed that EZ spheres included around 30% of Tuj1(+) neural cells. Therefore, the authors discussed the utilization of molecules for activation of mesodermal and myogenic signaling pathways such as BMPs and Wnts.

Taken together, it is likely that the induced cell populations from ESCs/iPSCs may contain other cell types such as neural cells or cardiac cells because neural cells share similar transcription factor gene expression with myogenic cells such as Pax3, and cardiac cells also develop from mesodermal cells. To overcome this limitation, Chal et al. treated ESCs/iPSCs with BMP4 inhibitor, which prevents ESCs/iPSCs from differentiating into lateral mesodermal cells [76, 77]. To identify what genes are involved in myogenic differentiation in vivo, they performed a microarray analysis which compared samples of dissected fragments in mouse embryos, which are able to separate tail bud, presomitic mesoderm, and somite regions. From microarray data, the authors focused on *Mesogenin1* (*Msgn1*) and *Pax3* genes. Importantly, they utilized three lineage tracing reporters, *Msgn1-repV* (*Mesogenin1-Venus*) marking posterior somitic mesoderm, *Pax3-GFP* marking anterior somitic mesoderm and myogenic cells, and *Myog-repV* (*Myogenin-Venus*) marking differentiated myocytes, allowing the authors to readily detect different differentiation stages during ESC/iPSC cultures. Treatment with GSK3β inhibitors and then BMP inhibitors in ESC cultures induced Msgn1(+) somitic mesoderm with 45 to 65% efficiencies, Pax3(+) anterior somitic mesoderm with 30 to 50% efficiencies, and myogenin(+) myogenic cells with 25 to 30% efficiencies. Furthermore, the authors examined differentiation of *mdx* ESCs into skeletal muscle cells and revealed abnormal branching myofibers. Current protocols were also published and described more details for hiPSC differentiation [77].

5. Induction of Skeletal Muscle Cells from iPSC-Derived Mesoangioblast-Like Cells

Some nonmuscle cell populations such as mesoangioblasts have the potential to differentiate into skeletal muscle [6]. Mesoangioblasts were originally isolated from embryonic mouse dorsal aorta as vessel-associated pericyte-like cells, which have the ability to differentiate into a myogenic lineage in vitro and in vivo [6, 78]. Mesoangioblasts possess an advantage for the clinical cell-based treatment because they can be injected through an intra-arterial route to systemically deliver cells, which is crucial for therapeutic cell transplantation for muscular dystrophies [79]. Tedesco et al. successfully generated human iPSC-derived mesoangioblast-like stem/progenitor cells called HIDEMs by stepwise protocols without FACS sorting [80, 81]. They displayed similar gene expression profiles as embryonic mesoangioblasts. However, HIDEMs do not spontaneously differentiate into skeletal muscle cells, and thus, the authors utilized overexpression

of *MyoD* to differentiate into skeletal muscle cells. Similar to mesoangioblasts, HIDEM-derived myogenic cells could be delivered to injured muscle via intramuscular and intra-arterial routes. Furthermore, HIDEMs have been generated from hiPSCs derived from limb-girdle muscular dystrophy (LGMD) type 2D patients and used for gene correction and cell transplantation experiments for the potential therapeutic application.

6. Enrichment of ESC/iPSC-Derived Myogenic Precursor Cells

Myogenic precursor cells derived from ESCs/iPSCs by various methods may contain nonmuscle cells. Therefore, further purification is mandatory for therapeutic applications. Barberi et al. isolated CD73(+) multipotent mesenchymal precursor cells from hESCs by FACS, and these cells underwent differentiation into fat, cartilage, bone, and skeletal muscle cells [82]. Barberi et al. also demonstrated that hESCs cultured on OP9 stroma cells generated around 5% of CD73(+) adult mesenchymal stem cell-like cells [83]. After FACS, these CD73(+) mesenchymal stem cell-like cells were cultured with ITS medium for 4 weeks and then gave rise to NCAM(+) myogenic cells. After FACS sorting, these NCAM(+) myogenic cells were purified by FACS and transplanted into immunodeficient mice to show their myogenic contribution to regenerating muscle.

It has been shown that many genes are associated with myogenesis. In addition, exhaustive analysis, such as microarray, RNA-seq, and single cell RNA-seq supplies much gene information in many different stages. Chal et al. showed key signaling factors by microarray from presomitic somite, somite, and tail bud cells [76]. They found that initial Wnt signaling has important roles for somite differentiation. Furthermore, mapping differentiated hESCs by single cell RNA-seq analysis is useful to characterize each differentiated stage [84].

As shown above, cell sorting of mesodermal progenitor cells, mesenchymal precursor cells, or myogenic cells is a powerful tool to obtain pure myogenic populations from differentiated pluripotent cells. Sakurai et al. have been able to induce PDGFRα(+)Flk-1(−) mesodermal progenitor cells by FACS followed by myogenic differentiation [85]. Chang et al. and Mizuno et al. have been able to sort SMC-2.6(+) myogenic cells from mouse ESCs/iPSCs [86, 87]. These SMC-2.6(+) myogenic cells were successfully engrafted into mouse regenerating skeletal muscle. However, this SMC-2.6 antibody only recognizes mouse myogenic cells but not human myogenic cells [86, 88]. Therefore, Borchin et al. have shown that hiPSC-derived myogenic cells differentiated into c-met(+)CXCR4(+)ACHR(+) cells, displaying that over 95% of sorted cells are Pax7(+) myogenic cells [72]. Taken together, current myogenic induction protocols utilizing small molecules and growth factors, with or without myogenic transcription factors, have been largely improved in the last 5 years. It is crucial to standardize the induction protocols in the near future to obtain sufficient myogenic cell conversion from pluripotent stem cells.

7. Epigenetic Myogenic Memory in Myoblast-Derived iPSCs

Recent work demonstrated that cells inherit a stable genetic program partly through various epigenetic marks, such as DNA methylation and histone modifications. This cellular memory needs to be erased during genetic reprogramming, and the cellular program reverted to that of an earlier developmental stage [16, 22, 89]. However, iPSCs retaining an epigenetic memory of their origin can readily differentiate into their original tissues [16–19, 90–100]. This phenomenon becomes a double-edged sword for the reprogramming process since the retention of epigenetic memory may reduce the quality of pluripotency while increasing the differentiation efficiency into their original tissues. DNA methylation levels are relatively low in the pluripotent stem cells compared to the high levels of DNA methylation seen in somatic cells [101]. Global DNA demethylation is required for the reprogramming process [102]. In the context of these observations, recent work demonstrates that activation-induced cytidine deaminase AID/AICDA contributing to the DNA demethylation can stabilize stem-cell phenotypes by removing epigenetic memory of pluripotent genes. This directly deaminates 5-methylcytosine in concert with base-excision repair to exchange cytosine in genomic DNA [103]. MicroRNA-155 has been identified as a key player for the retention of epigenetic memory during in vitro differentiation of hematopoietic progenitor cell-derived iPSCs toward hematopoietic progenitors [104]. iPSCs that maintained high levels of miR-155 expression tend to differentiate into the original somatic population more efficiently.

Recently, we generated murine skeletal muscle cell-derived iPSCs (myoblast-derived iPSCs) [23] and compared the efficiency of differentiation of myogenic progenitor cells between myoblast-derived iPSCs and fibroblast-derived iPSCs. After EB cultures, more satellite cell/myogenic progenitor cell differentiation occurred in myoblast-derived iPSCs than that in fibroblast-derived-iPSCs (unpublished observation and Figure 3), suggesting that myoblast-derived iPSCs are potential myogenic and satellite cell sources for DMD and other muscular dystrophy therapies (Figure 4). We also noticed that *MyoD* gene suppression by Oct4 is required for reprogramming in myoblasts to produce iPSCs (Figure 3) [23]. During overexpression of Oct4, Oct4 first binds to the Oct4 consensus sequence located in two MyoD enhancers (a core enhancer and distal regulatory region) [105–107] preceding occupancy at the promoter in myoblasts in order to suppress MyoD gene expression. Interestingly, Oct4 binding to the MyoD core enhancer allows for establishment of a bivalent state in MyoD promoter as a poised state, marked by active (H3K4me3) and repressive (H3K27me3) modifications in fibroblasts, one of the characteristics of stem cells (Figure 3) [23, 108]. It should be investigated whether the similar bivalent state is also established in Oct4-expressing myoblasts during reprogramming process from myoblasts to pluripotent stem cells. It remains to be elucidated whether Oct4-mediated myogenic repression only relies on repression of MyoD expression or is just a

general phenomenon of functional antagonism between Oct4 and MyoD on activation of muscle genes. Nevertheless, myoblast-derived iPSCs will enable us to produce an unlimited number of myogenic cells, including satellite cells that could form the basis of novel treatments for DMD and other muscular dystrophies (Figure 4).

8. Conclusions

There are pros and cons of transgene-free small molecule-mediated myogenic induction protocols. In the transgene-mediated induction protocols, integration of the transgene in the host genome may lead to risk for insertional mutagenesis. To circumvent this issue, there is an obvious advantage for transgene-free induction protocols. Some key molecules such as Wnt, FGF, and BMP have used signaling pathways to induce myogenic differentiation of ES/iPSCs. However, these molecules are also involved in induction of other types of cell lineages, which makes it difficult for ES/iPSCs to induce pure myogenic cell populations in vitro. By contrast, transgene-mediated myogenic induction is able to dictate desired specific cell lineages. In any case, it is necessary to intensively investigate these myogenic induction protocols for the efficient and safe stem cell therapy for patients.

For skeletal muscle diseases, patient-derived hiPSCs, which possess the ability to differentiate into myogenic progenitor cells followed by myotubes, can be a useful tool for drug screening and personalized medicine in clinical practice. However, there are still limitations for utilizing hiPSC-derived myogenic cells for regenerative medicine. For cell-based transplantation therapies such as a clinical situation, animal-free defined medium is essential for stem cell culture and skeletal muscle cell differentiation. Therefore, such animal-free defined medium needs to be established for optimal myogenic differentiation from hiPSCs. Gene correction in DMD patient iPSCs by TALENs and CRISPR-Cas9 systems are promising therapeutic approaches for stem cell transplantation. However, there are still problems for DNA-editing-mediated stem cell therapy such as safety and efficacy. Since iPSC-derived differentiated myotubes do not proliferate, they are not suited for cell transplantation. Therefore, a proper culture method needs to be established for hiPSCs in order to maintain cells in proliferating the myogenic precursor cell stage in vitro in order to expand cells to large quantities of transplantable cells for DMD and other muscular dystrophies. For other issues, it is essential to establish methods to separate ES/iPSC-derived pure skeletal muscle precursor cells from other cell types for safe stem cell therapy that excludes tumorigenic risks of contamination with undifferentiated cells. In the near future, these obstacles will be taken away for more efficient and safe stem cell therapy for DMD and other muscular dystrophies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Induction of Pluripotent Stem Cells from a Manifesting Carrier of Duchenne Muscular Dystrophy and Characterization of Their X-Inactivation Status

Yuko Miyagoe-Suzuki,[1] Takashi Nishiyama,[1] Miho Nakamura,[1] Asako Narita,[1] Fusako Takemura,[1] Satoru Masuda,[1] Narihiro Minami,[2] Kumiko Murayama,[2] Hirofumi Komaki,[3] Yu-ichi Goto,[4] and Shin'ichi Takeda[1]

[1]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[2]*Department of Laboratory Medicine, National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan*
[3]*Department of Child Neurology, National Center Hospital, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8551, Japan*
[4]*Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*

Correspondence should be addressed to Yuko Miyagoe-Suzuki; miyagoe@ncnp.go.jp

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Three to eight percent of female carriers of Duchenne muscular dystrophy (DMD) develop dystrophic symptoms ranging from mild muscle weakness to a rapidly progressive DMD-like muscular dystrophy due to skewed inactivation of X chromosomes during early development. Here, we generated human induced pluripotent stem cells (hiPSCs) from a manifesting female carrier using retroviral or Sendai viral (SeV) vectors and determined their X-inactivation status. Although manifesting carrier-derived iPS cells showed normal expression of human embryonic stem cell markers and formed well-differentiated teratomas in vivo, many hiPS clones showed bi-allelic expression of the androgen receptor (AR) gene and loss of X-inactivation-specific transcript and trimethyl-histone H3 (Lys27) signals on X chromosomes, suggesting that both X chromosomes of the hiPS cells are in an active state. Importantly, normal dystrophin was expressed in multinucleated myotubes differentiated from a manifesting carrier of DMD-hiPS cells with XaXa pattern. AR transcripts were also equally transcribed from both alleles in induced myotubes. Our results indicated that the inactivated X chromosome in the patient's fibroblasts was activated during reprogramming, and XCI occurred randomly during differentiation.

1. Introduction

X-linked Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene, which encodes the dystrophin protein required for stability of the sarcolemma. Most female carriers of *DMD* mutations are asymptomatic, but 3–8% of female carriers develop symptoms ranging from a DMD-like progression to a very mild Becker muscular dystrophy-like phenotype [1] due to skewed inactivation of X chromosomes in early development [2–4].

Human induced pluripotent (iPS) cells are embryonic stem- (ES-) like pluripotent cells derived from somatic cells by ectopic expression of a defined set of reprogramming factors [5, 6]. Patient-derived iPS cells are expected to be useful for disease modeling, but the effects of reprogramming by Yamanaka factors on X-inactivation in female iPS cells

remain controversial. A previous study showed that human iPS cells exhibit a nonrandom X chromosome inactivation (XCI) pattern because they reflect the XCI status of the single fibroblast from which they were derived [7]. Other groups reported two active X chromosomes in iPS cells derived from a patient with Rett syndrome [8]. For disease modeling of a manifesting carrier of DMD in vitro, the correct understanding of the XCI status of female iPS cells and hiPS-derived skeletal muscle is needed.

Here, we established iPS cells from one female DMD-manifesting carrier and one female DMD carrier with three X chromosomes and high serum creatine kinase (CK) levels by using an all-in-one retroviral vector or Sendai viral (SeV) vector and examined their X-inactivation status. Many hiPS clones showed a loss of X-inactivation-specific transcript (XIST) RNA and loss of biased methylation in exon 1 and bi-allelic expression of the *androgen receptor* (AR) gene. Interestingly, skeletal muscle cells differentiated from manifesting carrier of DMD-derived hiPSCs with XaXa patterns expressed dystrophin. Our results suggest that the inactivated X chromosome in the female manifesting carrier of DMD was activated during reprogramming, and XCI occurred randomly on differentiation.

2. Materials and Methods

2.1. Patient Fibroblasts. Patient 609 (41 years old) is a manifesting carrier of Duchenne muscular dystrophy. Dystrophin staining of muscle sections showed a mosaic pattern. Western blotting showed that the dystrophin protein level was 10% of the normal. Multiplex PCR revealed deletion of dystrophin exons 42-43 of the DMD gene (frame-shift mutation). Patient 386 is a 5-year-old girl with XXX trisomy. MLPA analysis revealed deletion of exons 13–44 in one X. The patient shows high levels of serum CK but no obvious muscle weakness. Patient 401 (1y2m, male) has duplication of exons 45–50 of the DMD gene. The generation and analysis of iPS cell lines and deposition of these cell lines in a public cell bank (RIKEN Cell Bank) were approved by the patients or their parents using consent forms and approved by NCNP Ethics Committees. Samples were anonymized upon leaving the clinic.

2.2. Reprogramming by Yamanaka Factors

2.2.1. Retroviral Vectors. Fibroblasts from patient 609 were infected with the human iPS cell generation all-in-one retroviral vector pDON-5 OKSNL (Takara Bio, Japan), encoding all five reprogramming factors (OCT4, KLF4, SOX2, LIN28, and NANOG), and then replated on STO cells. Virus particles were prepared using a retrovirus packaging kit Amph0 (Takara Bio) and a G3T-hi packaging cell line (Takara Bio). Human ES cell-like colonies were picked up at day 29 (Figure 1). Reprogramming efficiency (ALP + colonies/starting cell numbers) was 0.19%. Finally, five clones were selected based on their morphology and growth rates. STR analysis was performed to confirm that these iPS clones were derived from patient 609's fibroblasts. 401-8 iPS cells were obtained from 401 fibroblasts using the same protocol with 609 iPS clones.

2.2.2. Sendai Viral Vectors. To reprogram fibroblasts of patients 609 and 386, we used CytoTuneTM-iPS (DNAVEC, Tsukuba, Japan). Vector cocktails (*OCT3/4-SeV/TSΔF*, *SOX2-SeV/TSΔF*, *KLF4-SeV/TSΔF*, and *c-MYC(HNL)-SeV/TS15ΔF*) were used at an MOI of 3, according to the manufacturer's protocol. Human ES cell-like colonies were grown on SNL feeder cells, and picked up on days 14–28. RT-PCR for transgenes and the SeV genome were performed using the following primers: for SeV, forward: GGA TCA CTA GGT GAT ATC GAG C and reverse: ACC AGA CAA GAG TTT AAG AGA TAT GTA TC (181 bp); for c-MYC, forward: TAA CTG ACT AGC AGG CTT GTC G and reverse: TCC ACA TAC AGT CCT GGA TGA TGA TG (532 bp); for KLF4, forward: ACA AGA GAA AAA ACA TGT ATG G and reverse: CGC GCT GGC AGG GCC GCT GCT CGA C (529 bp); for SOX2, forward: ACA AGA GAA AAA ACA TGT ATG G and reverse: ATG CGC TGG TTC ACG CCC GCG CCC AGG (591 bp); for OCT3/4, forward: CCC GAA AGA GAA AGC GAA CCA G and reverse: AAT GTA TCG AAG GTG CTC AA (483 bp) (Integrated DNA Technologies, Inc.). Sendai iPS cells were immunostained with anti-Sendai virus polyclonal antibodies (MBL).

2.3. hiPS Cell Culture. hiPS cells were maintained on mitomycin C- (MMC-) treated SNL feeder cells (CiRA), MMC-treated mouse embryonic fibroblasts (MEFs), or SL10 feeder cells (ReproCELL) in a primate ES cell medium (ReproCELL) supplemented with bFGF (4 ng/ml). Human iPS cells, 201B7 [5], 253G1 [9], and 409B2 [10], were provided by S. Yamanaka of Kyoto University and used as control iPS cell lines.

2.4. Immunocytochemistry. Cells were fixed with 4% paraformaldehyde for 5 min, permeabilized with 0.1% Triton-X in PBS for 10 min, blocked with 5% goat (Cedarlane) or horse (Invitrogen) serum in 2% BSA for 15 min, and then incubated with anti-TRA-1-60 mouse monoclonal antibody (Millipore), anti-TRA-1-81 mouse monoclonal antibody (Millipore), anti-SSEA3 rat monoclonal antibody (R&D), anti-NANOG polyclonal antibody (R&D), anti-SOX2 antibody (6F1.2) (Millipore), anti-OCT4 mouse monoclonal antibody (C-10) (Santa Cruz Biotechnology), or anti-trimethyl-histone H3 (Lys27) (polyclonal, Millipore). The cells were then incubated with secondary antibodies labeled with Alexa-Fluor 488 or 568 (Molecular Probes) and DAPI. Images were photographed using a fluorescence microscope IX71 (Olympus, Tokyo, Japan) equipped with an Orca2 air-cooled CCD camera (Hamamatsu Photonics) and AQUACOSMOS software (Hamamatsu Photonics).

2.5. Fluorescence-Activated Cell Sorting (FACS). Cells were resuspended at a concentration of 1.0×10^6 cells/100 μl in PBS containing 2% FBS and incubated with anti-SSEA4 (MC-813-70, Millipore), anti-TRA-1-60 (Millipore), or anti-TRA-1-81 (Millipore) antibodies on ice for 30 min, then incubated with Alexa 488-labeled goat anti-mouse IgM (Invitrogen) or Alexa 488-labeled rabbit anti-mouse

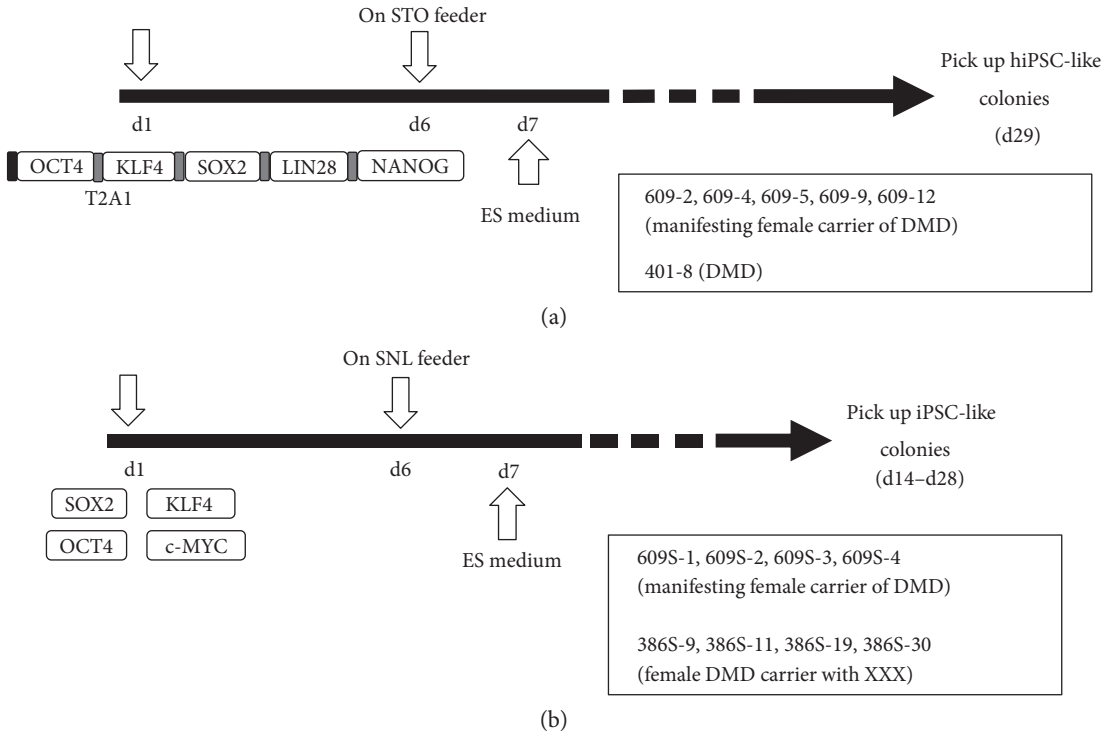

Figure 1: Time schedules of hiPS induction using an all-in-one retroviral vector (a) or four Sendai viral vectors (b). Fibroblasts from a manifesting female carrier of DMD (609) were infected with a retroviral vector, encoding five reprogramming factors. After replating onto MMC-treated STO feeder cells, colonies were picked up (a). Next, fibroblasts of 609 and a female DMD patient (386) were infected with four Sendai viral vectors on SNL feeders, and ES-like colonies were picked up 14–21 days after transduction (b).

IgG (Invitrogen). After extensive washing, cells were analyzed on an FACSAria flow cytometer (BD Bioscience).

2.6. RT-PCR and RT-PCR Array. Total RNA was extracted from cells using a MicroRNeasy kit (Qiagen). Complementary DNA (cDNA) was synthesized using a QuantiTect reverse transcription kit (Qiagen). For expression of DMD, four PCR primers were used: ex42F, AAT CAC TCA TGT CTC ACA AGC CCT A; ex43R, TCC GAC CTG AGC TTT GTT GTA; ex44F, TCC TGA GAA TTG GGA ACA TGC TA; and ex45R, CCA GTT GCA TTC AAT GTT CTG AC. For expression of AR, AR-F (TCCAGAATCTGTTCCAGAG CG TGC) and AR-R (CTCTACGATGGGCTTGGGGAGAA C) were used. For RT-PCR array, the gene expression was analyzed using a human embryonic stem cell RT2 profiler (SABiosciences, Qiagen) PCR array according to the manufacturer's protocol.

2.7. Karyotype G-Band Staining. Analysis was performed by Nihon Gene Research Laboratories Inc. (Sendai, Japan). The numbers of chromosomes were checked in fifty cells per group, then 20 cells were further analyzed by detailed investigation.

2.8. Androgen Receptor Exon 1 Methylation Analysis. Genomic DNA extracted from hiPS cells and parental fibroblasts were digested with HpaII or MspI. Then, digested DNA was amplified with Ar1-r-6FAM primer (6FAM TCCAGAA TCTGTTCCAGAGCG TGC) and Ar1-f2 primer (CTCTAC GATGGGCTTGGGGAGAAC). The PCR products were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems), and the ratios of signals were quantitated as described [11]. Male genomic DNA was used as a control for complete digestion of the unmethylated X chromosome (no signal). Since the difference between the two bands (a and b) was only 3 bp (23 CAG repeats and 24 CAG repeats, resp.) in patient 609, we detected the overlap of stutter bands. To revise the signal intensity, we used the following formula: true $b = b - a \times 0.31$. The calculation of 0.31 was based on our observation on 24 samples of 8 individuals.

2.9. Direct Sequencing of Androgen Receptor Transcripts. RNAs isolated from iPS cells were reverse-transcribed into cDNA and amplified with Ar1-r-6FAM and Ar1-f2 primers. Sequences of the PCR products were determined by direct sequencing. PCR products from induced myotubes were then cloned into a TA cloning vector (Invitrogen) and inserts were sequenced.

2.10. Fluorescence In Situ Hybridization (FISH) with XIST RNA Probe and X Chromosome Probe. FISH analysis was performed on parental fibroblasts and hiPS cells at Chromosome Science Labo Inc., Sapporo, Japan (http://www.chromoscience.jp). In brief, exon 1 (6 kb) and exon 6 (6 kb) of the XIST gene were amplified by PCR and labeled with Cy3 by nick translation. For the X chromosome, an FITC-labeled HXO-10 probe (Chromosome Science Labo Inc.) that hybridizes with the DXZ1 region was used. Cells

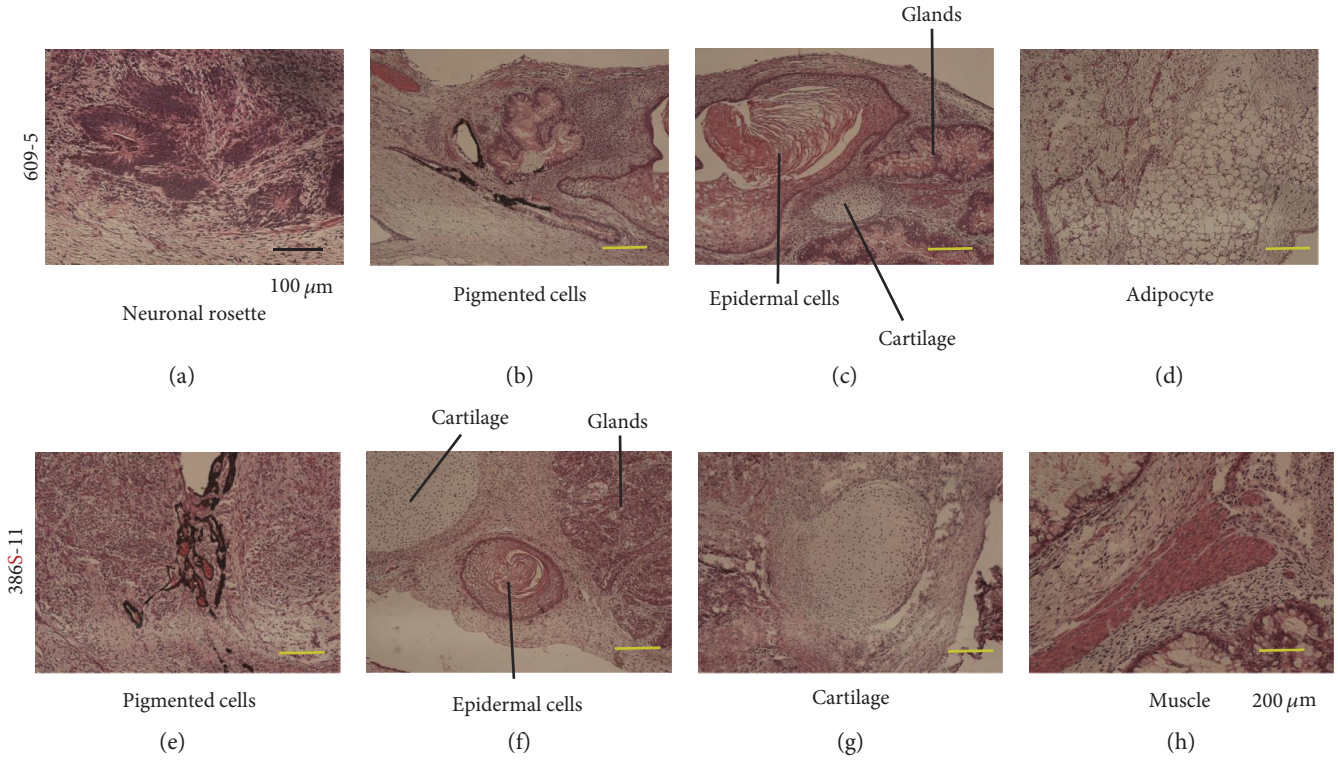

Figure 2: In vivo differentiation of 609-5 iPS cells (XX) and 386S-11 (XXX) iPS cells. Six months after transplantation into the testes of NOD/Scid mice, hiPS cells (609-5: (a–d), 386S-11: (e–h)) had formed teratomas, including mesodermal tissues (adipocytes, cartilage, and muscle), endodermal tissues (mucus-producing epithelium), and ectodermal tissues (neuronal rosette, epidermal cells, and pigmented cells).

on chamber slides were treated with 0.01% pepsin/0.1 N HCl for 4 min and then fixed in 4% paraformaldehyde/PBS. After denaturing at 70 °C for 5 min, cells were incubated with an XIST probe and X probe. After washing with 50% formamide/2xSSC, and 1xSSC, cells were stained with DAPI and mounted. The images were taken using a Leica DMRA2 camera and Leica CW4000 FISH software.

2.11. Teratoma Formation Assay. hiPSCs (1×10^6) were injected into the testes of 8- to 12-wk-old *NOD/Scid* mice. Three months after injection, tumors were dissected and fixed in 15% formalin, embedded in paraffin, cut by a microtome, and stained with hematoxylin and eosin (H&E).

2.12. Myogenic Induction by Inducible MyoD. hiPS cells were cultured on iMatrix-511 (Nippi) in StemFit AK02N culture medium (Ajinomoto) and induced into the mesoderm lineage using STEMDiff Mesoderm induction medium (Stem Cell Technologies). After 3 d culture, the cells were cultured as spheres [12]. After 6 wk culture, the cells were plated onto collagen-coated plates and infected with a lentiviral vector encoding a doxycycline (dox)-inducible mouse MyoD gene (pLVi(3G)-TagGFP-E2a-MYOD-Puro, Sirion Biotech). After 2 d selection with puromycin (1 μg/ml) (Calbiochem) and dox (1 μg/ml) (LKT Laboratories) selection, the cells were induced to form myotubes in the presence of dox in DMEM supplemented with 2% horse serum. Multinucleated myotubes were immunostained with Hoechst 33258 dye (nuclei), antidystrophin polyclonal antibody (Abcam), and antimyosin heavy chain (MF20) (R&D Systems), and then visualized with goat anti-mouse IgG2b-Alexa568 and goat anti-rabbit IgG-Alexa 488 (Life Technologies).

3. Results

3.1. Induction of iPS Cells from Fibroblasts of a Manifesting DMD Carrier. Fibroblasts from a manifesting carrier (patient 609) were first infected with an all-in-one retroviral vector expressing OCT4, KLF4, SOX2, LIN28, and NANOG and replated on STO feeder cells; five clones were obtained (609-2, 609-4, 609-5, 609-9, and 609-12), Next, the same fibroblasts were infected with four Sendai viral vectors encoding for SOX2, KLF4, OCT4, or c-MYC and replated on SNL feeder cells (Figure 1). We obtained four clones (609S-1, 609S-2, 609S-3, and 609S-4) using the Sendai viral vector. The appearance of ES-like colonies was faster (2-3 weeks) with SeV-mediated reprogramming than with retroviral vector-mediated reprogramming (3-4 weeks). SeV RNA (RT-PCR) or proteins were not detected as early as passage three (Supplementary Figure 2 available online at https://doi.org/10.1155/2017/7906843). The expression of human ES markers (TRA-1-60, TRA-1-81, SSEA3, NANOG, OCT3/4, and SOX2) in all hiPS cells was confirmed by immunocytochemistry (Supplementary Figure 2). FACS analysis confirmed the expressions of SSEA4, TRA-1-60, and TRA-1-81 on all hiPS cells tested (Supplementary Figure 3). Gene expressions of human ES markers and

Sample	Methylation of AR alleles		Gene expression of two AR alleles
	Xi allele 1* (%)	Xi allele 2* (%)	Direct sequencing of AR allele
609 fibroblasts	4.88	95.12	Allele 1 (23 CAG repeats) > allele 2 (24 CAG repeats)
609 blood	31.99	68.01	n.d.
609 skeletal muscle	15	85	n.d.
609-2 p31 (retro)	11.69	88.31	Equally expressed
609-4 p21 (retro)	13.65	86.35	Allele 1 > allele 2
609-5 p9 (retro)	24.07	75.93	Equally expressed
609-9 p7 (retro)	37.14	62.86	Equally expressed
609-12 p7 (retro)	16.61	83.39	Allele 1 > allele 2
609S-1 p6 (Sendai)	47.04	52.96	Equally expressed
609S-2 p5 (Sendai)	6.72	93.28	Allele 1 > allele 2
609S-3 p4 (Sendai)	40.77	59.23	Equally expressed
609S-4 p9 (Sendai)	50.86	49.14	Equally expressed

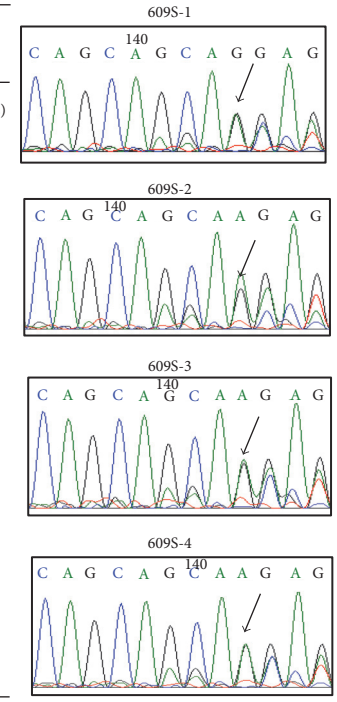

Figure 3: Methylation status and allele-specific expression of androgen receptor (AR) gene in the fibroblasts, skeletal muscle, and blood sample of a manifesting carrier of DMD (609) and derived iPS cells. *Left panel*: *% is calculated based on the assumption that one of two alleles is methylated, and signal intensities from allele 1 + allele 2 are shown as 100%. However, not all iPS clones have XCI. *Right panel*: representative images of direct sequencing of the *AR* gene.

differentiation markers were also examined by RT-PCR array (Supplementary Figure 3). Patient 609's iPSCs showed similar gene expression patterns to 201B7 and 253G4, which have already shown pluripotency in both in vitro differentiation assay and teratoma formation experiments [5, 9] (Supplementary Figure 3). FISH analysis using an X chromosome probe and G-band staining of parental fibroblasts and of hiPS cells (609-4 iPS and 609S-3 iPS cells) revealed that parental fibroblasts and iPS cells have normal karyotypes (46XX) and no gross chromosomal abnormalities (Supplementary Table 1 and Supplementary Figure 1). hiPS cells derived from 609 fibroblasts formed well-differentiated teratomas three months after transplantation into the testis (Figure 2).

3.2. Methylation of Exon 1 of AR Gene and Allelic Expression of AR Gene in Parental Fibroblasts and hiPS Cells. To determine the X chromosome-inactivation status of iPS cells, we first examined the methylation of exon 1 of the androgen receptor in muscle fibroblasts, skeletal muscle (biopsied sample), and blood according to the methods described by Allen et al. [13] (Figure 3). We digested genome DNA with HpaII (sensitive to methylation of CpG) or MspI (digests methylated CpG), and then amplified the exon 1 regions by PCR with specific primers. This assay is widely used for measuring XCI skewing of DMD homozygous carriers [1]. Patient 609 showed 22 and 23 repeats of CAG in the first exon of the androgen receptor gene (data not shown). Quantification of the signals of methylated and unmethylated alleles indicated skewing of XCI toward the same X chromosome in the skeletal muscle and muscle fibroblasts. The blood sample showed no obvious skewing. The data indicate that the AR gene with 23 CAG repeats and the mutated DMD gene are on the same X chromosome, and this allele (allele 1) is dominantly active in skeletal muscle. We next examined the methylation status of exon 1 of the androgen receptor in patient 609's iPS cells (Figures 3 and 4). All five retro-iPS clones inherited the nonrandom methylation pattern of *AR* exon 1 of the parental fibroblasts; allele 2 was preferentially methylated, although a theoretical pattern, 0% : 100%, was not observed. Surprisingly, three out of four SeV-iPS clones showed an almost nonskewing pattern, suggesting loss of XCI.

To know which allele of two AR genes is transcribed in iPS clones, the androgen receptor transcripts were reverse-transcribed into cDNA, amplified, and directly sequenced. Surprisingly, 6 iPS clones out of 9 iPS clones showed equally expressed patterns. Only three iPS clones (609-4, 609-12, and 609S-2) retained preferential expression of allele 1 (Figure 3 and Supplementary Figure 5).

3.3. XIST RNA Signals in Fibroblasts of Manifesting Carrier of DMD and Derived iPS Cells. Next, we examined XIST expression in fibroblasts, retro-iPS cells, and SeV-iPS cells. *XIST* is a nonprotein coding RNA that localizes to the inactive human X chromosome [14, 15]. RNA-FISH analysis revealed that more than 80% of retro-iPS cells in clones 609-2 and 609-4 retained XIST expression in one of the two X chromosomes (Figure 4 and Table 1). In contrast, no XIST signals were detected in 609S-1 iPS cells or 609S-2 iPSCs (Figure 4 and Table 1). Because 609S-2 iPS cells showed

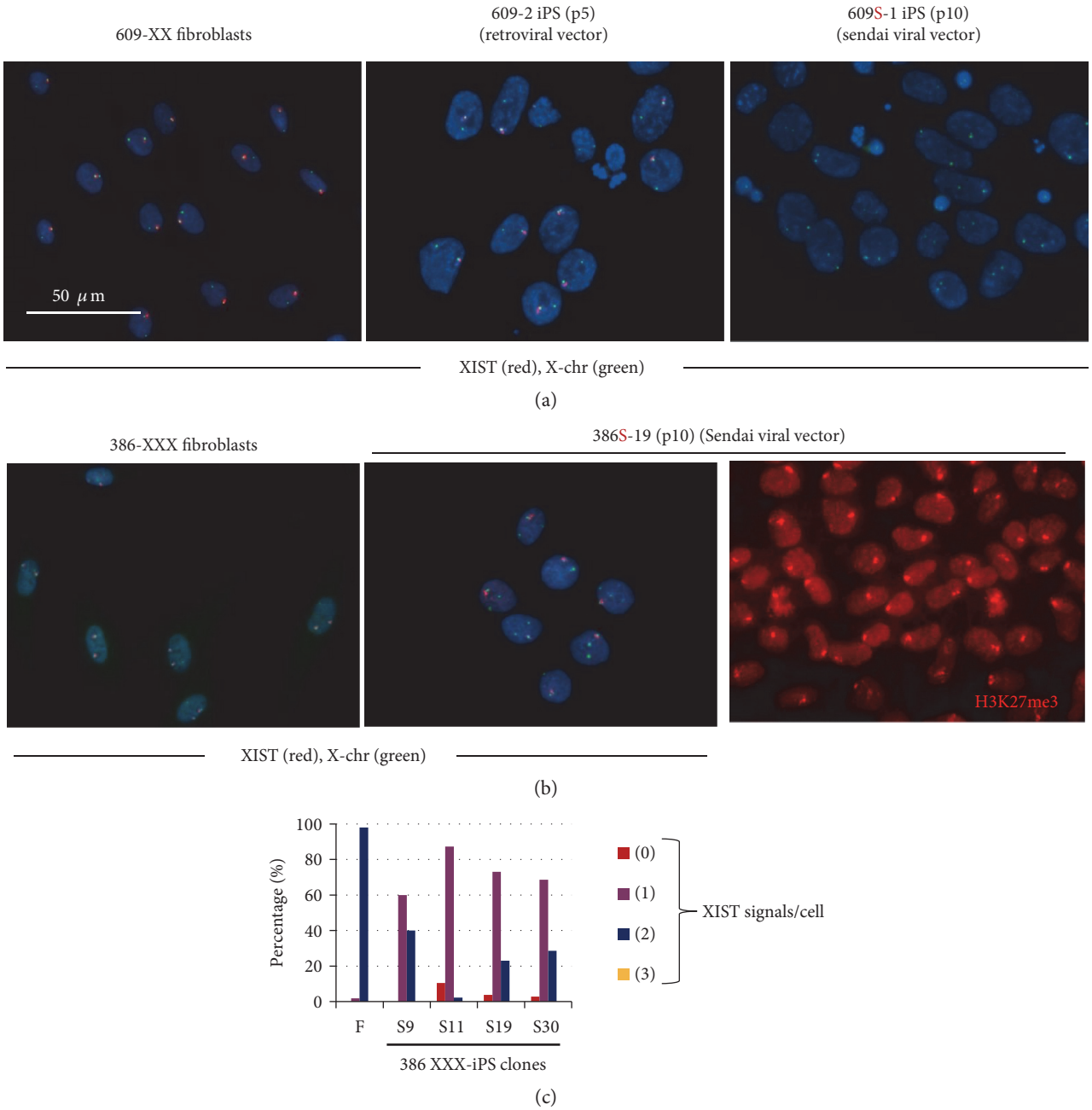

Figure 4: Loss of XIST signals in 609-iPS cells (XX) and 386-iPSCs (XXX). (a) Representative images of XIST RNA FISH (red)/X chromosome-DNA FISH (green) on 609 fibroblasts, 609-2 iPS cells (passage 5: p5), and 609S-1 iPS cells (p10). 609 fibroblasts have one XIST signal. Most 609-2 iPS cells retain XIST expression. In contrast, 609S-1 iPS cells, which were generated with Sendai viral vectors, had totally lost XIST expression. Quantitative data are also shown in Table 2. (b) Representative images of XIST RNA FISH (red)/X chromosome-DNA FISH (green) on 386 fibroblasts, and 386-S19 iPS cells. 386 fibroblasts have three X and two XIST signals. 386-S19 iPS cells, which were induced using Sendai viral vectors retained one XIST expression. Trimethyl-histone H3 (Lys27) (H3K27me3) signals (red) in 386-S19 iPS cells are shown. Most cells have just one H3K27me3 signal/cell. (c) XIST signals per cell in 386 fibroblasts and four 386 iPS cells. 386 fibroblasts have three X chromosomes and two XIST signals. In four 386 iPS clones, which were induced with Sendai viral vectors, most cells have one XIST signals.

skewed methylation of the AR exon 1 and dominant expression of one AR gene (Figures 3 and 4), we speculate that most 609S-2 iPS cells had lost XIST RNA but remained hypermethylated and transcriptionally suppressed. In contrast, our observations suggest that 609S-1 iPS cells reactivated the inactive X chromosome.

3.4. XCI of iPS Cells Derived from a Female Carrier of DMD with XXX Trisomy. To further examine the effects of reprogramming on XCI, we generated iPSCs from a female carrier with XXX trisomy (patient 386) by Sendai viral vectors. This patient has a large deletion of Ex13–Ex44 in the active X chromosome and high serum CK levels but shows no obvious

muscle weakness due to an in-frame mutation. In this case, immunostaining of dystrophin indicated that the other two X chromosomes are completely inactivated (data not shown). Induced iPS clones showed ES cell-like gene expression patterns (Supplementary Figure 4) and triple XXX karyotypes (Supplementary Figure 1). XIST FISH analysis also showed that parental fibroblasts have three X chromosomes and that two of them are coated with XIST RNA. In contrast, the majority of patient 386's iPS cells showed only one XIST signal (Figure 4(b)). H3K27me3 is a repressive chromatin mark on inactive X chromosomes. Signals of H3K27me3 were also found on only one X chromosome in most 386 iPS cells (Figure 4(b)). In spite of such an aberrant XCI status, 386-iPSCs programmed by SeV vectors formed well-differentiated teratomas in the testes of NOD/Scid mice (Figure 2).

3.5. Dystrophin Expression in DMD-Manifesting Carrier hiPSC-Derived Myotubes. To determine whether patient 609's iPS cells indeed reactivated the X chromosome on which the normal dystrophin was encoded, we induced her iPS cells to differentiate into skeletal muscle lineage by using doxycycline-inducible MyoD expression and examined dystrophin expression by immunocytochemistry. Myotubes derived from 609S-2, 609S-3, and 609S-4 iPSCs expressed normal dystrophin at undistinguishable levels from myotubes derived from control 409B2 (Figure 5). RT-PCR for *dystrophin* confirmed that the normal *dystrophin* gene was actively transcribed. Direct sequencing of CAG-repeats of AR transcripts also indicated that both alleles are equally expressed in myotubes induced from 609S-3 and 609S-4 iPSCs. In 609S-2 myotubes, the AR gene with 23 CAG repeats on allele 1 was still preferentially expressed (Figure 5).

4. Discussion

4.1. Reactivation of Inactivated X Chromosome in hiPS Cells Is a Common Phenomenon. Inactivation of the X chromosome in human iPS cells has been well examined in normal female iPSCs [16] and Rett syndrome iPS cells [8, 17–20]. Three groups reported nonrandom XCI status of Rett iPS clones. In contrast, Marchetto et al. and Kim et al. reported that XCI was erased in some, but not all, reprogrammed RTT-iPSC clones, and again XCI occurred randomly during differentiation into neurons. The XCI of patient 609's iPS clones are similar to their RTT-iPSCs clones. We also found that XXX-iPS clones reprogrammed by SeV vectors have only one XIST signal, while the parental fibroblasts have two, suggesting activation of one of two inactive X chromosomes. Barakat et al. reported that many XXX-iPS cells derived from a triple X patient did not show Xi markers in their experiments [21]. Therefore, although we speculated that hiPS cells with three active X chromosomes cannot survive in an overdose of X chromosome genes, XaXaXa hiPS cells might survive in different culture conditions. To explain the contradictory results, further investigation would be required.

Table 1: XIST signals on the X chromosomes in the nuclei of fibroblasts isolated from a DMD-manifesting carrier (609) and four hiPS cells induced from 609 fibroblasts (609-2, 609-4, 609S-1, and 609S-2).

Cell (counted cells)	XIST (+) cells	XIST (−) cells
609 fibroblasts ($n = 168$)	164 (97.6%)	4 (2.4%)
609-2 p5 iPSCs ($n = 151$)	114 (75.5%)	37 (24.5%)
609-4 p6 iPSCs ($n = 72$)	62 (86.1%)	10 (13.9%)
609S-1 p10 iPSCs ($n = 150$)	0 (0%)	150 (100%)
609S-2 p8 iPSCs ($n = 142$)	0 (0%)	142 (100%)

N: number of cells counted; p: passage number.

4.2. Do Reprogramming Methods Affect XCI of Female DMD-iPSCs? We found a difference in XCI status between retro-iPSCs and Sendai-iPSCs, but the mechanisms by which different reprogramming methods produce hiPS cells with different XCI statuses is unclear. One possibility is that high levels and long duration of the expression of reprogramming factors by Sendai viral vectors cause a loss of XCI in hiPS cells. Tomoda et al. reported that the "Kyoto method," in which LIF-producing SNL cells are used as feeders, contributes to reactivation of Xi during the reprogramming process [22]. Although how LIF erases XCI remains to be determined, our results are in agreement with their hypothesis; we used STO cells (parental cells of SNL cells) for retroviral reprograming, and SNL cells for Sendai viral-mediated reprogramming. We also compared the effects of two feeders, that is, MEFs and SL10, on reprogramming of normal female fibroblasts. Interestingly, reprogramming by MEFs tended to retain trimethyl-histone H3 (Lys27) compared with iPSCs established on SL10, again suggesting that the feeder cell is one factor that affects the XCI state (Supplementary Figure 6). Combination of reprogramming factors is another possible factor which influenced XCI in hiPSCs in our study (Figure 1). How both X chromosomes are activated during reprogramming and XaXa status was stably kept in culture should be determined in future studies.

4.3. Human iPSC Derived from a Manifesting Carrier of DMD Differentiated into Myotubes and Expressed Normal Dystrophin. Because many iPSC clones derived from a manifesting carrier of DMD showed XaXa-like patterns in methylation of the AR gene locus, expression of two *AR* alleles (Figure 3), XIST signals (Table 1, Figure 4), and H3K27me3 immunostaining (Figure 4), we expected the inactive X chromosome to be reactivated during reprogramming, resulting in a reset of skewed XCI in differentiated cells. Unexpectedly, "XaXa" hiPSCs derived from a DMD-manifesting carrier differentiated into multinucleated myotubes, and showed robust expression of dystrophin like the control. Our observation suggests that XaXa hiPSC clones reset the skewing of XCI to express dystrophin in differentiated cells. Bi-allelic *AR* expression in myotubes confirmed that XCI was randomly induced during differentiation from XaXa hiPSCs to myotubes (Supplementary Figure 7).

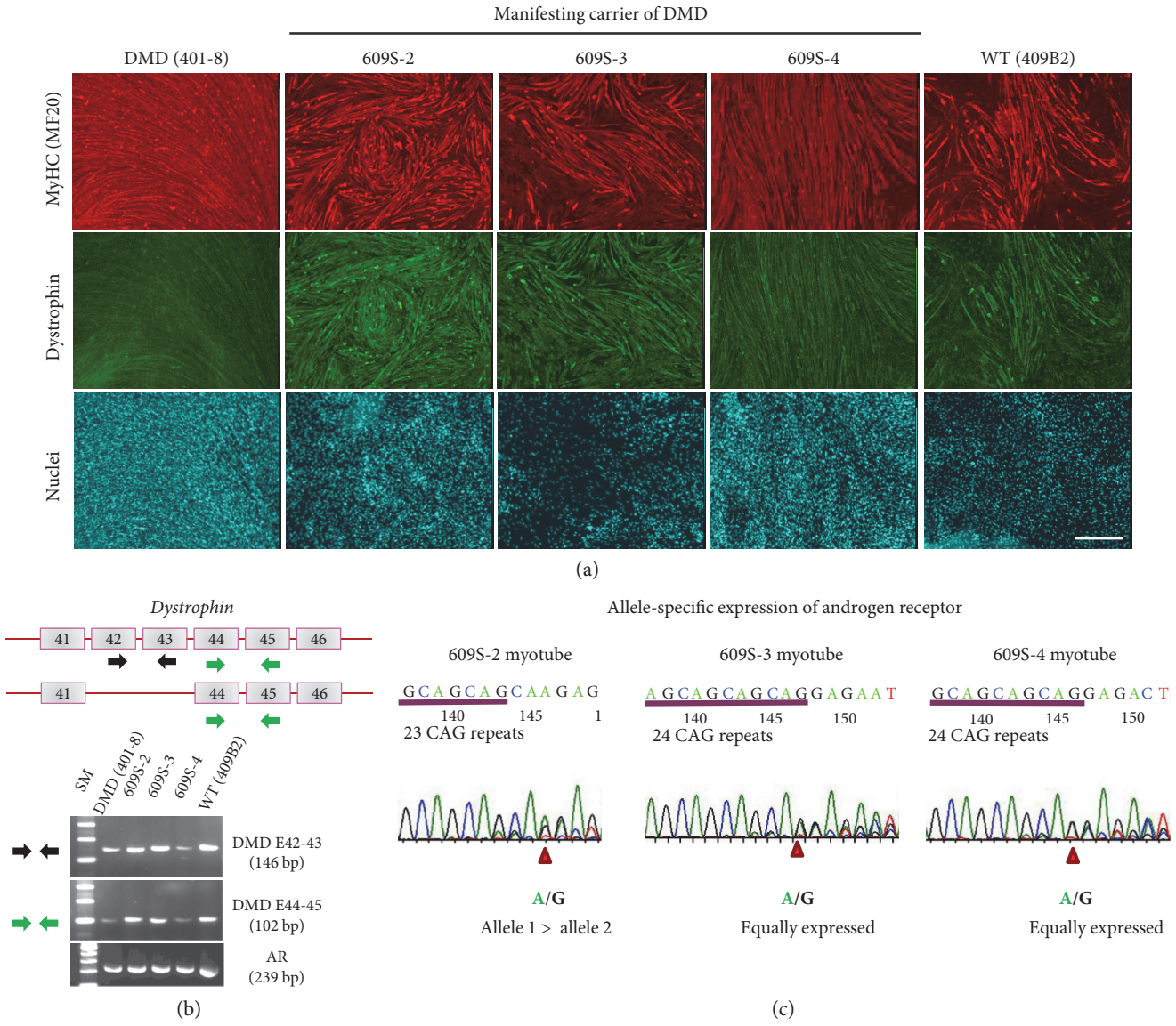

Figure 5: Myotubes derived from XaXa 609-hiPS cells restore normal dystrophin expression and nonskewed expression of the androgen receptor gene. (a) 409B2 iPSCs (normal), DMD-iPSCs (401-8, duplication of exons 45–50 of the dystrophin (*DMD*) gene), and manifesting carrier of DMD-iPS clones (609S-2, 609S-3, and 609S-4) were induced to differentiate into multinucleated myotubes. Normal iPS-derived myotubes expressed dystrophin. DMD-iPS-derived myotubes were negative for dystrophin. 609 iPSC-derived myotubes expressed dystrophin at comparable levels with wild-type myotubes. Scale bar: 500 μm. (b) RT-PCR analysis for *dystrophin* (*DMD*) and *androgen receptor* (*AR*) in myotubes derived from 409B2 iPSCs (normal), DMD-iPSCs (401-8), and manifesting carrier of DMD-iPSCs (609S-2, 609S-3, and 609S-4). Primer positions and directions for dystrophin transcripts are indicated by arrows. (c) Direct sequencing of CAG repeats of AR transcripts in myotubes derived from manifesting carrier of DMD-iPS clones (609S-2, 609S-3, and 609S-4). 609S-2 myotubes show allele 1(23 CAG repeats)—dominant expression. In 609S-3 and 609S-4 myotubes, two alleles (23 and 24 CAG repeats) are equally expressed.

5. Conclusion

(1) Many iPS clones derived from a manifesting female carrier of DMD had two active X chromosomes (XaXa) or mixed patterns (XaXa/XaXi).

(2) Redifferentiation of female manifesting carrier of DMD-iPSCs with two active X chromosomes into myotubes restored dystrophin expression due to acquirement of nonskewed XCI.

Conflicts of Interest

There is no conflict of interests regarding the publication of this manuscript.

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Review Article

Epigenetic Manipulation Facilitates the Generation of Skeletal Muscle Cells from Pluripotent Stem Cells

Tomohiko Akiyama, Shunichi Wakabayashi, Atsumi Soma, Saeko Sato, Yuhki Nakatake, Mayumi Oda, Miyako Murakami, Miki Sakota, Nana Chikazawa-Nohtomi, Shigeru B. H. Ko, and Minoru S. H. Ko

Department of Systems Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan

Correspondence should be addressed to Tomohiko Akiyama; akiyama617@keio.jp and Minoru S. H. Ko; ko.minoru@keio.jp

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Human pluripotent stem cells (hPSCs) have the capacity to differentiate into essentially all cell types in the body. Such differentiation can be directed to specific cell types by appropriate cell culture conditions or overexpressing lineage-defining transcription factors (TFs). Especially, for the activation of myogenic program, early studies have shown the effectiveness of enforced expression of TFs associated with myogenic differentiation, such as PAX7 and MYOD1. However, the efficiency of direct differentiation was rather low, most likely due to chromatin features unique to hPSCs, which hinder the access of TFs to genes involved in muscle differentiation. Indeed, recent studies have demonstrated that ectopic expression of epigenetic-modifying factors such as a histone demethylase and an ATP-dependent remodeling factor significantly enhances myogenic differentiation from hPSCs. In this article, we review the recent progress for in vitro generation of skeletal muscles from hPSCs through forced epigenetic and transcriptional manipulation.

1. Introduction

The characteristics of cells are principally determined by patterns of gene expression. During the course of development, the generation of various cell types consisting of our body are driven by the dynamic alteration of gene expression patterns. Human pluripotent stem cells (hPSCs) such as embryonic stem cells (ESCs) [1] and induced pluripotent stem cells (iPSCs) [2, 3] express a specific set of genes ("pluripotency genes") that generate the gene regulatory network for pluripotency [4, 5]. Differentiation of hPSCs involves the suppression of these pluripotency genes such as POU5F1, SOX2, and NANOG, which maintain hPSCs in undifferentiated state, and the activation of early developmental genes, followed by the activation of tissue-specific genes [6–8]. Conversely, it is conceivable that forcibly altering the gene expression patterns from the pluripotent state to the cell-type-specific state would lead to the differentiation of hPSCs to the desired cell types in vitro.

The changes of gene expression patterns during myogenic differentiation have been well characterized. Several myogenic transcription factors (TFs) are identified as markers for specific stages in differentiation [9, 10]. The paired box transcription factors—Pax3 and Pax7—are specifically expressed in myogenic progenitors, such as satellite cells and myoblasts, and downregulated upon differentiation. The basic helix-loop-helix TFs—MyoD and Myf5—are activated in committed satellite cells and regulate skeletal muscle specification and differentiation. Using these markers as a guide, in vitro differentiation protocols have been developed: culturing hPSCs with other types of cells and in media supplemented with suitable growth factors [11, 12]. However, in most cases, the protocols require long-term, complicated steps, yet the efficiency of differentiation is rather low. To overcome these limitations, the forced ectopic expression of myogenic TFs in hPSCs has been effectively used [13–16]. Furthermore, recent studies have revealed that ectopic expression of epigenetic modifying

factors such as a histone demethylase and an ATP-dependent remodeling factor significantly enhances the TF-mediated myogenic differentiation from hPSCs [17, 18].

In this review, we discuss the current methods for differentiating skeletal muscle cells from hPSCs through enforced epigenetic and transcriptional manipulation that can directly activate the myogenic gene expression program.

2. Forced Expression of TFs Leads to Direct Myogenic Differentiation of hPSCs

The first attempts to direct myogenic differentiation with forced expression of TFs have been performed using mouse ESCs (mESCs). Darabi et al. demonstrated that overexpressing Pax3 or Pax7 during the embryoid body (EB) formation of mESCs induces efficient myogenic differentiation [19–21]. When the mESC-derived myogenic progenitors are transplanted in dystrophic mice, these cells are engrafted in muscle and restore muscle function. Using the same approach, the authors subsequently showed that hESC- and hiPSC-derived mesodermal cells after PAX7 overexpression can give rise to myogenic progenitors with a high engraftment capacity in cardiotoxin-injured mouse muscle [14]. Furthermore, other studies have reported that forced expression of the myogenic regulator—MYOD1—also drives the myogenic differentiation of hESCs and hiPSCs. Goudenege et al. have demonstrated that the ectopic expression of MYOD1 converts mesenchymal cells derived from hESCs and hiPSCs (MB1-hPSCs) into engraftable myoblast-like cells [15]. Tedesco et al. have also reported efficient myogenic conversion of hiPSC-derived mesoangioblast-like progenitors (HIDEM) by the overexpression of MYOD1 [16].

In these experiments, the introduction of TFs was performed with lentiviral or adenoviral vectors, and their overexpression was not directly carried out in hESCs or hiPSCs but rather in mesodermal and mesenchymal-like cells derived from hESCs/hiPSCs. Indeed, the efficiency of myogenic differentiation is low when adenoviruses encoding MYOD1 are directly transduced to hESCs [15]. Albini et al. have also shown that lentivirus-mediated MYOD1 overexpression fails to induce myogenic conversion in hESCs, whereas comparable levels of MYOD1 expression efficiently induce myogenic differentiation from human fibroblast cells [18]. However, when a piggyBac vector system is used to express MYOD1, the direct myogenic conversion of hiPSCs can be successfully achieved [13], suggesting that a large amount of MYOD1 protein is required to activate skeletal myogenesis in hPSCs. These findings indicate that hPSCs have considerable resistance to MYOD1-mediated myogenic conversion.

3. Epigenetic Barrier of hPSCs against Differentiation

The functional activities of TFs require their physical access to the genome, which is tightly enclosed in chromatin structure consisting of DNA and histone complexes. Especially, the chromatin structure around the TF-binding sites plays critical roles in the regulation of gene expression [22–25]. The N-terminal tails of histone proteins are subject to various posttranslational modifications, including acetylation, methylation, phosphorylation, and ubiquitylation that result in changes of the chromatin structure [26–28].

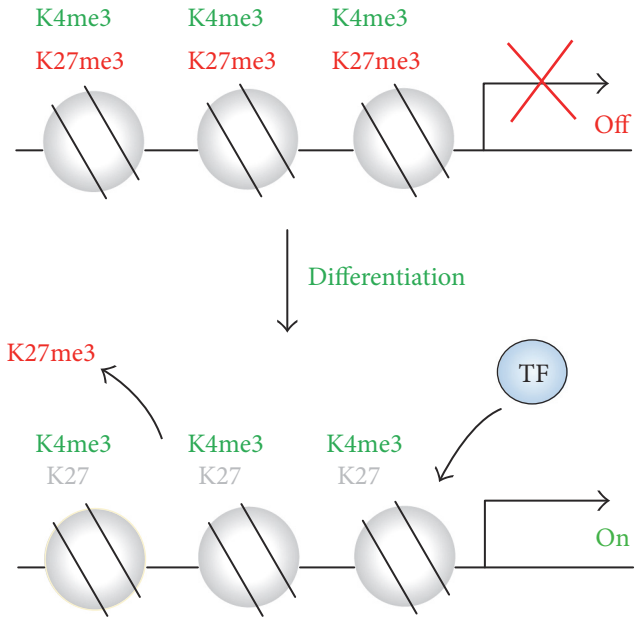

Figure 1: The chromatin regulation of developmental genes in PSCs. In PSCs, developmental genes are marked by bivalent domains containing both H3K4me3 and H3K27me3, which are associated with the transcriptional silencing in the undifferentiated state. The removal of H3K27me3 and the binding of transcription factors (TFs) allow rapid transcriptional activation upon differentiation stimuli.

Pluripotent ESCs/iPSCs possess unique chromatin signatures to be prepared for differentiation. In ESCs/iPSCs, lineage-affiliated genes are transcriptionally poised by "bivalent" histone modifications, consisting of H3 Lys-4 trimethylation (H3K4me3) and H3 Lys-27 trimethylation (H3K27me3) [29–32]. H3K4me3 is generally localized in the gene regulatory regions such as promoters and associated with transcriptional activation, whereas H3K27me3 is generally associated with inactive gene promoters [22]. However, in ESCs/iPSCs, both H3K4me3 and H3K27me3 are enriched in the promoter regions of genes associated with lineage differentiation (Figure 1). When differentiation is stimulated, ESCs/iPSCs initiate developmental programs by removing repressive H3K27me3 marks from lineage-affiliated gene promoters. Rapid gene expression can occur, because active H3K4me3 mark remains in those promoters. Loss of H3K27me3 by knocking out responsible histone enzymes results in derepression of developmental regulatory genes in mESCs and hESCs [33, 34]. Importantly, H3K27me3 represses the gene expression by impeding the binding and/or function of TFs and/or RNA polymerase [35, 36]. These results indicate that H3K27me3 functions as an "epigenetic barrier" against ESC/iPSC differentiation.

In hESCs, more than 1500 genes are categorized as bivalent "H3K4/K27me3-modified genes" [30, 31, 37], which

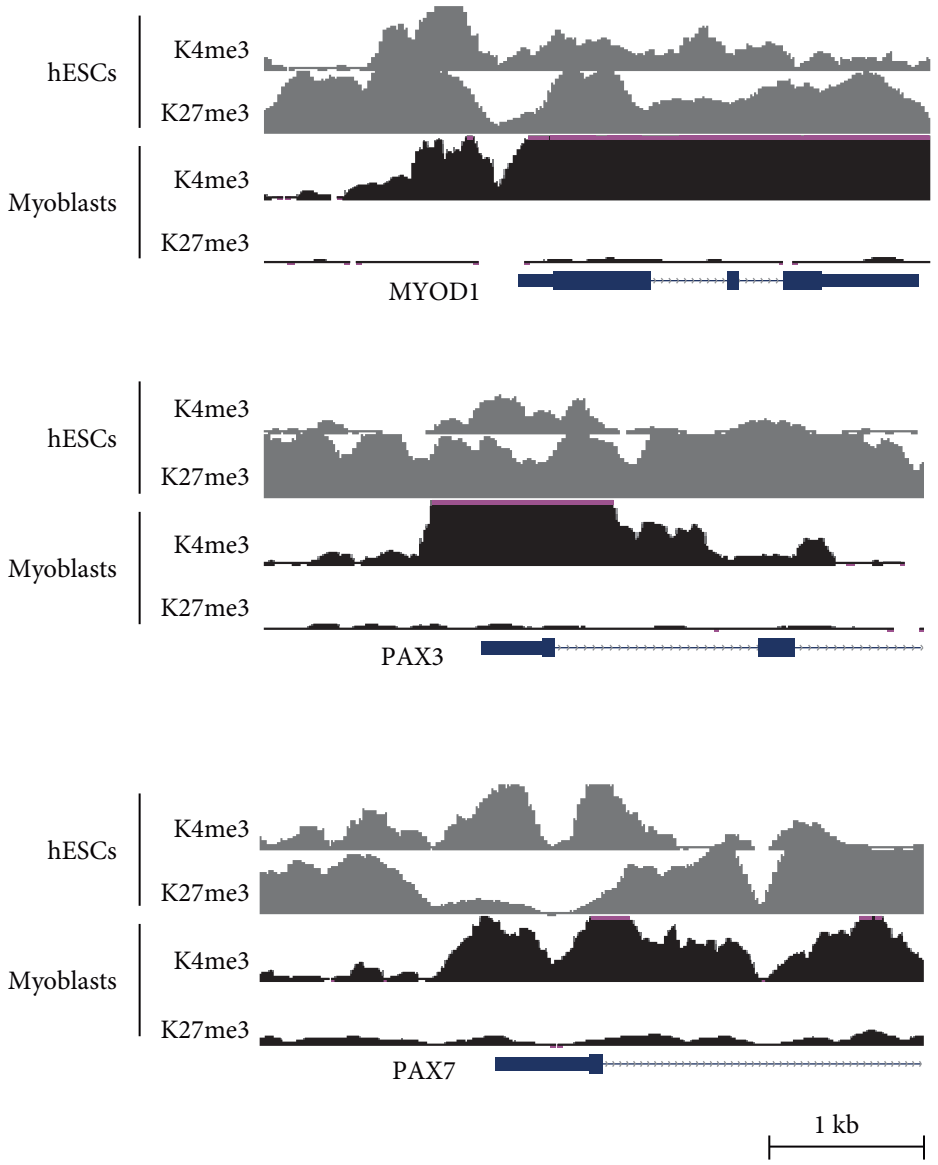

Figure 2: The states of H3K4me3 and H3K27me3 near myogenic genes in hESCs and myoblasts. The ChIP-sequencing peaks of H3K4me3 and H3K27me3 at the MYOD1, PAX3, and PAX7 genes are shown. The data were generated in the Bernstein laboratory [59].

include myogenic regulatory genes, such as PAX3, PAX7, and MYOD1. Chromatin immunoprecipitation analysis revealed that both H3K4me3 and H3K27me3 are enriched in their promoters of hESCs, whereas only H3K4me3 is enriched in those of human myoblasts (Figure 2). These epigenetic states correspond to the expression states of the genes: they are repressed in hESCs and activated in myoblasts. These results suggest that the removal of H3K27me3 is crucial to switch the gene expression patterns of ESCs to those of muscle cells and to direct myogenic differentiation of ESCs/iPSCs.

4. Specific Enzymes Remove H3K27me3 during Differentiation

Histone methylation is dynamically regulated by two kinds of enzymes, histone methyltransferases and demethylases, which add and remove the histone lysine methylation, respectively. The addition of H3K27me3 is mediated by the Polycomb repressive complex containing the histone methyltransferase—EZH2—as the enzymatic subunit [38–40]. On the other hand, the removal of H3K27me3 is mediated by the Jumonji C (JmjC) domain containing demethylases—UTX and JMJD3 [41–43]. Knockdown and knockout experiments have shown that UTX and JMJD3 are required for the differentiation of mPSCs and hPSCs into endoderm, ectoderm, and mesoderm lineages [44–51]. Interestingly, although both UTX and JMJD3 are the specific demethylases of H3K27me3, their expression levels and patterns are completely different during the differentiation of hESCs. For instance, the expression of UTX is high in hESCs, whereas the expression of JMJD3 is quite low in these cells. Moreover, comparative transcriptome analysis between undifferentiated and differentiated ESCs has revealed that JMJD3 is significantly

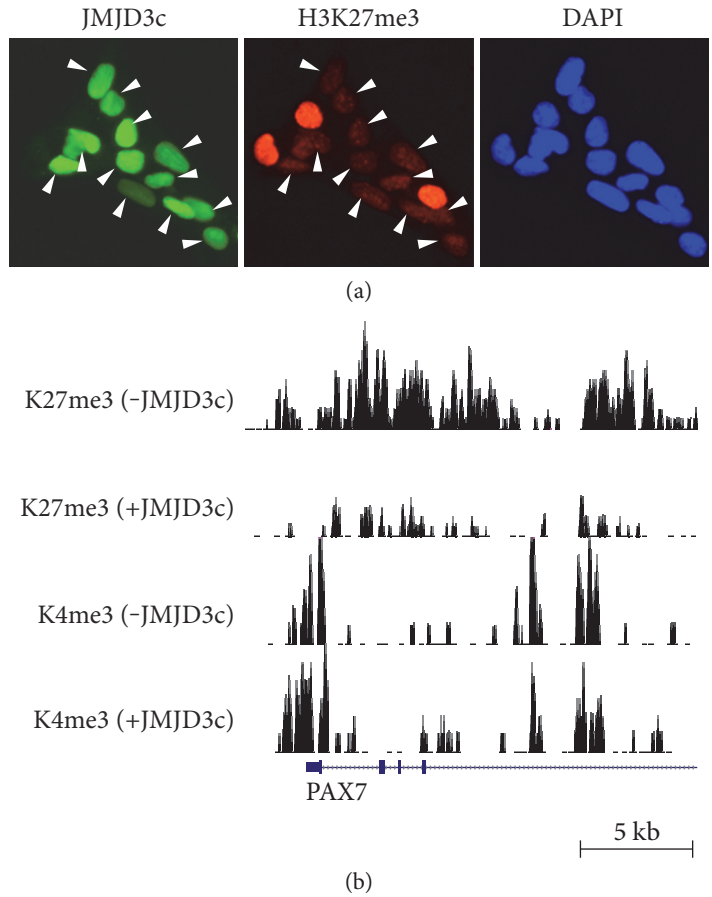

Figure 3: H3K27me3 demethylation in hESCs by forced expression of JMJD3c. (a) Immunostaining analysis showing the H3K27me3 demethylation in whole nuclei of hESCs by JMJD3c overexpression (arrows). (b) ChIP-sequencing analysis showing the significant reduction of H3K27me3 at the PAX7 gene in hESCs by JMJD3c overexpression. H3K4me3 enrichment remains at the PAX7 gene after JMJD3c overexpression.

upregulated upon hESC differentiation into the three germ layers, whereas UTX is downregulated [17]. These findings suggest that JMJD3 upregulation is important for inducing H3K27me3 demethylation during the differentiation of hESCs.

5. Demethylation of H3K27me3 Facilitates MYOD1-Mediated Myogenic Differentiation

Manipulating JMJD3 expression has the potential to change the epigenetic status of hPSCs toward differentiation. Indeed, Akiyama et al. have revealed that the forced expression of JMJD3 results in genome-wide demethylation of H3K27me3 in hESCs [17]. Furthermore, the overexpression of its C-terminal region containing catalytic JmjC domain (named "JMJD3c") leads to more significant reduction of H3K27me3 compared to the full length of JMJD3 (Figure 3). When UTX, another H3K27 demethylase, is overexpressed instead of JMJD3, the demethylation of H3K27me3 does not occur. These results suggest that JMJD3 is a specific epigenetic modifier for generating the chromatin characteristics of differentiated cells.

The forced expression of JMJD3c enables the hESCs to upregulate developmental genes that are accompanied by H3K27me3 demethylation [17]. In this condition, genes associated with meso/endoderm differentiation are strongly activated compared to neuroectodermal genes. In addition to meso/endodermal TFs such as Brachyury (T) and SOX17, BMP and Wnt-signaling-related genes are also activated by the JMJD3c overexpression. As BMP and Wnt/β-catenin signaling is responsible for meso/endodermal differentiation [52, 53], ectoderm differentiation may be inhibited by JMJD3c overexpression through the mesoendoderm gene network.

JMJD3c overexpression also activates the PAX3 and PAX7 genes, but not MYF5 or MYOD1 [17], indicating that H3K27me3-deficient ES cells have a propensity to differentiate into myogenic progenitor cells. The chromatin states generated by JMJD3c overexpression may be similar to those of mesenchymal cells or mesoangioblast-like cells such as MB1-hPSCs or HIDEMs. Although MB1-hPSCs and HIDEMs are generated through signal transduction in response to changes in the culture conditions for differentiation, H3K27me3-deficient ES cells directly alter their gene expression patterns, resulting in exiting from the pluripotent

state and upregulating developmental genes, even when the culture conditions for the hPSCs are not changed. Indeed, the activation of the PAX3 and PAX7 genes occurs within only a few days even in a medium that promotes the maintenance of an undifferentiated state.

The chromatin structure established in H3K27me3-deficient ES cells provides a suitable state for MYOD1-mediated myogenic differentiation. Akiyama et al. have shown that JMJD3c overexpression followed by MYOD1 overexpression significantly upregulates markers for skeletal muscle differentiation—MYOG, MEF2C, CKM, and SIX1 [17]. The myogenic gene expression program is quickly activated through the epigenetic changes. By 4 days after JMJD3c and MYOD1 overexpression, hESCs show expression patterns similar to the skeletal myotubes. JMJD3c cooperates with MYOD1 to activate the myogenic genes by changing the chromatin structure at their promoters. After JMJD3 and MYOD1 overexpression in hESCs, the MYOG and MEF2C promoters are enriched in active epigenetic marks—H3K4me3 and H3K27 acetylation.

6. Synthetic mRNA-Based Myogenic Differentiation of hPSCs

In previous studies, the overexpression of TFs was performed by viral or transposon vectors such as lentivirus, adenovirus, and piggyBac transposons. These vectors can effectively induce the expression of exogenous genes in hPSCs, but they have considerable limitations in terms of therapeutic applications: for example, possible insertional mutagenesis may occur due to random integration of the vectors into the host genome.

Synthetic mRNAs (synRNAs) encoding developmental regulator genes is one of the most promising approaches for directing the differentiation of hPSCs. This approach eliminates the risk of genomic DNA integration and insertional mutagenesis and is, thus, considered suitable for therapeutic applications. It has been shown that the transfection of synRNAs encoding reprogramming TFs into fibroblast cells can efficiently generate hiPSCs [54]. Furthermore, synRNAs encoding lineage-defining TFs such as Myod1, Hnf4a, and Ascl1 can differentiate mESCs into skeletal muscles, hepatocytes, and neurons, respectively [55]. However, in hPSCs, the efficiency of synRNA-mediated differentiation is low. Indeed, transfection of synRNA-encoding MYOD1 in hPSCs can generate only ~10% of myocyte-like cells. When hiPSCs were cultured in a fibroblast medium for 4 weeks and then transfected with synRNA-encoding MYOD1, ~40% of the cells became myogenic cells [54].

Akiyama et al. have demonstrated that transfection of JMJD3c-synRNAs prior to MYOD1-synRNAs dramatically increases the efficiency of myogenic differentiation of hPSCs [17]. The majority (>60%) of hESCs can be differentiated into myosin heavy chain- (MHC-) positive cells with myotube-like morphology in several days (Figure 4). By 4 days after transfection, some of the differentiated cells express a mature myogenic marker, creatine kinase-M, and possess the capacity for fusion with mouse C2C12 myoblast cells. These results suggest that the myotube-like cells induced by the JMJD3c and MYOD1 synRNAs have the potential to become mature skeletal muscles in vitro.

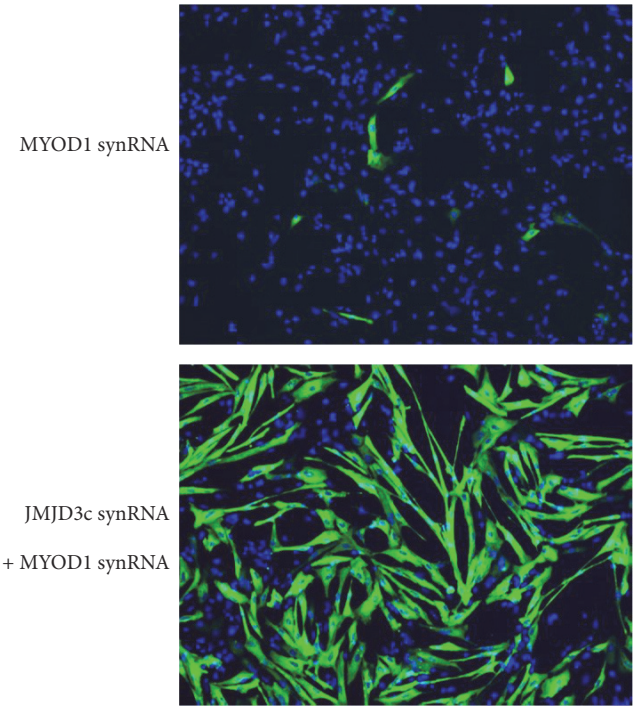

Figure 4: Efficient myogenic differentiation of hESCs by synRNA-mediated overexpression of JMJD3c and MYOD1. Transfection of synRNAs encoding JMJD3c and MYOD1 directly converts hESCs into MHC-positive myogenic cells for 5 days post differentiation. The differentiation efficiency was much higher in the JMJD3c/MYOD1-overexpressing hESCs compared with the MYOD1-overexpressing hESCs. MHC, green. DAPI, blue.

7. SWI/SWF Chromatin Remodeling Factor Enhances MYOD1-Mediated Myogenic Differentiation

A recent study showed that the SWI/SNF chromatin remodeling component BAF60C also promotes MYOD-mediated myogenic conversion in hESCs [18]. There are three variants of the BAF60 proteins, which are encoded by different genes: BAF60A (SMARCD1), BAF60B (SMARCD2), and BAF60C (SMARCD3). BAF60C is expressed in skeletal muscle cells but repressed in hESCs. The expression levels of one of the BAF60C isoforms, BAF60C2, significantly increase during embryoid body (EB) formation of hESCs. Albini et al. reported that sequential infection of BAF60C2 and MYOD1 lentiviruses enhances the activation of myogenic program in hESCs. BAF60C2 and MYOD1-overexpressing hESCs can be converted into MHC-positive cells with high efficiency (~60%) through changes of cell culture conditions: floating aggregates, followed by dissociation into single cells that are subsequently cultured in standard myogenic differentiation medium [18]. Infection of BAF60C2 lentivirus alone cannot activate the myogenic program in the absence of MYOD1. BAF60C2 facilitates the recruitment of MYOD1

Table 1: Methods for the skeletal myogenic differentiation of hPSCs.

Overexpressed genes	Methods	Conditions	Maturation	Time	% MHC + cells	References
PAX7	Lentivirus	Doxycyclin-inducible overexpression in hPSC-derived mesodermal cells	Progenitors	2~3 weeks	>90%**	[14]
MYOD1	Adenovirus	Infection in hPSC-derived mesenchymal cells	Mature cells	1~2 weeks	60%	[15]
MYOD1	Lentivirus	Tamoxyfen-inducible overexpression in mesoangioblast-like progenitors derived from hPSCs	Mature cells	3~4 weeks	>90%	[16]
MYOD1	piggyBac transposase	Doxycyclin-inducible overexpression in hPSCs	Mature cells	1~2 weeks*	>90%	[13]
MYOD1	synRNA	Transfection of MYOD1 in hPSC-derived fibroblasts	Mature cells	5 weeks	40%	[54]
JMJD3c and MYOD1	synRNA	Transfection of JMJD3c followed by MYOD1 in hPSCs	Mature cells	5 days	60%	[17]
BAF60C and MYOD1	Lentivirus	Infection of BAF60C followed by MYOD1 in hESCs	Mature cells	1~2 weeks	60%	[18]

* includes the procedure for generating stable cell lines.
** Differentiated cells from FACS-sorted PAX7-positive cells.

and polymerase II to the target promoters by enhancing the chromatin accessibility. Interestingly, mesodermal genes such as Brachyury (T), MESOGENIN, and MESP1 are not upregulated by BAF60C2/MYOD1 overexpression, indicating that BAF60C2/MYOD1 can directly convert hESCs into the skeletal myogenic cells without the transition through the mesodermal stage. When BAF60C2/MYOD1-overexpressing hESCs are continuously cultured as floating clusters, they become contractile three-dimensional myospheres composed of skeletal myotubes.

8. Conclusion

In this review, we have provided an overview of the current status of skeletal muscle generation from hPSCs using epigenetic and transcriptional manipulation (Table 1). Direct differentiation of hPSCs hardly occurs with the ectopic expression of TFs alone. The forced introduction of epigenetic-modifying factors in hPSCs can facilitate the TF-mediated myogenic differentiation by bypassing or rapidly proceeding with the mesoderm stage. The combinatory approach using chromatin modifying factors and TFs will enhance the efficiency and robustness of RNA-based differentiation systems: an ideal method for generating footprint-free differentiated cells. Moreover, epigenetic variations are thought to be the main cause of significant variation in the differentiation capacities of different hiPSC lines [56–58]. Manipulating epigenetic states by using chromatin-modifying factors will allow the alteration of the epigenetic patterns of even low-potential hiPSC lines and improve their differentiation capacity.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Myogenic Differentiation from *MYOGENIN*-Mutated Human iPS Cells by CRISPR/Cas9

Koki Higashioka,[1,2] Noriko Koizumi,[2] Hidetoshi Sakurai,[3] Chie Sotozono,[1] and Takahiko Sato[1]

[1]*Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[2]*Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan*
[3]*Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan*

Correspondence should be addressed to Takahiko Sato; takahiko@koto.kpu-m.ac.jp

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It is well known that myogenic regulatory factors encoded by the *Myod1* family of genes have pivotal roles in myogenesis, with partially overlapping functions, as demonstrated for the mouse embryo. *Myogenin*-mutant mice, however, exhibit severe myogenic defects without compensation by other myogenic factors. MYOGENIN might be expected to have an analogous function in human myogenic cells. To verify this hypothesis, we generated *MYOGENIN*-mutated human iPS cells by using CRISPR/Cas9 genome-editing technology. Our results suggest that MYOD1-independent or MYOD1-dependent mechanisms can compensate for the loss of MYOGENIN and that these mechanisms are likely to be crucial for regulating skeletal muscle differentiation and formation.

1. Introduction

In vertebrate embryos, skeletal muscles of the trunk and limbs are derived from the somites, from the dermomyotome which gives rise to myogenic progenitor cells that are directed into the skeletal muscle programme by four myogenic bHLH transcription factors, Myf5, Myod1, Mrf4, and Myogenin (Myog) [1–3]. The myogenic differentiation process in vertebrate embryos is regulated by these factors leading to the formation of multinucleated myotubes and subsequently to the regeneration of skeletal muscle by a reserve of myogenic stem cells in adulthood [4, 5].

Single or compound knockout mice for the genes encoding these myogenic factors have been created to identify their function in myogenesis [3]. Single *Myf5*- or *Myod1*-deficient mice revealed no striking skeletal muscle phenotype [6–8], pointing to overlapping functions between these myogenic determination factors [9–11]. *Mrf4*, which is coexpressed with *Myf5* at the onset of myogenesis, also acts as an early myogenic determination factor [11]. Double and triple mutants for these genes demonstrate their role in determining muscle cell fate. The fourth member of this gene family, *Myog*, is expressed at the onset of muscle cell differentiation. Single *Myog*-deficient mice exhibit severe defects of skeletal muscle formation during development, at a stage when Myod1 and, in many muscles, Mrf4 are also present. This therefore demonstrates that Myog is required for embryonic muscle differentiation, and no redundant or compensatory mechanisms replace its function, unlike for the other myogenic regulatory factors [10, 12–15].

In this study, we have tackled the question of whether these myogenic factors have analogous interrelationships in human myogenesis and, in particular, whether human MYOGENIN (MYOG) is also essential for muscle cell differentiation and muscle fiber formation in myogenic cells derived from human induced pluripotent stem (hiPS) cells. To perform functional experiments, we have used versatile genome-editing technology, with the CRISPR/Cas9 system [16, 17].

Table 1: Candidates and oligos for *MYOGENIN* exon1 target positions for CRISPR/Cas9.

Position		Target sequence	Sequence information		Number of target sites		
Start–end	+/−	20 bp + **PAM**	GC (%)	Tm (°C)	20 bp	12 bp	8 bp
143–165	−	**cct**gcctgtccacctccagggct	70	84	1	61	5171
147–169	−	**cct**gtccacctccagggcttcga	65	80	1	38	92,481
152–174	−	**cca**cctccagggcttcgaaccac	65	79	1	89	10,513
155–177	−	**cct**ccagggcttcgaaccaccag	65	79	1	8	11,331
156–178	+	ctccagggcttcgaaccacc**agg**	65	79	1	1	4654
158–180	−	**cca**gggcttcgaaccaccaggct	65	81	1	7	21,861
166–188	+	tcgaaccaccaggctacgag**cgg**	60	77	1	10	12,349
170–192	**+**	accaccaggctacgagcgga**cgg**	65	82	1	1	3812
171–193	−	**cca**ccaggctacgagcggacgga	70	82	1	8	1537
174–196	−	**cca**ggctacgagcggacggagct	70	83	1	3	479
191–213	+	**gga**gctcaccctgagccccgagg	75	84	1	43	382

pX458-*hMYOG*+189_F primer: CACCaccaccaggctacgagcgga. pX458-*hMYOG*+189_R primer: AAACtccgctcgtagcctggtggt.

2. Materials and Methods

2.1. Gene Targeting with Human iPS Cells. The *hMYOG*-targeting plasmid vector, pX458-*hMYOG*+189, was constructed using the pX458 vector (Addgene #48138, Cambridge, USA) [17] with ligating oligos (Table 1) as described, and plasmid DNA was introduced into HEK293- or Hu5/KD3-immortalized human myogenic cells [18], with ViaFect reagent (Promega, Madison, USA). The electroporator NEPA21 (NEPA GENE, Chiba, Japan) was used for introducing plasmids into hiPS cells [19].

2.2. Cell Culture and Myogenic Differentiation. The hiPS cells were maintained on SNL feeder cells, treated with 10 μg/ml of mitomycin (Sigma, St. Louis, USA) in DMEM (Wako, Osaka, Japan) supplemented with 10% of fetal bovine serum (GIBCO, Grand Island, USA), or expanded in Primate ES cell medium (ReproCELL, Kanagawa, Japan) supplemented with 10 ng/ml of recombinant human FGF2 (bFGF; Wako, Osaka, Japan) and 100 μg/ml of G418 (Nacalai Tesque, Kyoto, Japan). *MYOG*-deficient iPS cells were maintained on SNL feeder cells or iMatrix-511 (Nippi, Tokyo, Japan)-coated plates with StemFit AK03N (Ajinomoto, Tokyo, Japan) under a feeder-free culture system [20].

For the derivation of myogenic cells from hiPS cells, the detailed protocol of Tanaka et al., based on MYOD1 induction [21], was followed. In brief, single iPS cells carrying an inducible MYOD1 activation system were expanded in Primate ES cell medium without bFGF and with 10 μM of Y-27632 (Nacalai Tesque, Kyoto, Japan) for 24 hours and then induced into myogenic cells by adding 500 ng/ml of doxycycline (Dox; Tocris, Bristol, UK). After 24 hours, culture medium was changed into myogenic differentiation medium composed of alpha-MEM (Nacalai Tesque, Kyoto, Japan) with 5% of KSR (GIBCO, Grand Island, USA) and 500 ng/ml of Dox. After 6 days, culture medium was changed into muscle maturation medium, DMEM/F12 (Nacalai Tesque, Kyoto, Japan), with 5% of horse serum (Sigma, St. Louis, USA), 10 ng/ml of recombinant human insulin-like growth factor 1 (IGF-1; PeproTech, Hartford County, USA), and 200 μM of 2-mercaptoethanol (2-ME; Sigma, St. Louis, USA).

To obtain myogenic cells derived from embryonic mesodermal cells, single iPS cells were expanded in StemFit AK03N supplemented with 10 μM of Y-27632. After 2 days, the culture medium was changed into modified mesodermal differentiation medium as described by Loh et al. [22]. Cultured cells were passaged 12 days later and cultured in mesoderm differentiation medium with 10 μM of Y-27632 for 2 days. To initiate myogenic differentiation, medium SF-O3 (EIDIA, Tokyo, Japan), supplemented with 10 ng/ml of bFGF, 10 ng/ml of IGF-1, 10 ng/ml of HGF (PeproTech, Hartford, USA), and 200 μM of 2-ME, was used and changed into myogenic differentiation medium with IGF-1 after 4 days and then with IGF-1 and HGF after 3 days [23]. To obtain more mature myogenic differentiation, culture medium was changed into DMEM/F12 supplemented with 2% of horse serum, 10 ng/ml of IGF-1, and 200 μM of 2-ME 2 weeks later and induced cells were harvested at day 60 [23].

2.3. Cell Sorting. Cultured cells transfected with pX458-*hMYOG*+189 were dissociated with TrypLE select (GIBCO, Grand Island, USA) at 37°C for 5 min for detecting transfected cells. Dissociated cells were resuspended with 1% bovine serum albumin in PBS. Cell debris were eliminated with a cell strainer (35 μm; BD, New Jersey, USA), and suspensions were stained with propidium iodide (Molecular Probes, Eugene, USA) to exclude dead cells. Cells were analyzed and collected by a cell sorter using FACSJazz (BD, New Jersey, USA).

2.4. Quantitative PCR Analyses. Total RNAs from sorted or cultured cells were extracted using the RNeasy micro kit (QIAGEN, Hilden, Germany). For quantitative PCR analyses, single strand cDNA was prepared using a SuperScript VILO kit (Invitrogen, Carlsbad, USA) as in the manufacturer's protocol. All RT-qPCR reactions were carried out in triplicate using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan), normalized to the mRNA expression level of

Table 2: Primer sequences for T7 endonuclease assay and quantitative RT-PCR.

Genes	Sequences	Amplicon size
Primer for T7 EI assay		
MYOG gDNA_F	5′-GGCCGCCCAGCTAGGAGTAATTGA-3′	786
MYOG exon1_R	5′-CGCTCGATGTACTGGATGGCACTG-3′	
Primer for RT-qPCR		
*RPL13A*_F	5′-CCCTGGAGGAGAAGAGGAAA-3′	91
*RPL13A*_R	5′-ACGTTCTTCTCGGCCTGTTT-3′	
*MYOG*_F	5′-GCTCAGCTCCCTCAACCA-3′	94
*MYOG*_R	5′-GCTGTGAGAGCTGCATTCG-3′	
*MYOD1*_F	5′-GCACTACAGCGGCGACTCC-3′	118
*MYOD1*_R	5′-GTAGGCGCCTTCGTAGCAG-3′	
Endo-*MYOD1*_F	5′-CACTCCGGTCCCAAATGTAG-3′	180
Endo-*MYOD1*_R	5′-TTCCCTGTAGCACCACACAC-3′	
*MRF4*_F	5′-GGCCAAGTGTTTCCGATCAT-3′	89
*MRF4*_R	5′-AAGGCTACTCGAGGCTGACG-3′	

ribosomal protein L13A (RPL13A). Primer sequences (5′ to 3′) are listed in Table 2.

2.5. Immunofluorescence Assay. Cultured cells were fixed in 4% paraformaldehyde for 10 min at 4°C, permeabilized with 0.2% Triton and 50 mM NH_4Cl. Fixed samples were pretreated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 30 min at RT and incubated with anti-MYOGENIN (diluted 1 : 100, Santa Cruz Biotechnology, California, USA), anti-MYOSIN HEAVY CHAIN (MYHC, diluted 1 : 200, Santa Cruz Biotechnology, California, USA), anti-TRA-1-81 (diluted 1 : 200, Cell Signaling Technology, Massachusetts, USA), anti-SSEA4 (diluted 1 : 200, Cell Signaling Technology, Massachusetts, USA), anti-OCT4A (diluted 1 : 200, Cell Signaling Technology, Massachusetts, USA), and anti-NANOG (diluted 1 : 200, Cell Signaling Technology, Massachusetts, USA) antibodies in 5% of Blocking One in PBS with 0.1% Tween20 (PBST) overnight at 4°C. After three washes with PBST, cells were incubated with Alexa488-, Alexa594-, or Alexa647-conjugated secondary antibodies (diluted 1 : 500, Molecular Probes, Eugene, USA). Cells were washed with PBST three times and mounted in SlowFade Diamond antifade mountant with DAPI (Molecular Probes, Eugene, USA). Fluorescent images were collected on the software of BZ-X700 (Keyence, Osaka, Japan). Cultured cells were analyzed from triplicate experiments.

3. Results

3.1. Human MYOGENIN Genomic DNA Editing with the CRISPR/Cas9 System. To generate *MYOG*-mutated hiPS cells by double-strand break in *MYOG* exon1 which includes coding sequence (Figure 1(a)), we selected several sequences bound to single guide RNA for targeting by nuclease Cas9 from the CRISPRdirect website as candidates (http://crispr.dbcls.jp, Table 1) [24] and ligated them into the pX458 vector to create the pX458-*hMYOG*+189-editing vector, which targets a unique 20 bp sequence in *hMYOG* exon1 (position 170–192; accaccaggctacgagcgga, Figure 1(b)). The effect of a double-strand break in *hMYOG* genomic sequences was evaluated by heteroduplex PCR fragments, involving the sequences targeted by the pX458-*hMYOG*+189-editing vector, monitored in HEK293 cells and T7 endonuclease I (T7EI). Enzymatic digested PCR bands of 500 bp and 300 bp were observed in T7EI-treated genomic DNA (Figure 1(c)). The data suggested that nuclease Cas9 and single guide RNA target *hMYOG* genomic sequences of exon1. The expression of *MYOG* is initiated in differentiating myogenic cells. To check the amount of *MYOG* transcripts produced from this Cas9 construct, immortalized Hu5/KD3, human myoblasts, transfected with or without the pX458-*hMYOG*+189 vector were differentiated in medium with 2% horse serum for 48 hours. The transcriptional level of *MYOG* was attenuated in differentiated Hu5/KD3 cells (Figure 1(d)). This CRISPR/Cas9 construct for *hMYOG* sequences may not only be effective because of its genomic double-strand break which knocks out *MYOG* expression but may also affect the remaining *MYOG* transcription level.

3.2. Generation of MYOGENIN-Mutated hiPS Cells. In order to generate *MYOG*-mutated hiPS cells, we used hiPS cells carrying a *MYOD1* expression construct which is inducible with Dox to activate the myogenic programme (Figure 2(a)) [21]. The iPS cells were expanded on SNL feeder-coated plates after electroporation with pX458-*hMYOG*+189 vector for 48 hours, and GFP-positive cells were collected by cell sorting (Figures 2(b) and 2(c)). These cells were plated out to form colonies which were individually picked up. Each clone was screened for further analyses.

We were able to identify 25 clones, which were lacking the wild-type *MYOG* sequences (wild type: 19.4%, heterozygotes; 64.5%, homozygotes; and 16.1%, total screened clones $n = 31$) by checking genomic sequences around the targeted *MYOG* region. Selected clone number 28 or clone number C3 was confirmed to have biallelic on-target frameshift

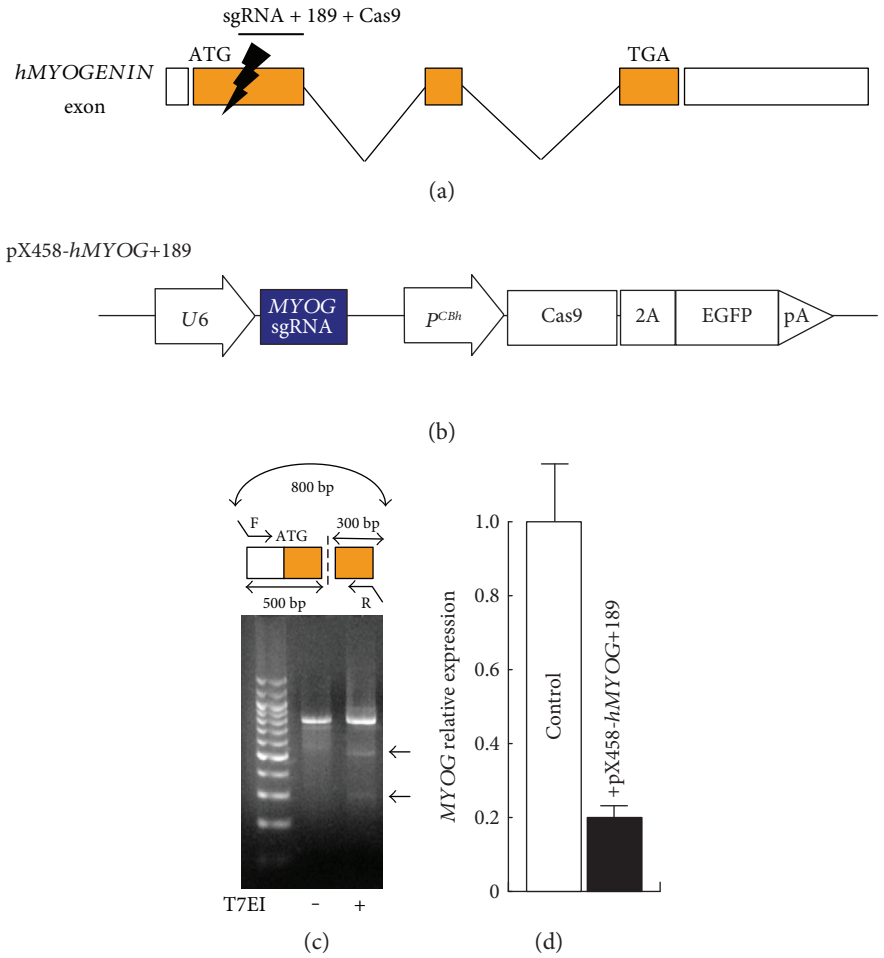

Figure 1: Effect of single guide sequence for *hMYOGENIN* by the CRISPR/Cas9 system. A schematic representation of *MYOG* exons and introns. A candidate position for Cas9 targeting of *MYOG* exon1 (a). pX458-*hMYOG*+189, a construct for driving single guide RNA bound to *MYOG* exon1 and bicistronic expression of both Cas9 and GFP (b). T7 endonuclease I assay for Cas9-mediated cleavage (arrows, 500 bp and 300 bp) on an agarose gel, showing comparable modification of the targeted human *MYOG* genomic fragment in HEK293T cells (c). Relative expression of *MYOG* in Hu5-immortalized human myoblast cells transfected with or without the pX458-*hMYOG*+189 vector. All error bars indicate ±SEM ($n = 3$).

mutations, 5 bp of deletion, and an extra 1 bp of integration in the *hMYOG*-sgRNA and Cas9-targeted region as shown in Figure 2(d). These data suggest that this targeting CRISPR/Cas9 system is sufficiently efficient to knockout both alleles of *MYOG* directly by introducing out-of-frame mutations (lower images in Figure 2(f)). *MYOG*-mutated hiPS cells (clone number 28) were immunostained with undifferentiated pluripotent markers, anti-SSEA4, anti-OCT3/4, anti-TRA1-80, and anti-NANOG antibodies, to evaluate the undifferentiated pluripotent state, and these markers were detected positively in *MYOG*-mutated hiPS cells (Figure 2(e)). To confirm the translation of truncated MYOG protein from these mutated sequences, myogenic cells differentiated from Dox-treated hiPS cells for 7 days were immunoreacted with antibodies against human MYOG N-terminus and C-terminus relatively because *MYOG* mRNAs are transcribed with the extra stop codon, which results from the *MYOG* gene targeting. Myogenic cells derived from wild-type hiPS cells were detected by both of these MYOG antibodies; however, the C-terminus of MYOG was not detected in *MYOG*-mutated hiPS cells (Figure 2(f)).

3.3. Skeletal Myogenic Differentiation by MYOD1 Induction. To investigate human MYOG function during myogenic differentiation, MYOD1 was overexpressed in hiPS cells by administrating Dox as shown in Figure 3(a). *MYOD1* expression mimics bicistronic mCherry fluorescence after Dox treatment (Figure 3(b)). Induced myogenic cells derived from hiPS cells were cultured in vitro under differentiation conditions and immunostained for MYHC expression as an indicator of their ability to differentiate into skeletal muscle fibers (Figure 3(c)). Although the rate of myoblast fusion in *MYOG*-mutated hiPS cell clone number 28 was slightly less than that of wild type (Figure 3(d)), terminal differentiation is similar.

To further characterize the differentiation of these myogenic cells, RNA expression of myogenic factors was analyzed by quantitative RT-PCR. The transcript for *MYOG* was downregulated as shown in Figure 1(d) with unknown mechanisms; however, other myogenic factors, notably

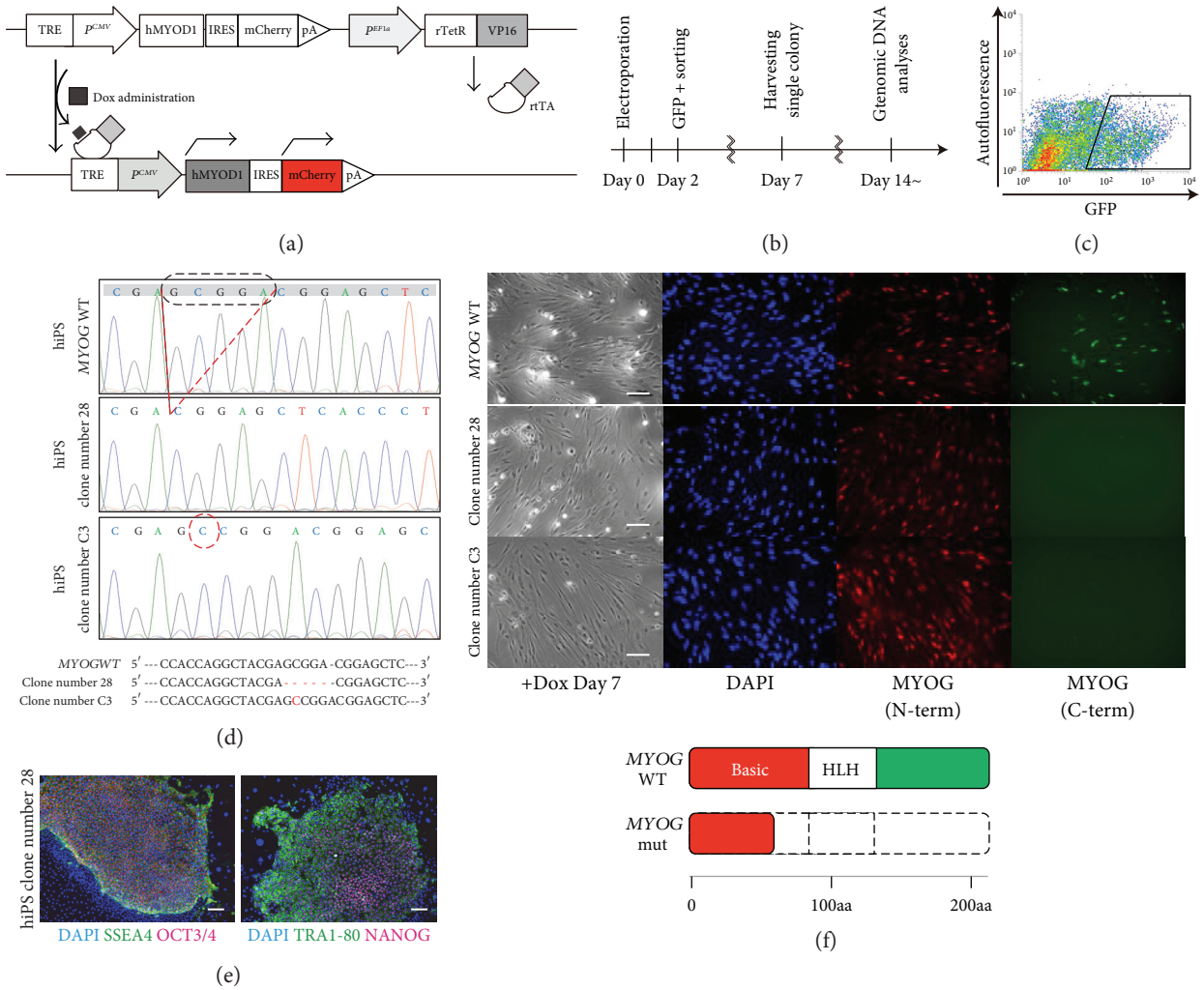

FIGURE 2: Generation of *hMYOGENIN*-mutated hiPS cells. A schematic model for the induction of myogenic cells derived from hiPS cells by overexpression of *MYOD1* marked with mCherry (red) after administrating Dox (a). A flowchart of the time course for the identification of *MYOG*-mutated hiPS cells (b). FACS analyses to isolate hiPS cells after the introduction of the pX458-*hMYOG*+189 vector (c). Genomic sequence data around the region targeted by pX458-*hMYOG*+189. 5 bp of deletion in clone number 28 and 1 bp of insertion in clone number C3 (dashed lines, (d)). Established hiPS cells were immunostained with undifferentiated pluripotent cell markers, anti-SSEA4 (green in left panel), anti-OCT3/4 (red in left panel), anti-TRA1-80 (green in right panel), and anti-NANOG (red in right panel) antibodies. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 100 μm (e). Differentiated myogenic cells after Dox treatment for 7 days were immunostained with anti-MYOG N-terminus (N-term, red) and C-terminus (C-term, green) antibodies. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 100 μm (upper in (f)). Putative MYOG protein structures both in wild-type (*MYOG* WT) and mutated cells (*MYOG* mut) (lower in (f)).

transcripts of *MYOD*1 or *MRF4*, were upregulated under conditions where *MYOG* is mutated in human myogenic cells (Figures 3(e)–3(g)).

3.4. Skeletal Muscle Differentiation via Mesodermal Differentiation In Vitro. Transient overexpression of *MYOD1* might have overcome the effect of MYOG deficiency because artificially high MYOD1 may compensate the inactivation of the *MYOG* gene in human myogenic cells. To avoid excessive MYOD1 levels, myogenic cells were induced from mesodermal precursors derived from hiPS cell clone number 28, without administration of Dox as shown in Figure 4(a).

The percentage of mesodermal induction marked by DLL1 [22] was shown by FACS analyses and was similar irrespective of *MYOG* mutation (Figure 4(b)). In myogenic cells derived from mesodermal precursors, total *MYOD1* transcripts did not accumulate, in contrast to Dox-treated hiPS cells, including lower level of endogenous *MYOD1* expression (Figure 4(c)). Under these conditions, MYHC-positive differentiated myofibers derived from both MYOG-positive and MYOG-negative hiPS cells were identified to a similar extent (Figure 4(d)). To analyze myogenic differentiation potential from mesodermal cells, transcripts of myogenic regulatory factors were monitored in these cells. The level of *MYOG* transcript was attenuated; however, *MYOD1* or *MRF4* transcripts were not much changed in wild-type and *MYOG*-mutated myogenic cells, as upregulated in *MYOG*-mutated cells during periods of cell culture (Figure 4(e)).

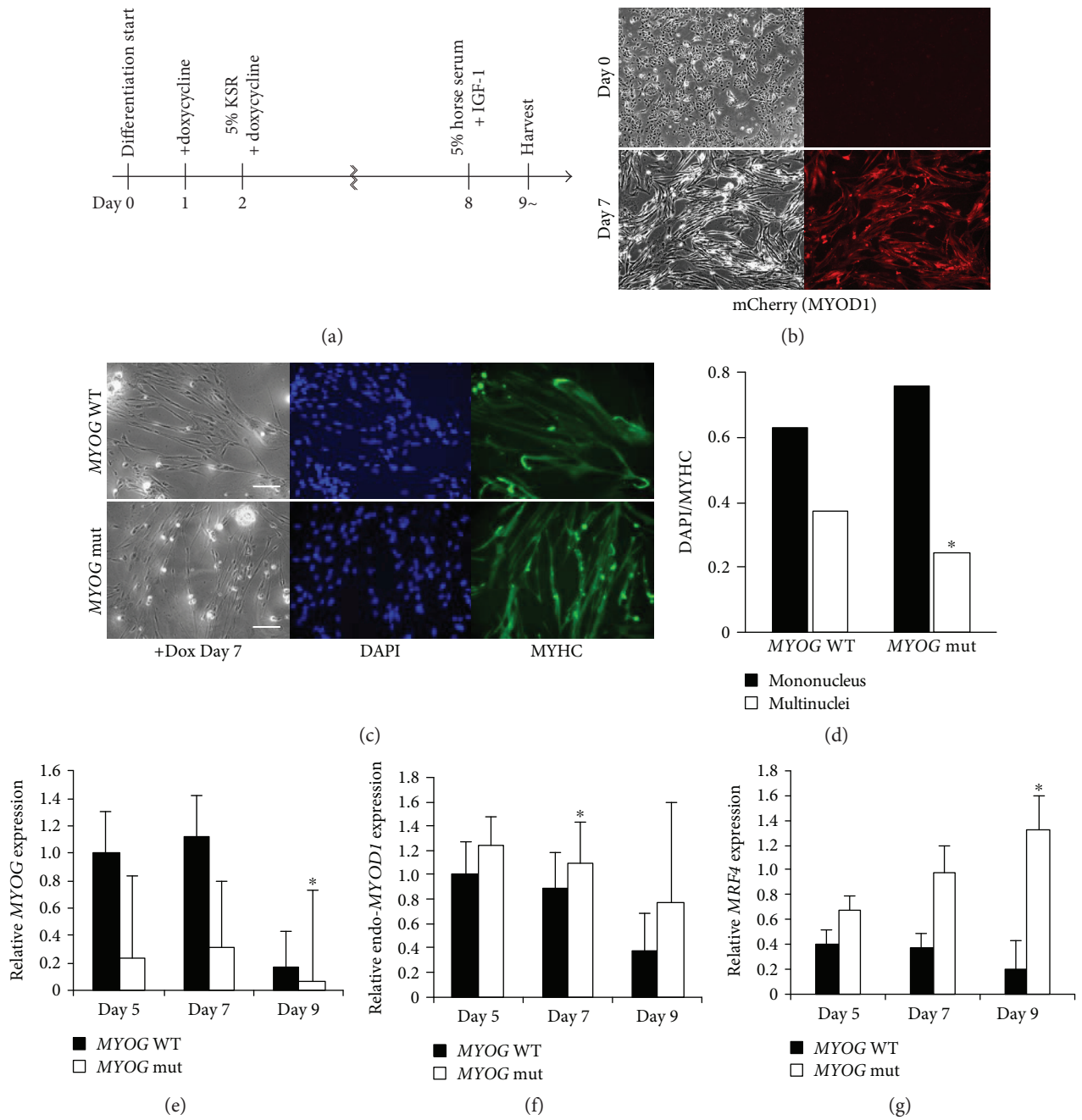

Figure 3: Myogenic differentiation in skeletal muscle cells derived from *MYOGENIN*-deficient hiPS clone number 28 cells. Myogenic differentiation flowchart of the time course (a). Morphological changes and mCherry fluorescent expression after treatment with Dox (b). Differentiated myogenic cells derived from hiPS cells with or without MYOG by Dox treatment for 7 days were immunostained with anti-MYOSIN HEAVY CHAIN (MYHC, green) antibody. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 100 μm (c). The ratio of DAPI-positive mono or multiple nuclei staining present in single MYHC-positive myofibers derived from wild-type or *MYOG*-mutated hiPS cells (d). Relative expression of transcripts for myogenic regulatory factors, *MYOG* (e), endogenous *MYOD1* (f), and *MRF4* (g), in differentiated myogenic cells treated with Dox for 5, 7, and 9 days. All error bars indicate ±SEM ($n = 3$). P values are determined by a t-test from a two-tailed distribution. $^*P < 0.05$.

4. Discussion

Here, we report the generation of *MYOGENIN*-deficient hiPS cells and the impact on human myogenic differentiation using CRISPR/Cas9 technology. This bacterial system has emerged as an effective tool for gene targeting through non-homologous end joining (NHEJ); however, it has been reported to be inefficient for precise editing of genomic sequences. In this study, we selected the sequence of *MYOG* exon1-targeted sgRNA with the Cas9 complex as a unique

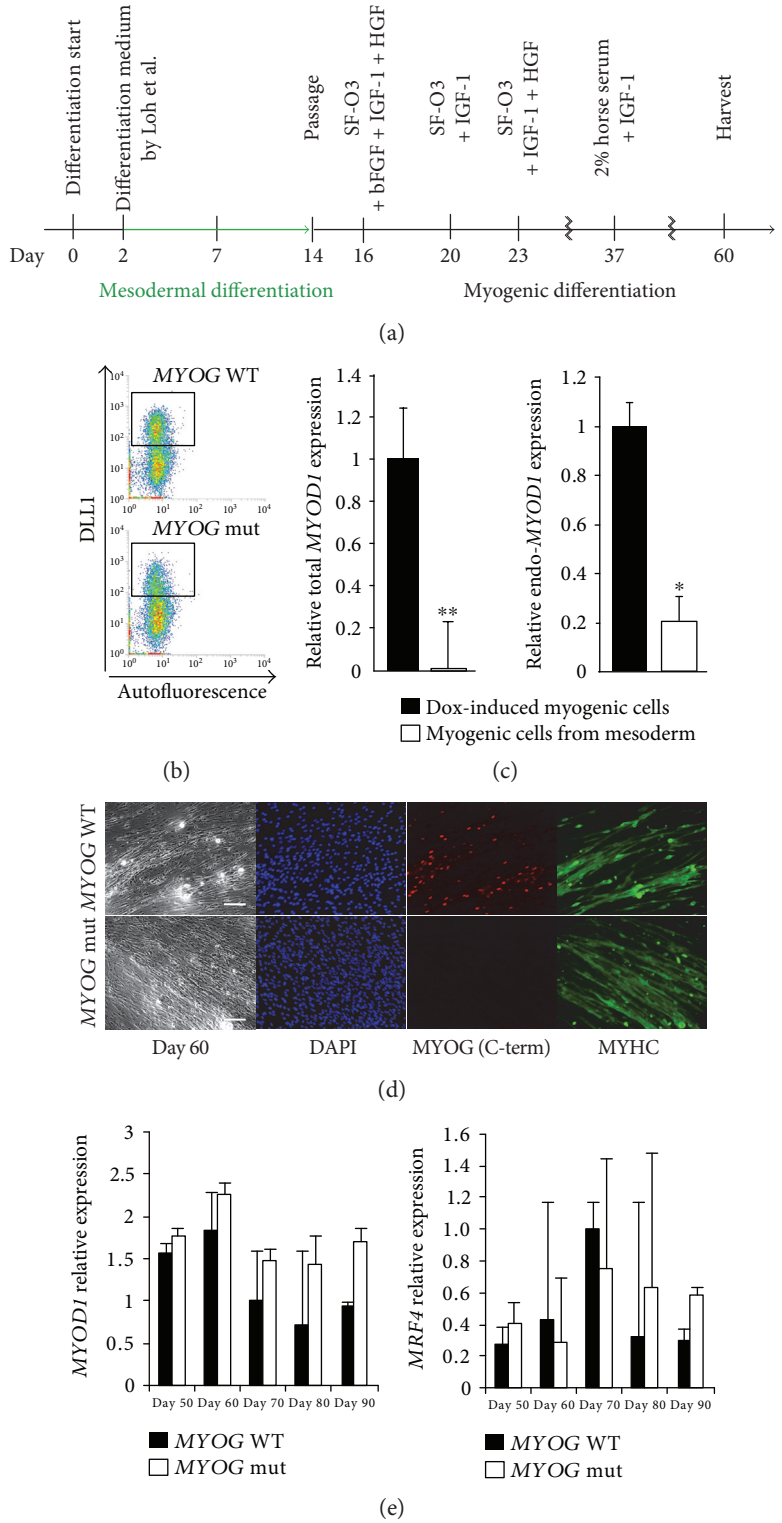

Figure 4: Myogenic differentiation from mesodermal precursors derived from *MYOGENIN*-deficient hiPS clone number 28 cells. Mesodermal and myogenic differentiation flowchart of the time course for muscle cells derived from hiPS cells (a). FACS analyses of DLL1-positive mesodermal cells derived from hiPS cells (b). Relative expression of total *MYOD1* and endogenous *MYOD1* (c). Differentiated myogenic cells derived from mesodermal cells with or without MYOG for 60 days were immunostained with anti-MYOSIN HEAVY CHAIN (MYHC, green) antibody. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 100 μm (d). Relative expressions of *MYOD1* and *MRF4* transcripts in wild-type or *MYOG*-mutated myogenic cells derived from mesodermal cells (e). All error bars indicate ±SEM ($n = 3$). P values are determined by a t-test from a two-tailed distribution. $^{*}P < 0.05$, $^{**}P < 0.01$.

in genomic sequence, which targeted *MYOG* by the T7EI assay not with high efficiency; however, the result of genomic editing in hiPS cells showed high efficiency for knocking out the *MYOG* gene, including in heterozygotes with an efficiency of over 80%. This was not changed with additional azidothymidine, which has been reported to increase the efficiency for NHEJ [25] (not shown).

While knockout mice of *Myog* exhibit a lethal deficiency of differentiated skeletal myofibers, there are nevertheless residual myofibers in *Myog* mutants [12, 13]. The possible differences between in vivo and in vitro situation of *Myog* mutants could be explained by the selection of a particular route to muscle cell differentiation from Myog-independent lineage in vitro, potentially controlled by MyoD1 and Mrf4 because Mrf4 can drive early myogenic differentiation in the myotome when Myog protein is not initially accumulated [10, 14]. Alternatively, there may be a threshold level of total myogenic regulatory factors required in myoblasts to trigger the terminal differentiation program. We have not observed any deficiencies of myogenic differentiation with *MYOG*-mutated cells under two different conditions, either with overexpression of MYOD1 or through medium conditions that promote mesodermal cell progression towards myogenesis. Mutated hiPS cells without not only *MYOG* but also other myogenic factors, *MYF5*, *MYOD1*, and *MRF4*, would be necessary for further analyses to identify the relationships of human myogenic regulatory factors because we observed the upregulation of other myogenic factors in *MYOG*-mutated cells which might compensate MYOG functions in vitro, and triple knockout of *Myog*, *Mrf4*, and *Myod1* or *Myf5*, *Myod1*, and *Mrf4* exhibited impaired ability to terminally differentiate into myofibers, not double knockout of *Myod1* and *Mrf4* [8, 10]. Moreover, there is also other possibility that Myog via skeletal muscle affects systemic factors in vivo [13] and that this feeds back on myofiber formation.

Taken together, these results demonstrate that *MYOG*-mutated human iPS cells have the capacity for myogenic differentiation and can form terminally differentiated myofibers, under differentiation conditions, in contrast to results on developing mouse *Myog* mutants.

Conflicts of Interest

No competing financial interests exist.

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