Chapter 7

Making Skeletal Muscle from Human Pluripotent Stem Cells

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Abstract

Human pluripotent stem cells (hPSCs) proliferate in vitro for long periods without losing pluripotency and can be induced to differentiate into various cell types including skeletal muscle cells (SMCs). Human embryonic stem cells (hESCs) are generated from a preimplantation-stage embryo. Human-induced pluripotent stem cells (hiPSCs) are derived from somatic cells of both healthy donors and patients with muscle diseases of any age using reprogramming factors. Currently, there are two kinds of protocols to induce skeletal muscle from hPSCs. One type utilizes overexpression of a potent myogenic master regulator, MyoD, to directly induce skeletal muscle. Stepwise induction of skeletal muscle has also been reported by many research groups, but hiPSC-based cell therapy for muscular dystrophy is still experimental. On the other hand, hiPSCs derived from patients with muscle disease are widely used for disease modeling in vitro. Here, we review the recent literature on derivation of skeletal muscle from human pluripotent stem cells and discuss their application.

Keywords: muscular dystrophy, myoblasts, skeletal muscle, pluripotent stem cells, iPSC, Pax7, MyoD, myogenic differentiation, cell therapy, disease modeling

1. Introduction

When injured, skeletal muscle regenerates by activation and proliferation of its own stem cells: muscle satellite cells. Therefore, muscle satellite cells were expected to be a cell source for cell therapy for devastating muscular dystrophies. However, clinical trials in the 1990s

were unsuccessful [1], possibly because myoblasts that had been expanded in vitro lost the high ability to fuse with the host's injured myofibers, indicating that improvement of muscle function requires a large quantity of myogenic progenitors with regenerative potential. Human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) [2] have almost unlimited proliferative potential and the ability to differentiate into the skeletal muscle lineage (reviewed in [3, 4]). Therefore, they are a promising source of new cells for cell therapy of muscle diseases such as muscular dystrophy (DMD). They are also useful for biological and physiological studies of human skeletal muscle.

Disease-specific hiPS cells are generated from patients' somatic cells (usually blood cells or skin fibroblasts) with almost the same efficiency and quality as cells from healthy donors [5]. Therefore, disease-specific-induced pluripotent stem (iPS) cells are being widely used to study the molecular mechanisms of these diseases and to screen potential drugs. As a method to directly derive skeletal muscle cells from hPSCs, MyoD-mediated reprogramming was established and is widely used. MyoD induces muscle cells in a relatively short period with high efficiency. In this chapter, we review the literature on derivation of skeletal muscle from human PSCs and discuss the next steps toward clinical applications. In the last part, we review the recent reports on successful disease modeling in vitro using patient iPS cells and discuss future directions.

1.1. Muscle stem cells

Muscle satellite cells were first identified by electron microscopy as a mononuclear cell between myofibers and the basal lamina and named by Mauro in 1961 [6]. Later, muscle satellite cells were shown to be skeletal muscle-specific stem cells in postnatal muscle [7]. Muscle satellite cells are activated upon muscle injury, proliferate as myoblasts, and fuse with each other or with regenerating myofibers to repair damaged muscle [8]. In 1989, Partridge et al. reported successful recovery of dystrophin expression in dystrophin-deficient mdx mice after direct injection of wild-type myoblasts [9]. Based on this finding, myoblast transfer therapy was performed on DMD patients in several hospitals. Unfortunately, the clinical trials failed to recover the muscle function of these patients. The majority of injected cells seemed to be lost within 48 h [1]. The results were unexpected at that time because endogenous muscle satellite cells themselves have high regenerative activity in situ and repair damaged muscle quickly. Later, researchers started to search for multipotent stem cells, which can be delivered systemically, engraft in muscle, and differentiate into myofibers. One of these cells is the mesoangioblalst, which showed an amazing ability to recover dystrophin expression in the muscles of dystrophic dogs after intra-arterial injection [10]. Mesenshymal stem/progenitor cells (MSC/MPC) are also expected to be a tool for regenerative medicine. They themselves do not differentiate into myofibers, but support muscle regeneration by paracrine effects [11]. The history of direct reprogramming in the muscle field is long, starting with the discovery of MyoD by Weintraub and his colleagues [12, 13]. MyoD powerfully converts non-muscle cells to skeletal muscle cells, but it is difficult to induce Pax7+ myogenic progenitors using MyoD alone. Recently Ito et al. reported that a combination of transcription factors (*Pax3*, *Mef2b*, and *Pitx1* or *Pax7*, *Mef2b*, and *Pitx1* for embryonic fibroblasts, and *Pax7*, *Mef2b* plus *MyoD* for adult fibroblasts) successfully induced transplantable myogenic progenitors from mouse fibroblasts [14]. Whether the same set of reprogramming factors can induce myogenic progenitors from human fibroblasts remains to be seen. Human iPS cells are relative newcomers in the muscle stem cell field. hiPSCs are pluripotent stem cells with almost

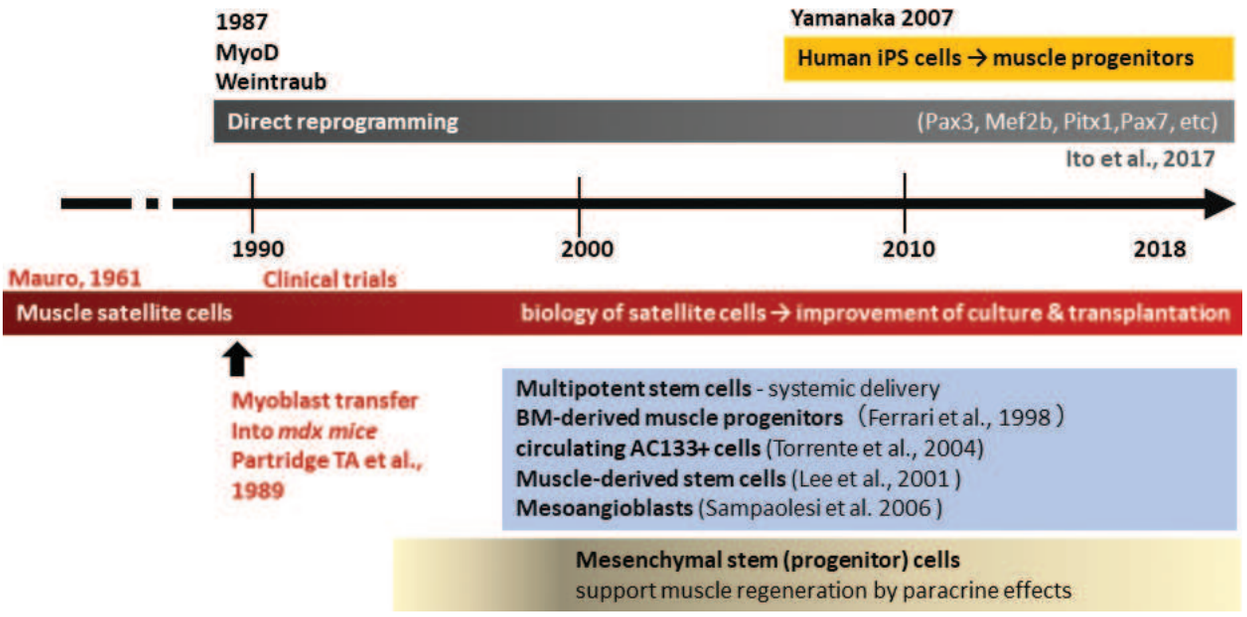

Figure 1. History of research on muscle stem cells and cell therapy for muscular dystrophies [2, 6, 10, 15–18].

equivalent properties to human ES cells, but can be derived from somatic cells such as skin fibroblasts [2]. Successful derivation of muscle cells from hiPS cells opened a new era of regenerative medicine for muscular dystrophies (**Figure 1**).

Muscle satellite cells were identified and named by Mauro in 1961 [6]. Direct reprogramming was reported for the first time in the skeletal muscle field in 1987 [15], but MyoD alone cannot induce myogenic progenitors. After a surprising report of BM-derived myogenic cells by Ferrari in 1998 [16], researchers looked for multipotent stem cells that can be delivered via the circulation. MSCs are modulators of muscle regeneration and widely used in regenerative medicine. Human iPS cells are a relative newcomer in the muscle stem cell field.

2. Myogenic induction by overexpression of myogenic transcription factors

MyoD: Weintraub and colleagues showed that overexpression of MyoD, a muscle-specific basic helix-loop-helix transcription factor, converted non-myogenic cells to muscle cells [13]. In 2014, Abujarour et al. reported efficient conversion of hiPSCs into muscle cells using a lentiviral vector-mediated doxycycline (DOX)-inducible MyoD overexpression system [19]. Sakurai and his colleagues used a PiggyBac transposon system to overexpress DOX-inducible MyoD in hiPSCs [20]. Importantly, the induced myotubes contracted on electrical stimulation [20]. MyoD induces skeletal muscle cells in a short period with high efficiency, but it cannot induce PAX7(+) myogenic progenitors. MyoD-induced skeletal muscle is now widely used for in vitro modeling of inherited muscle diseases.

Darabi et al. [21] reported derivation of engraftable muscle progenitors from hiPSCs by using a lentiviral vector encoding DOX-inducible PAX7. PAX7 expression was induced transiently in differentiating cells in a monolayer culture after a 7-day embryoid body (EB) culture [21].

Pax7-expressing GFP-positive cells purified by fluorescence-activated cell sorting (FACS) were then transplanted into the skeletal muscle of immune-deficient dystrophin-deficient mice, *NSG-mdx*^{4Cv}, and improved the muscle function of the mice. Whether myogenic cells induced by overexpression of Pax7 are suitable for cell transplantation therapy remains to be determined because transgenes have a risk of tumor formation. As far as the integration sites of the expression units in the genome and the expression of PAX7 are strictly regulated, myogenic progenitors expanded by PAX7 successfully regenerated damaged muscle of patients [22].

3. Stepwise induction of skeletal muscle by mimicking development

The majority of stepwise muscle induction protocols recently reported for human ESCs/iPSCs utilize a GSK3b inhibitor in common, myogenic growth factors (HGF, IGF-1, bFGF, EGF, etc.), and a serum-free medium (**Table 1**; [3]). For example, Chal et al. treated hiPS cells with CHIR-99021, which activates Wnt signaling, and LDN-193189, which prevents hiPSCs from differentiation into lateral mesoderm and induces differentiation into paraxial mesoderm. Treatment with these molecules of hESC cultures induced myogenin(+) myogenic cells with 25–30% efficiencies [34, 36, 39].

EB culture and sphere culture are often used to induce muscle progenitor cells (**Table 1**). Hosoyama et al. reported that hESCs/hiPSCs cultured as floating cell aggregates (termed EZ

Authors	Year	Journals	Cell types	Factors	Engraftment	Culture method/FACS sorting
Barberi et al.	2005	Plos Med	hESC	OP9 and C2C12 coculture		CD73(+) MSC sorting
Barberi et al.	2007	Nat Med	hESC	OP9 coculture, insulin		CD73(+) MSC sorting NCAM(+) cell sorting
Teng et al.	2010	J Cell Biochem	hESC	TGFβ inhibitor		EB culture
Ryan et al.	2012	Stem Cell Rev and Rep	hESC	FGF-2, All-trans RA		EB culture
Awaya et al.	2012	PloS One	hESC/hiPSC		yes	EB culture
Sakurai et al.	2012	PLoS One	hESC	LiCl, BMP4, ActivinA		2D culture
Xu et al.	2013	Cell	hiPSC	GSK3β inhibitor, FGF-2, forskolin	yes	EB culture
Leung et al.	2013	Biomacromolecules	hESC	C-PLC nanofibers, Wnt3a		2D culture
Borchin et al.	2013	Stem Cell Reports	iPSC	GSK3β inhibitor		2D culture c-met(+) cell sorting
Hosoyama et al.	2013	Stem Cell Transl Med	hESC/hiPSC	FGF-2, EGF		EZ sphere culture
Shelton et al.	2014	Cell Reports	hESC/hiPSC	GSK3β inhibitor, BMP, VEGF, Inhibinβ, FGF-2		EB culture
Chal et al.	2015	Nat Biotechnol	hESC/hiPSC	GSK3β inhibitor, BMP inhibitor		2D culture
Swartz et al	2016	Stem Cells Transl Med.	hiPSC	Transferrin, 1-thioglycerol, insulin, FGF-2, BMP4, PI3-kinase inhibitor , GSK3β inhibitor		2D culture
Chal et al.	2016	Nat Protoc	hiPSC	GSK3β inhibitor, BMP inhibitor, FGF-2, HGF, IGF-1	yes	2D culture
Caron et al.	2016	Stem Cell Transl Med	hESC	GSK3β inhibitor, Ascorbic acid, Alk5 inhibitor, Dex, EGFm insulin		2D culture
Hicks et al.	2018	Nat Cell Biol.	hiPSC	GSK3β inhibitor, BMP, VEGF, Inhibinβ, FGF-2 TGF-β inhibitor	yes	2D culture ERBB3(+) NGFR(+) cell sorting

Table 1. Papers reporting myogenic induction without transgenes from hPSCs [23–38].

spheres) in a medium developed for neural stem cells supplemented with bFGF and EGF efficiently differentiated into myogenic cells. After a 6-week floating culture, 40–50% of cells expressed PAX7, MyoD or myogenin [32]. Because dissociation of sphere cells into single cells during the induction process drastically reduces myogenic activity, direct cell-cell interaction might be essential for commitment of hPSCs to the skeletal muscle lineage.

4. Mimicking the muscle microenvironment

4.1. Importance of the extra-cellular matrix for myogenesis

The extra-cellular matrix (ECM) is an interactive environment having, apart from its simple role as a mechanical support or physical barrier, a role in signaling and providing a niche for the stem cell [40].

Collagens, laminins, fibronectin, or Matrigel have been used for in vitro studies in order to mimic the ECM of muscle cells in 2D or 3D culture systems because ECM is indispensable for skeletal muscle development [41]. Interestingly, native ECM obtained by decellularization of skeletal muscle stimulated muscle differentiation and a more rapid cell organization compared to single matrix glycoprotein culture [42], indicating again that ECM has functions in myogenesis. The use of a 3D fibrin-based hydrogel culture after production of myogenic cells with transient overexpression of Pax7 was recently reported to generate functional biomimetic skeletal muscle tissues from hiPSC-derived paraxial mesoderm cells for the first time [43].

The stiffness of the microenvironment has also been reported to regulate differentiation of myogenic cells [44, 45]. More precisely, in an in vitro context, 12 kPa stiffness was shown to give optimal results for muscle stem cells, compared to the harsh 10^6 kPa stiffness of a regular polystyrene plastic culture dish [45]. The impact of stiffness can be partially explained by the activation of pathways such as N-RAP, FAK, or PKC [44].

Some studies showed improvement of myoblast migration and differentiation through MMP-1 treatment [46], migration with MMP-13 [47], and fusion through MMP-7 overexpression [48], suggesting the importance of remodeling of the ECM for migration and differentiation of myogenic cells even in vitro.

4.2. Co-culture, exosomes, and miRNA

The interaction between myogenic cells and non-myogenic cells in muscle tissue may influence myogenesis either by interacting directly or by secretion of paracrine factors (**Figure 2**). Motor neurons were used to form neuro-muscular junctions [49] and further adapted to a 3D culture system [50]. The presence of motor neurons had positive effects on myotube maturation [50]. Co-culture with mesenchymal stem cells (MSCs) significantly enhanced the proliferation of muscle cells [51]. Co-culture of fibroblasts with myoblasts improved the alignment of the formed myotubes, but reduced differentiation [52]. Another paper, however, reported that the myoblasts formed longer, thicker myotubes with a mature phenotype in the presence of fibroblast-conditioning media, compared with control media [53].

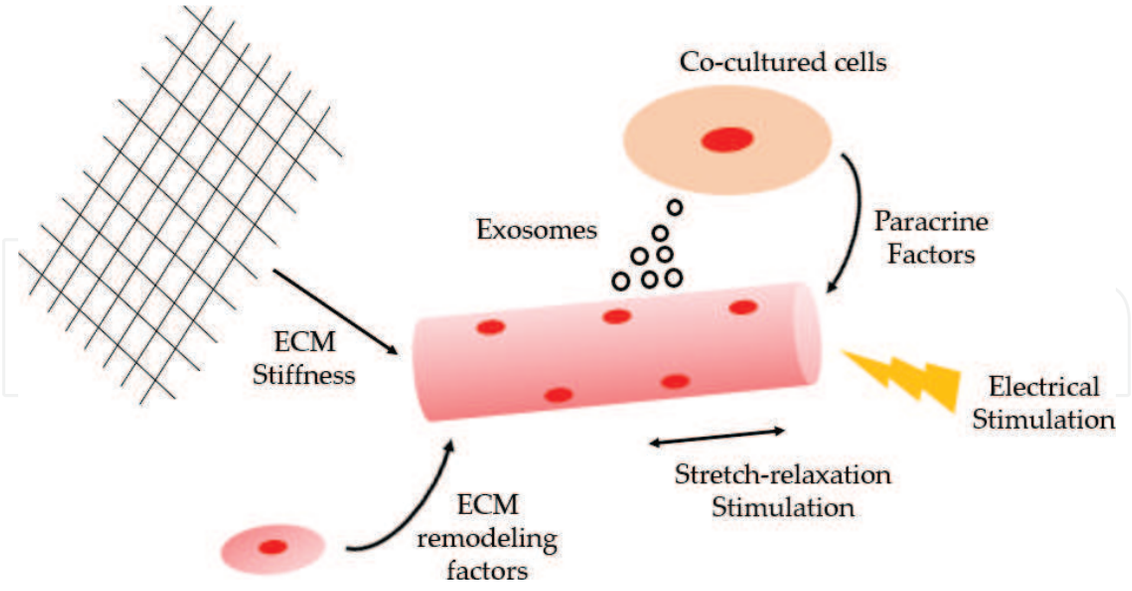

Figure 2. Summary of environmental inputs that promote differentiation of muscle cells.

Exosomes are major tools of cross talk between cells throughout the body and miRNAs are one of the elements they transport. Some miRNAs, such as miR-206, have been identified as promoters of skeletal muscle development and differentiation [54]. Therefore, miRNA-mediated induction of skeletal muscle from human iPSCs is a topic of great interest.

4.3. Differentiation of skeletal muscle cells through stimulation

Electrical: Another aspect of the muscle cell environment is the stimulation they are subjected to during the final steps of myogenesis. Exercise has been shown to have a great impact on muscle growth and differentiation in mice [55], and when electrically stimulated in vitro, C2C12 cells, a mouse myogenic cell line, showed similar responses to those of exercised skeletal muscle in vivo [56]. In the last couple of years, studies showed improvement of cardiac muscle differentiation of hiPSC by using electrical stimulation [57, 58].

Mechanical: Mechanical or stretch-relaxation stimulation has been shown to promote muscle growth and alignment in an electrical stimulus-independent manner [59]. Stretch-relaxation cycles have been shown to promote differentiation, muscle growth, fibers alignment, and overall organization [59–61]. Whether mechanical stimulation improves muscle induction from human iPS cells and their maturation remains to be determined even though results using cardiomyocytes are encouraging [58].

By mimicking the muscle microenvironment, it might be possible to increase the overall quantity, quality, and functionality of skeletal muscle cells produced from hiPSC.

5. Purification of myogenic cells

Most published protocols for muscle induction from human iPS cells generate a heterogenous cell population, including myogenic cells, undifferentiated cells, and non-myogenic cells. Non-myogenic cells are in many cases of neuronal lineage. Undifferentiated cells proliferate

actively and form tumors in the host muscle. Therefore, purification of myogenic progenitors is an important step for clinical use. Purification of myogenic cells would also facilitate the study of molecular pathogenesis and drug screening. To enrich myogenic cells by FACS, several iPS cell lines where the expression cassette of fluorescent proteins are inserted in the locus of myogenic regulators such as PAX7 [62] or Myf5 [63]. Combinations of myogenic cell-specific surface markers to enrich myogenic cells are also reported, for example, CD56 and CD82 [64], or CXCR4 (CD184) and C-MET [31]. We identified CD271 (NGFR) as a myogenic marker (submitted). M-cadherin antibody is also useful when the cells are dissociated into single cells by non-enzymatic treatment. Recently, Hicks et al. [38] identified ERBB3 (HER3) as a cell surface marker that enriches transplantable hiPSC-derived myogenic cells. To exclude neurogenic cells, CD57(HNK-1) is useful [31].

6. Myogenic progenitors for cell therapy

Cell source: Although cell therapy is a promising therapeutic approach to DMD and other muscular dystrophies, myoblast transfer therapy (MTT) in the early 90s failed to improve muscle function of DMD patients, possibly due to expansion of satellite cells *in vitro*. It was proposed that expansion of myoblasts in culture dishes reduced the regenerative activity of the injected cells. Incomplete immune suppression was also suggested as a cause of unsuccessful transplantation [1]. Because human iPSCs are highly proliferative, it might be possible to derive engraftable myogenic progenitors from hiPSCs on a large scale. iPSC-based cell therapy also allows the use of the patient's own cells (autologous cell transplantation) to avoid an immune response against engrafted cells. However, it takes a long time and is expensive to custom-make iPSCs from each patient and correct the disease-causing gene mutation. To solve this problem, HLA-homozygous donor-iPSC stocks are now being prepared (e.g., https://www.cira.kyoto-u.ac.jp/e/research/stock.html). It is expected that HLA-matched cells will greatly lower the risk of rejection. In addition, the safety of the cells in an iPS cell bank can be carefully examined in advance.

Tumorigenicity: A major concern of the use of hiPSCs for cell therapy is the tumorigenicity of hiPSC-derived cells. Tumor-forming cells could be roughly divided into two categories: residual undifferentiated cells and transformed cells. For undifferentiated iPS cells, purification of the differentiated cells with lineage-specific markers and elimination of undifferentiated cells using iPS markers would be effective. To avoid transformed cells, integration of the transgenes used for reprogramming into the genome should be carefully ruled out. Checking the integrity of the whole genome of parental iPS cells using genome-wide sequencing might be necessary. Prolonged culture of iPS cells should be avoided because long-term culture causes genomic abnormalities. Some groups propose, as a final line of defense, the use of a specific hiPSC line containing a HSV-tk gene [65] or an inducible Caspase-9 gene as a suicide gene, which would allow eradication of hiPSC-derived tumors in vivo after transplantation [66–69].

Quality control: For clinical use, (1) stable induction of myogenic progenitors in large quantity, (2) reduction of culture period and cost, and (3) establishment of a reliable system to monitor the quality and safety of the cells are all required. The monitoring system is especially important, because the cells change their properties during culture and it is difficult to keep the myogenic potential of the FACS-sorted cells high during culture.

7. Disease modeling in vitro using patient-derived iPSCs

During the past decades, researchers have generated numerous mouse models, such as knockout mice to analyze the pathogenesis of muscle diseases. However, mouse models often fail to reproduce the phenotypes of patients. For example, dystrophic *mdx* mice, which carry a DMD-type mutation in the dystrophin gene, exhibit much milder dystrophic phenotypes than DMD patients. It also has happened that drugs proven to be effective in a mouse model have much less effect in human patients. This is why human iPS cells are expected to become a tool for disease modeling.

These days, iPSCs from many kinds of muscular diseases have been established. For example, Abujarour et al. generated iPSC lines from patients with DMD or Becker muscular dystrophies (BMD) [19]. Shoji et al. established DMD-iPSCs and reported abnormal calcium ion influx in DMD myotubes. Importantly, dystrophin expression was restored to DMD myotubes by an exon-skipping technique, and the calcium ion overflow was suppressed [70]. Choi et al. [71] reported that DMD-iPS cells showed aberrant expression of inflammation or immune-response genes and collagen genes, increased BMP/TGFβ signaling, and reduced fusion competence. Tanaka et al. [20] established iPSC lines from patients with Miyoshi myopathy. Patient-iPSC-derived myotubes showed defective membrane repair, and the authors rescued the phenotype by expression of full-length DYSFERLIN. Snider et al. [72] showed the expression of full length DUX4 in embryoid bodies from iPSCs with facioscapulohumeral dystrophy (FSHD). Caron et al. reported that FSHD1 myotubes were thinner, and the genes involved in cell cycle control, oxidative stress response, and cell adhesion were differentially regulated [37]. Du et al. and Ueki et al. [73, 74] reported that the CTG-CAG triplet repeats were expanded by passaging iPSCs derived from myotonic dystrophy type1 (DM1) patients. Yoshida et al. [75] generated iPSCs from a patient with infantile-onset Pompe disease that showed lysosomal glycogen accumulation, which was dose-dependently rescued by rhGAA.

These successful in vitro disease modelings using patient-iPSCs are encouraging and would be useful for screening new drugs. Because hiPSCs have unlimited proliferative potential, one can perform experiments repeatedly and screen potential drugs extensively even if it is a rare disease. It should be, however, also recognized that skeletal muscle cells derived from hPSCs are much more immature in gene expression, morphology, and function than real myofibers in the body. Therefore, to what extent patient-derived iPSCs can reproduce a disease phenotype in vitro is important. In addition, there are variations in differentiation propensity among hiPS cell lines [76], and it is important to confirm the reproducibility of the results. Patient-iPS cells whose mutated genes were corrected by genome-editing technique [77] would serve as good controls and help to validate the findings.

8. Conclusion

Skeletal muscle can be induced from hPSCs by direct reprogramming or a stepwise differentiation method. Disease modeling using patient-derived iPSCs is now widely used to elucidate disease mechanisms and to screen for drugs. For successful disease modeling, it is important

to induce mature myofibers. For cell-based therapy, the protocols for induction of myogenic progenitors from hiPS cells and their purification have been almost completely established. However, to eliminate the risk of tumor formation by engrafted cells, more study is needed.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Notes/Thanks/Other declarations

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