Generation of skeletal muscle cells from embryonic and induced pluripotent stem cells as an in vitro model and for therapy of muscular dystrophies

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Abstract

Muscular dystrophies (MDs) are a heterogeneous group of inherited disorders characterized by progressive muscle wasting and weakness likely associated with exhaustion of muscle regeneration potential. At present, no cures or efficacious treatments are available for these diseases, but cell transplantation could be a potential therapeutic strategy. Transplantation of myoblasts using satellite cells or other myogenic cell populations has been attempted to promote muscle regeneration, based on the hypothesis that the donor cells repopulate the muscle and contribute to its regeneration. Embryonic stem cells (ESCs) and more recently induced pluripotent stem cells (iPSCs) could generate an unlimited source of differentiated cell types, including myogenic cells. Here we review the literature regarding the generation of myogenic cells considering the main techniques employed to date to elicit efficient differentiation of human and murine ESCs or iPSCs into skeletal muscle. We also critically analyse the possibility of using these cellular populations as an alternative source of myogenic cells for cell therapy of MDs.

Keywords: myoblast • embryonic stem cell • induced pluripotent stem cell • muscular dystrophy • protocol

Introduction

Muscular dystrophies (MDs) are a heterogeneous group of inherited disorders characterized by progressive skeletal muscle weakness and degeneration [1]. Of the MDs, Duchenne muscular dystrophy (DMD) and limb girdle muscular dystrophy (LGMD) are the most frequent forms.

DMD is a genetic X-linked recessive disorder that affects 1 in 3500 male births [2], caused by mutations in the gene encoding dystrophin [3], a protein normally localized in the cytoskeleton and implicated in the stability of the skeletal muscle myofibre membrane [4]. The LGMDs are a group of muscular disorders, characterized by muscle degeneration resulting from a defect in specific skeletal muscle proteins. These diseases show wide genetic and phenotypic inter- and intra-family heterogeneity, and one of their possible clinical manifestations is the involvement of limb girdles [5]. The most frequent are LGMD2B and LGMD2A. LGMD2B or dysferlinopathy is an autosomal recessive disease

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caused by mutations in the gene encoding dysferlin, a protein implicated in the process of membrane repair in skeletal muscle cells through a mechanism of vesicle fusion [6, 7]. LGMD2A or calpainopathy results from mutations in the gene coding for calpain-3, a non-lysosomal calcium-dependent cysteine protease. This protein seems to interact with several cytoskeletal proteins including dysferlin. Its absence results in abnormal sarcomeres that eventually generate muscle fibre death [8].

The majority of MDs are caused by mutations in genes coding for proteins associated with the muscular membrane. The lack of these proteins causes mechanical fragility and alterations in muscle fibre contraction [9, 10]. This change leads to muscle inflammatory cell infiltration and satellite cell activation [11], promoting muscle regeneration. The dystrophic muscle is subjected to continuous cycles of degeneration and regeneration of muscle fibres, until, in the terminal stages of illness, the endogenous satellite cell pool is exhausted. This outcome leads to a full replacement of muscle tissue by fibrotic and adipose tissue, compromising normal muscle function [12]. Different adult multi-lineage progenitor cell populations contribute to skeletal muscle regeneration and have myogenic potential; these include satellite cells (MuSCs), muscle-derived stem cells, muscle side-population cells, mesangioblasts [13] and muscle-derived CD133+ progenitors [14]. In particular, there are many studies investigating CD133+ circulating cells that display a potential to commit into haematopoietic, endothelial and myogenic lineages [15]. These cells are capable of generating myotubes when they are co-cultured in vitro with C2C12 and to display a potential to commit into haematopoietic, endothelial and myogenic lineages [16]. These cells are capable of generating myotubes when they are co-cultured in vitro with C2C12 and to form in vivo new myofibres when they are transplanted in mouse models [17]. In addition to these cells, bone marrow-derived cells appear to contribute to skeletal muscle regeneration, as demonstrated by Luth and his colleagues [17]. They suggest that a CD45+ bone-marrow side-population, a cluster enriched in haematopoietic stem cells, contains precursor cells (CD45−/Sca-1−/desmin+ cells) that can be isolated by FACS. Their progeny is able to differentiate into a myogenic lineage following transplantation [17].

Moreover, all cited cell populations have shown regenerative ability when employed as therapy for skeletal muscle disorders to replace the intrinsic stem cell population that is unable to repair damaged muscle tissue [18].

As a consequence of these events, MD pathogenesis can be linked also to the loss of the ability of the resident cells, such as MuSCs, to activate the cellular repair cycle and regenerate muscle; consequently, the severe muscle atrophy/dystrophy often coincides with the decline in their regenerative capacity. These data seem to indicate that the progression of MDs is partially the result of the failure of resident cells to maintain the repair cycle after damage initiated by a specific protein deficiency [19, 20].

Currently, there are no effective therapies for the MDs, which have enormous personal and socioeconomic consequences for the lives of thousands of patients and their caregivers. The study of mutant proteins involved in these diseases has led to the development of potential treatments, none of which, however, have proved curative to date. These include, for example, corticosteroids, which have shown a good therapeutic effect for DMD patients [21]. Alternative therapeutic approaches have involved the use of agents modulating muscle growth, anti-inflammatory agents [22], or antisense oligonucleotides that induce exon-skipping [23, 24] or abolish the stop codon mutations [25, 26]. Numerous studies over the past two decades have exploited the potential of cell-based therapies to promote muscle regeneration. Initial work involved transplantation of adult myoblasts derived from satellite cells to develop new or hybrid muscle fibres [27]. As an alternative, adult myoblasts can be obtained through transdifferentiation experiments in which a specialized cell type such as primary dermal fibroblasts, chondroblasts, smooth muscle, or retinal pigmented epithelial cells is reprogrammed into another type without reversion to pluripotent cells [28–31]. However, the transplantation of adult myoblasts has not led to remarkable results because of the reduced survival and limited migratory capacity of transplanted cells in early clinical trials [19].

In recent years, myogenic stem cells have proved a valid resource primarily for the good ability to in vitro differentiate, as well as, for their ability to self-renew and the possibility of maintaining them in vitro for a long time without genetic alterations [32]. The myogenic stem cells, defined as primary cells originating from satellite cells (post-natal committed stem cells of skeletal muscles), are better candidates than myoblasts because of their pluripotent characteristics that allow the derivation of several populations of myogenic precursors with different degree of differentiation. Using primitive myogenic precursors can improve engraftment ability based on their more efficient homing capacities to sites of degeneration and highly efficient cell motility in the target tissue as demonstrated by different in vivo cell transplantation studies. Recent data have suggested the viability of using embryonic stem cells (ESCs) as a source for cellular therapies for muscle regeneration [13] as well as for the treatment of various neurological diseases [33]. Limits to their use relate to ethical issues regarding human embryos, the fact that they can lead to heterologous immuno-rejection, and the risk of teratoma formation. An alternative to ESCs is induced pluripotent stem cells (iPSCs), reprogrammed from adult somatic cells with a defined set of pluripotency factors [34–38]. iPSCs exhibit the main characteristics of ESCs. They can form all three germ layers, give rise to teratomas and generate chimeras. The advantage of these cells is that they do not originate from embryos and would enable generation of autologous patient-specific stem cells.

Pluripotent stem cells could represent an interesting source for generating myogenic cells and an innovative research tool, providing an in vitro disease model for investigating cellular and molecular mechanisms in the pathogenesis of human disorders, drug screening and eventually transplantation.

**Transdifferentiation of somatic cells in skeletal myoblasts**

Before considering the generation of myogenic cells from pluripotent stem cells, we review another reprogramming strategy that

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leads to the direct conversion of somatic cells into myoblasts without passing through a pluripotent state, a process that we designate as ‘transdifferentiation’. In fact, most of the molecular strategies that have been used for this transdifferentiation (well before the discovery of iPSCs) can also be employed in the generation of myogenic cells from pluripotent cells in addition to being useful by themselves. Indeed, the direct reprogramming of somatic cells into another phenotype, like fibroblasts into neurons [39], has recently emerged as a powerful alternative strategy to the production of differentiated cells from iPSCs.

In 1987, Davis et al. described for the first time that the treatment of fibroblasts with 5-azacytidine (5-AZA) leads to the conversion of these cells into myoblasts [40]. Subsequently, Tapscott et al. identified MyoD as the master gene for this myogenic reprogramming. In fact, the expression of a cDNA encoding the mouse MyoD1 in a variety of fibroblast and adipoblast cell lines converts them to myogenic cells [41]. A few years later, with the use of transfection with a DNA vector or a retrovirus, MyoD was expressed in pigment, nerve, fat, liver and fibroblast cell lines of several species, converting them in muscle. The expression of muscle-specific proteins has suggested that no additional factors other than MyoD are needed to activate the terminal muscle differentiation program. These data were also confirmed by Choi and colleagues in 1990 [28, 42]. The obtained myotubes showed an elongated morphology, a number of nuclei and a myofibril density similar to normal control muscles, but MyoD-converted cultures of myogenic cells presented a reduced capacity for replication, suggesting that the overexpression of MyoD limits their proliferative capacity [28, 40]. Another strategy for obtaining a high-efficiency myogenic conversion is the infection of human fibroblasts with an E1-deleted adenoviral vector carrying a retroviral long terminal repeat-promoted MyoD cDNA. MyoD-converted cell populations were infused into the regenerating muscles of mice, causing the appearance of new muscle fibres, thus validating the feasibility of this protocol [30].

To better control the fibroblast–myogenic transdifferentiation, our group previously described the possibility of using an inducible promoter, silent in the presence of tetracycline, to drive the transfection of MyoD on demand [31]. Because the transplantation efficiency of the obtained muscle cells is not very high, it would be useful to modulate the expression of muscle master genes, such as Pax3/7, Myf-5 or Myf-4, as well as MyoD, in non-committed cells to increase the myogenic stem cell phenotype.

Myogenic cell induction from ESCs

The most recent alternative to transdifferentiation is based on skeletal myoblast induction from human and murine ESCs. Several methods for skeletal myoblast induction have been proposed (Tables 1 and 2); here, we summarize two of them (Figs 1 and 2). The first consists of the derivation of multipotent mesenchymal precursors (MMPs) from ESCs and then of the differentiation of MMPs into myogenic cells (Table 1). The second method employs the formation of embryoid bodies (EBs) from ESCs and their differentiation towards a myogenic fate (Table 2).

| Table 1 Methods of myocyte differentiation of human and murine ESCs by MMP generation |
|---------------------------------------------------------------|
| **The first method: multipotent mesenchymal progenitors (MMPs)** |
| Reference | Barberi et al., 2005 [51] | Barberi et al., 2007 [52] |
| Starting cells | hESCs | hESCs |
| MMP acquisition | CD73 + cells | CD73 + cells |
| Media | αMEM, 20% inactivated FBS | DMEM/F12 |
| Factors | - | ITS |
| Duration (days) | ~20 | |
| Myogenic cell differentiation | NCAM + cells |
| (1) Media | αMEM, 20% inactivated FBS | Serum-free/N2 |
| Factors | - | Insulin |
| Duration (days) | 14–21 | 14–21 |
| (2) Media | αMEM, 3% HS, 1% FBS |
| Factors | C2C12 co-culture |
| Duration (days) | 1 |
| **Reference** | Sakurai et al., 2009 [43] |
| Starting cells | mESCs |
| MMP differentiation | |
| Media | Serum-free SF-O3 |
| Factors | β-ME, BMP4 |
| Duration (days) | - |
| Myogenic cell differentiation | PDGF-α low/ECD low |
| Media | Serum-free SF-O3 |
| Factors | LIL1, IGF-1, HGF and bFGF |
| Duration (days) | 18 |
plates with murine embryonic fibroblasts, or on dishes coated with type IV collagen because the survival of single stem cells plated in feeder-free conditions is very low.

In Sakurai’s protocol, cells were maintained with a serum-free culture medium (SF-O3) supplemented with the human recombinant bone morphogenetic protein 4 (BMP4) to induce a mesenchymal fate [43]. In fact, the administration of recombinant BMP4 to ESC culture induced the expression of primitive mesoderm-specific genes (Tbx6 and VEGFR2) [44–46], allowing acquisition of haematopoietic cells [47], endothelial cells [48], cardiomyocytes [49] and other intermediate mesodermal derivatives [50].

As an alternative, Barberi et al. used a serum-containing medium with insulin, transferrin and selenium (ITS), without the addition of exogenous growth factors, suggesting that mesenchymal progenitors derive from a spontaneous process. After the expansion phase, cells were subjected to FACS sorting to isolate mesenchymal precursors expressing the CD73 surface antigen [51, 52]. This antigen is a typical marker of mesenchymal progenitor cells together with others such as CD44, CD90, CD166 and CD105 [53].

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### Table 2
Methods of myocyte differentiation of human and murine ESCs by EB generation

| The second method: embryoid bodies (EBs) | Rohwedel et al., 1998 [60] | Zheng et al., 2006 [61] |
| --- | --- | --- |
| Reference | mESCs | hESCs |
| EB acquisition | Hanging drop | Hanging drop |
| Duration (days) | 5 | 4–6 |

#### Myogenic cell differentiation

| (1) Media | DMEM, 15% DCC-FCS | DMEM, 10% FBS |
| Factors | L-glutamine, NEAA, β-ME, sodium selenite, transferrin | ITS, L-glutamine, EGF |
| Duration (days) | 9 | 14/28 |

| (2) Media | IMDM differentiation medium | DMEM, 2% HS |
| Factors | Glutamine | |
| Duration (days) | 9 | 14/28 |

| (3) Media | DMEM, 10% HS, 10% FBS |
| Factors | Glutamine, 5-AZA |
| Duration (days) | 14/28 |

#### Reference
Darabi et al., 2008 [62]

| Starting cells | iPAX3 mESCs |
| EB acquisition | Hanging drop |
| Duration (days) | 5 |

#### Myogenic cell differentiation

| (1) Media | DMEM low-glucose, 2% HS |
| Factors | Doxycycline |
| Duration (days) | 7 |

### Fig. 1
Differentiation of ESCs into myocytes by MMP generation. Schematic procedures for the in vitro differentiation of mouse and human ESCs by MMP generation. (A) mESCs are differentiated into mesoderm by adding BMP4 and then into MMPs (PDGFRα low, ECD low) with the simultaneous addition of LiCl and subtraction of BMP4, as described by Sakurai et al. [43]. (B) hESCs are differentiated into mesoderm by supplying ITS and then into MMPs (CD73+) before the final differentiation into myocytes (NCAM+), as shown by Barberi et al. [52].

### Differentiation into skeletal myoblasts

Once mesenchymal precursors are obtained, several strategies can be used to achieve myoblast differentiation. In Sakurai’s protocol [43], the induction of MMPs is gained by the removal of BMP4 and the exposure to lithium chloride (LiCl), which is sufficient to stimulate myogenic differentiation because it inhibits glycogen synthase kinase-3β activity [54]. Then MMPs are isolated by FACS sorting based on the low positivity for the α-receptor for platelet-derived growth factor (PDGFR-α) and the undifferentiated cell marker E-cadherin (ECD) [49, 55]. This population (PDGFR-α low/ECD low cells) can differentiate into mature skeletal muscle...
cells in the presence of insulin-like growth factor (IGF-1), hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF) in serum-free media. Indeed, these factors are known to induce proliferation of Myf5/H11001/MyoD/H11001 myoblasts [56]. Actually, IGF-1 could stimulate myogenin expression independently; however, with the addition of both HGF and bFGF, myogenin expression is enhanced, and MRF4 expression is stimulated [56].

In Barbieri’s work, different protocols were used [51, 52]. MMPs are maintained with inactivated foetal bovine serum (FBS) or co-cultured with the mouse skeletal myoblast line C2C12; the co-culture allows a more rapid differentiation induction, promoting cell fusion [51]. A modification to the last protocol provides a number of changes after acquisition of MMPs, including cellular growth without C2C12, purification of the skeletal myoblasts by a second FACS analysis for the neural cell adhesion molecule (N-CAM, a marker specific for embryonic skeletal muscle [57]) and their expansion in a serum-free N2 medium with insulin [52].

The advantage to use the MMPs’ methods is the possibility of obtaining, a pure commissioned myogenic cell population by simple FACS sorting without previous genetic manipulation from both, murine and human stem cells. However, a significant number of cells and a rather long timescale for the differentiation are required (Table 4).

The second method: EB generation and differentiation into skeletal muscle

Formation of EBs
A crucial step at the beginning of in vitro human and murine ESC differentiation is the formation of EBs, floating three-dimensional embryo-like aggregates [58]. ESCs are cultured on murine fibroblasts feeder in the presence of leukaemia inhibitory factor (LIF) to remain in an undifferentiated state [58]. The formation of EBs through the ‘hanging drop’ method starts after the detachment of ESCs from the feeder cells and the removal of LIF [58]. Then EBs are cultivated as free-floating spheres in dishes or flasks for 2 days in the same medium used for ESCs without LIF and fibroblast growth factor [58]. To allow for a greater proliferation and an increase in their size, they are transferred into dishes, cultured for another 5 days, and finally plated onto multi-well culture plates for the definitive differentiation [58].

EBs generated by the ‘hanging drop’ method are well defined regarding the cell number and size. In particular, the number of ESCs aggregated in a hanging drop can be controlled by varying the number of cells in the initial suspension to be hung as a drop from the lid of the dish. Moreover, several factors influence the developmental potential of ESCs in culture: the number in the initial suspension, the medium, the quality of serum, the additive growth factors, and the time of EB growth [59]. The obtained EBs can be differentiated into a variety of specialized cell types such as cardiogenic, myogenic, and neuronal cells.

Differentiation of EBs into skeletal myoblasts
The myogenic differentiation of human and murine EBs can be induced by changing the composition of growth medium [60, 61] or by transfection of key genes [62]. Regarding human EBs, Zheng et al. achieved generation by varying the concentration of serum [61]. Serum decrease permits a progressive proliferative decline and stimulates myogenic differentiation through cellular fusion. This cellular mechanism is also enhanced by addition of some specific factors such as dexamethasone, ITS, glutamine, epidermal...
growth factor (EGF), and 5-AZA [61]. The specific contribution of these substances to the differentiation process is not completely understood, although some mechanisms have been identified. For example, dexamethasone, a potent synthetic member of the glucocorticoid class, enhances myogenic differentiation through the synthesis of dysferlin and sarcoplemmal and structural proteins in addition to the up-regulation of specific myogenic transcription factors, as shown in experiments with the C2C12 cell line [63]. 5-AZA, a chemical analogue of cytidine, can enhance cell differentiation into skeletal muscle by causing hypomethylation of muscle regulatory loci. In fact, the mouse embryonic cell line CSH10T1/2, treated with 5-AZA, is converted into skeletal muscle. This process is accompanied by changes in protein patterns unique for each specific cell type, suggesting that a few hypomethylation events are sufficient to activate myogenic differentiation [64].

For the murine ESCs, in Rohwedel’s work [60], the differentiation was stimulated by the addition of specific factors such as glutamine and transferrin, also used by Zheng et al., plus non-essential amino acids (NEAA), β-mercaptoethanol (β-ME), and sodium selenite. Compared to Zheng et al.’s work, Rohwedel’s group did not reduce the serum concentration but replaced the conventional serum with foetal calf serum free of growth factor (DCC-FCS) to stimulate cell proliferation and diminish the differentiation potential. Moreover, Rohwedel et al. tried to cultivate the EBs in Iscove’s modified Dulbecco’s medium (IMDM) differentiation medium instead of Dulbecco’s modified Eagle medium (DMEM). However, the use of DCC-FCS and IMDM did not improve myogenic differentiation compared to treatment with the specific factors. Overall, Rohwedel’s method allowed induction of myogenic differentiation more quickly than did Zheng’s: in the first case, the myoblasts were obtained after 9 days while in the second, they could be detected after 14–28 days. The more rapid differentiation approach was based on the use of ESCs transfected with the Pax3 gene [62], which plays an essential role in myogenesis by acting upstream of myogenic regulatory factor genes, including Myf5, MyoD and Myog, which encodes myogenin [65].

Darabi and colleagues produced an ESC line in which Pax3 expression was induced by administration of doxycycline [62]. EBs, obtained using the hanging drop method, were cultured with DMEM low-glucose, 2% horse serum (HS) plus doxycycline. After only 3 days, they were sorted by FACS using antibodies to PDGF-αR and foetal liver kinase-1 (Flk-1) to isolate the early myogenic progenitors (PDGFαR+/Flk-1−) [66, 67]. The myogenic potential of PDGFαR−/Flk-1− cells was demonstrated by the expression of myogenic markers such as Myf5, MyoD, myogenin and myosin heavy chain (MHC). The overexpression of Pax3 allowed full maturity of the cells, as shown by the appearance of myotubes and characterized by a greater number of cells positive for later myogenic markers such as myogenin and MHC.

Compared to the other described methods, this last approach, based on the expression of genes essential for myogenesis, is more efficient in driving differentiating cells to become skeletal muscle and decreasing the number of uncommitted or non-myogenic cells. In fact, the derivation of skeletal myogenic progenitors from ESCs has proven to be less efficient in vitro because of the absence of somitogenesis or paraxial mesoderm patterning, two essential mechanisms in normal muscle differentiation. This failure can be compensated by the overexpression of Pax3, which promotes myogenesis in vitro and generates a high-quality population of myogenic progenitors [68].

In comparison with the MMPS’ methods, the generation of myogenic cells through the formation of EBs in combination with either transfection or by using factors that stimulate the differentiation, is certainly faster. It shall be shown that a possible outcome of transfection could be changes of cell genetics and high mortality, but providing of more efficient in driving differentiating cells (Table 4).

### Protocols for myogenic cell induction from iPSCs

In addition to the methods described above and based on ESCs, myoblasts can also be obtained by approaches that exploit the in vitro differentiation of iPSCs. The iPSCs can be obtained through direct reprogramming of different human somatic cells to a pluripotent state with viral and/or non-viral methods [37]. The most commonly used cell type for reprogramming are fibroblasts, because it is easy to obtain them from a skin biopsy and to cultivate in vitro [34–38]. Recent studies have revealed the possibility to reprogram other cell types such as myoblasts. For example, Watanabe and colleagues derived iPSC lines from mouse myoblasts by infection with retroviral vectors expressing well-defined factors including Oct4. This factor is needed for the early reprogramming step as it is able to suppress MyoD, a myogenic master gene [69]. These data are also confirmed by Ahmed’s studies in which skeletal myoblast have been successfully reprogrammed to iPSCs expressing markers similar to ESCs. These cells are able to differentiate spontaneously into cardiomyocytes, providing a donor cell source to treat a mouse model of acute myocardial infarction and also having the interesting feature of reducing tumour formation after transplantation [70].

Regarding the type of cells used for iPSCs’ production, some groups have managed to successfully obtain skeletal myoblasts from murine iPSCs, while the production of skeletal myogenic lineages from human iPSCs is in its early stages. Here, we report two protocols aimed at the production of myoblasts from murine iPSCs (miPSCs) (Table 3 and 4; Fig. 2) [71, 72].

In Darabi’s work, the method used to differentiate miPSCs into myocytes was similar to a previous protocol applied to ESCs [62, 71]. The specific features of this approach involve three consecutive steps: the transfection of miPSCs with a plasmid expressing Pax7 [73], a key myogenic gene, under the control of doxycycline; the production of EBs by the hanging drop method; and the isolation by FACS of early myogenic progenitors (PDGFαR+/Flk-1−) with high myogenic potential. Mizuno’s work has similarities with this approach: first of all, the starting cell population is a miPSC...
line reprogrammed from murine fibroblasts; second, there is the generation of EBs by the floating drop method; and third, it involves the separation by FACS of cells with a strong capability to differentiate into myofibres in vitro [72].

Mizuno et al., however, did not induce the expression of crucial myogenic genes, resulting in longer times to myoblast appearance, about 27 days compared to only 7 in Darabi's work. Another important difference concerns the use of an anti-satellite cell antibody, SM/C-2.6, a cell surface marker for murine skeletal muscle [74]. The kinetics of SM/C-2.6 expression correlates with the maturity of myofibres and with the up-regulation of the skeletal myogenesis-related gene expression. The use of this antibody allows FACS selection of a SM/C-2.6 + cell fraction with good expression of myogenic stem/progenitor cell-related surface markers and a high myogenic activity. Regarding the media applied in these protocols, they are the same both for miPSCs maintenance in culture and for EBs formation: DMEM plus LIF. In contrast, different media are used during myogenic differentiation; Darabi et al. employed DMEM supplemented with horse serum, while Mizuno et al. used DMEM with FCS, HS, NEAA and β-ME.

Concerning the use of miPSCs, only adding differentiation factors to the medium, allows greater cell viability compared to the drastic reduction observed with the master muscle gene transfection. However, as mentioned above, the latter consents a shorter time range to obtain myogenic cells and it is more competent in directing cell differentiation (Table 4).

Greater efficiency of myogenic differentiation can be achieved by generating iPSCs from sorted mesangioblasts (MABs), a subgroup of muscle-derived pericytes, as shown by Quattrocelli's work. Surprisingly, in this study, a simple transient transfection with PAX3 and PAX7 is sufficient to trigger a strong myogenic commitment of MAB-iPSCs, without addition of exogenous factors. These cells also undergo a complete muscle differentiation without particular media or matrices or variation in serum concentration. Quattrocelli concludes that MAB-iPSCs contain an intrinsic and strong cell memory compared to iPSCs obtained from fibroblasts (iPSCs) [75].

### Table 3 Methods of myocyte differentiation of miPSCs

| Reference          | Darabi et al., 2011 [71] | Mizuno et al., 2010 [72] |
|--------------------|--------------------------|--------------------------|
| Starting cells     | iPAX7-miPSCs             | miPSCs                   |
| EB acquisition     | Hanging drop             | Hanging drop             |
| Duration (days)    | 5                        | 4–6                      |
| Myogenic cell differentiation | DMEM, 2% HS            | DMEM, 10% FCS, 5% HS    |
| Media              | NEAA, β-ME               |                          |
| Duration (days)    | 7                        | 27                       |

### Mouse and human-pluripotent derived myocytes as an in vitro model of MDs

Disease-specific pluripotent cells, capable of differentiation into the various tissues affected, might provide the opportunity to broaden our understanding of neuromuscular disorders by permitting analysis in a human system, under controlled conditions in vitro, using a large number of genetically modifiable cells, and in an approach specific to the genetic lesions in each whether known or unknown. Different in vitro models that offer new insight into human pathologies have already been generated with pluripotent cells, as previously described for human motor neuron diseases [76]. Similar approaches could be applied for a better understanding of the molecular mechanisms underlying MDs. In particular, myocytes obtained from mouse or hESCs may mimic in vitro some specific features of muscle disorders and therefore represent an excellent model for further studies.

For example, one of the pathogenetic aspects of these pathologies is the alteration of important proteins for proper muscle function. Pluripotent stem cells, manipulated in vitro to express mutant genes or silenced in genes linked to MDs and subsequently differentiated into skeletal muscle, may help to clarify the involvement of these proteins in cellular changes of muscle tissue.

Park and colleagues have reported the derivation of hiPSC lines from patients with a range of human genetic diseases, including DMD and Becker MD patients. Dermal fibroblasts or mesenchymal cells obtained from these patients were used to establish disease-specific lines of hiPSCs by transduction with either four (OCT4, SOX2, KLF4 and c-MYC) or three reprogramming factors (lacking c-MYC). iPSC lines were analysed to verify their pluripotency and multi-lineage development and then were induced to differentiate into EBs, showing their potential to give rise to all three embryonic germ layers [37, 38]. No further differentiation into skeletal muscle was assessed. However, the generation of these cell lines could be the starting point for further in vitro experiments of muscle differentiation and for obtaining a model of these neuromuscular disorders. Overall, these patient-specific cell lines not only offer an exceptional opportunity to summarize pathologic human tissue development in vitro but also provide a new tool for drug screening.

### Therapeutic development based on ESC- or iPSC-derived cells

Several cell sources have been used for therapeutic purposes in MDs, such as satellite cells, mesangioblasts and adult mesenchymal stem cells [13, 14]. As an alternative, the successful use of hESC-iPSC-derived myocytes for therapeutic applications makes it necessary to control the process of in vitro differentiation and the consequent specific isolation of the target cell populations.
| Cell type | Intermediate step | Advantages | Disadvantages | References |
|-----------|-------------------|------------|---------------|------------|
| hESC      | MMP               | Pluripotent| Ethical problems | Barberi et al., 2005, 2007 [51, 52] |
|           |                   | Sorting to select a pure myogenic committed cellular population | Longer time range for obtaining myogenic cells |           |
|           |                   | Addition of some differentiation factors without gene transfection | Obtaining an adequate amount of cells for sorting |           |
| hESC      | EB                | Pluripotent| Use of ES cells poses ethical problems | Zheng et al., 2006 [61] |
|           |                   | More rapid differentiation protocol respect to MMP approach | Addition of some differentiation factors without gene transfection |           |
| mESC      | MMP               | Pluripotent| Ethical problems | Sakurai et al., 2009 [43] |
|           |                   | Sorting to select a pure population of myogenic committed cells | Longer time range for obtaining myogenic cells |           |
|           |                   | Addition of some differentiation factors without gene transfection | Obtaining a sufficient quantity of cells for sorting |           |
| mESC      | EB                | Pluripotent| Use of ES cells poses ethical problems | Rohwedel et al., 1998 [60] |
|           |                   | More rapid differentiation protocol respect to MMP approach | Addition of some differentiation factors without gene transfection |           |
| iPAX3 mESCs| EB             | Pluripotent| Use of ESC poses ethical problems | Darabi et al., 2008 [62] |
|           |                   | Sorting to select a pure population of myogenic committed cells | Obtaining a sufficient quantity of cells for sorting |           |
|           |                   | Short time range to obtain myogenic cells through master muscle gene transfection | Possible change of cell genetics as a result of transfection |           |
|           |                   | More efficient in driving differentiating cells | High mortality as a possible outcome in cell transfection |           |
| miPSCs    | EB                | Pluripotent| Longer time range for myogenic differentiation respect to iPAX7-miPSCs | Mizuno et al., 2010 [72] |
|           |                   | Obtained through direct reprogramming of different human somatic cells | Addition of some differentiation factors without gene transfection |           |
| iPAX7-miPSCs | EB        | Pluripotent| Possible alteration of cell genetics as a result of transfection | Darabi et al., 2011 [71] |
|           |                   | Obtained through direct reprogramming of different human somatic cells | High mortality as a possible outcome in cell transfection |           |
|           |                   | Short time range to obtain myogenic cells through master muscle gene transfection | More efficient in driving differentiating cells |           |
Within this perspective, Barberi and colleagues have recently described a method for generating mesenchymal precursors from hESCs without applying mouse stroma or human telomerase reverse transcriptase, facilitating the use of these cells in clinical applications [52]. In these conditions, hESC-derived myoblasts have shown a good ability to form myotubes in vitro and an excellent survival after transplantation in a muscle injury model [52]. These data, in association with the absence of teratoma formation and the evidence of long-term engraftment of myoblasts, represent an optimal starting point for further pre-clinical development of this strategy. Despite these interesting data, the immune privilege of hESCs is still under debate [77] and, in addition, the cellular rejection after transplantation may occur even after the progression towards myogenic differentiation [78]. On the other hand, iPSCs can represent a valid alternative to ESCs, eliminating the ethical and immunological issues. However, before using of iPS cells for future therapeutic applications in MDs, it will be basic to consider both, their ability as well as their aptitude to make functional therapeutic myogenic progenitors in vivo in an animal models. The ability of iPSCs to regenerate damaged tissues has been described by Darabi et al. [71]. The authors generated a miPSC line with the conditional expression of Pax7, obtaining a cell population able to differentiate in a myogenic direction. Once transplanted into dystrophic mice, myogenic progenitors have shown a good capacity for engraftment and for improving the contractility of treated muscles [71].

The use of iPSC-derived myoblasts resolves a negative aspect associated with ESCs, the potential immunological rejection of transplanted cells. In fact, the iPSCs can be produced from the same patient, allowing the generation of autologous myocytes. Patient-specific iPSC lines have been obtained for several genetic disorders [79, 80] including MDs [37, 38]. These cells are not only an excellent tool to model genetic diseases, but as they also open the possibility of ex vivo correction of autologous cells for a patient specific personalized treatment. In the case of MDs caused by known genetic defects, the patient-specific iPSCs could be corrected by gene therapy, then induced to differentiate into myocytes and subsequently transplanted into the patients with the aim of complementing the genetic defect. In this context, Kazuki and colleagues have obtained a complete genetic correction of DMD-iPSCs using a gene transfer strategy. These cells can be used as a source for transplantation into patients after their differentiation into myocytes [81]. iPSCs, derived from fibroblasts of dystrophin-deficient mice (mdx) and DMD patients, have been corrected by microcell-mediated chromosome transfer of a human artificial chromosome that includes a genomic dystrophin sequence. Once transplanted into mice, these cells have been observed in all examined tissues, with tissue-specific expression of dystrophin [81].

These approaches hold great potential for future therapeutic applications, but many milestones have to be overcome yet: definition of the optimal cell administration protocol to significantly repopulate muscle compartments; improvement of the method to generate an efficient myogenic cell population from human pluripotent stem cells; activation of muscle key genes to induce myogenic differentiation without affecting cell genetics. Hence additional studies are required before the clinical use of pluripotent stem cell-derived myocytes.

Conclusion
At present, no cures or efficacious treatments are available for MDs. Several studies have exploited the potential of cell-based therapies to promote muscle regeneration. An important source for cell therapy can be represented by pluripotent stem cell-derived myocytes. When we affirmed that pluripotent stem cell-derived myocytes have a great potential for future therapeutic applications, we mean that the not yet terminally differentiated myocytes obtained by differentiation from pluripotent stem cells, have more potential to treat MDs in term of muscle fibre regeneration and tissue repair compared to myoblasts directly obtained from the post-natal muscle tissue. In fact, stem cell-derived myocytes were proven to be a valid cell source, primarily because of their good ability to differentiate in vitro, but also because of self-renewing ability that allows to maintain them in vitro for a long time without genetic alterations [32]. In this work, we have summarized the advantages and the disadvantages of several protocols (Table 4) and results from studies regarding the use of ESCs and iPSCs for myocyte generation. The differentiation of human skeletal myogenic cells from iPSCs has yet to be demonstrated, and we believe that this is an important step that requires a research focus. iPSCs can be a precious research tool for creating a model to investigate cellular and molecular mechanisms underlying MD and also a valuable source for cell-based therapies.

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Conflicts of interest
The authors confirm that there are no conflicts of interest.
References

1. Emery AE. The muscular dystrophies. Lancet. 2002; 359: 687–95.
2. Palmieri B, Tremblay JP. Myoblast transplantation: a possible surgical treatment for a severe pediatric disease. Surg Today. 2010; 40: 902–8.
3. Hoffman EP, Brown RH Jr, Konkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 1987; 51: 919–28.
4. Durbeej M, Campbell KP. Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. Curr Opin Genet Dev. 2002; 12: 349–61.
5. Broglio L, Tentorio M, Cotelli MS, et al. Limb-girdle muscular dystrophy-associated protein diseases. Neurologist. 2010; 16: 340–52.
6. Wein N, Avril A, Bartoli M, et al. Efficient bypass of mutations in dysferlin deficient patient cells by antisense-induced exon skipping. Hum Mutat. 2010; 31: 136–42.
7. Urtizberea JA, Bassez G, Leturcq F, et al. Dysferlinopathies. Neurol India. 2008; 56: 289–97.
8. Gallardo E, Saenz A, Illa I. Limb-girdle muscular dystrophy 2A. Handb Clin Neurol. 2011; 101: 97–110.
9. McArdle A, Edwards RH, Jackson MJ. How does dystrophin deficiency lead to muscle degeneration? – evidence from the mdx mouse. Neuromuscul Disord. 1995; 5: 445–56.
10. Tanabe Y, Esaki K, Nomura T. Skeletal muscle pathology in X chromosome-linked muscular dystrophy (mdx) mouse. Acta Neuropathol. 1986; 69: 91–5.
11. Watkins SC, Cullen MJ. A quantitative study of myonuclear and satellite cell nuclear size in Duchenne's muscular dystrophy, polymyositis and normal human skeletal muscle. Anat Rec. 1988; 222: 6–11.
12. Matsumura K, Campbell KP. Deficiency of dystrophin-associated proteins: a common mechanism leading to muscle cell necrosis in severe childhood muscular dystrophies. Neuromuscul Disord. 1993; 3: 109–18.
13. Negroni E, Vallesse D, Vilquin JT, et al. Current advances in cell therapy strategies for muscular dystrophies. Expert Opin Biol Ther. 2011; 11: 157–76.
14. Péault B, Rudnicki M, Torrente Y, et al. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther. 2007; 15: 867–77.
15. Meregalli M, Farini A, Belicchi M, et al. CD133(+) cells isolated from various sources and their role in future clinical perspectives. Expert Opin Biol Ther. 2010; 10: 1521–8.
16. Torrente Y, Belicchi M, Sampaolesi M, et al. Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. J Clin Invest. 2004; 114: 182–95.
17. Luth ES, Jun SJ, Wessen MK, et al. Bone marrow side population cells are enriched for progenitors capable of myogenic differentiation. J Cell Sci. 2008; 121: 1426–34.
18. Otto A, Collins-Hooper H, Patel K. The origin, molecular regulation and therapeutic potential of myogenic stem cell populations. J Anat. 2009; 215: 477–97.
19. Vilquin JT. Myoblast transplantation: clinical trials and perspectives. Acta Myol. 2005; 24: 119–27.
20. Collins CA, Olsen I, Zammit PS, et al. Stem cell function, selfrenewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell. 2009; 122: 289–301.
21. Hyser CL, Mendell JR. Recent advances in Duchenne and Becker muscular dystrophy. Neurol Clin. 1988; 6: 429–53.
22. Biggar WD, Harris VA, Elisaph L, et al. Long-term benefits of deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade. Neuromuscul Disord. 2006; 16: 249–55.
23. Forrest S, Meloni PL, Muntoni F, et al. Personalized exon skipping strategies to address clustered non-deletion dystrophin mutations. Neuromuscul Disord. 2010; 20: 810–6.
24. Hoffman EP, Bronson A, Levin AA, et al. Restoring dystrophin expression in Duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. Am J Pathol. 2011; 179: 12–22.
25. Finke RS. Read-through strategies for suppression of nonsense mutations in Duchenne/Becker muscular dystrophy: aminoglycosides and ataluren (PTC124). J Child Neurol. 2010; 25: 1158–64.
26. Malik V, Rodino-Klapac LR, Viollet L, et al. Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy. Ann Neurol. 2010; 67: 771–80.
27. Brussee V, Tardif F, Roy B, et al. Successful myoblast transplantation in fibrotic muscles: no increased impairment by the connective tissue. Transplantation. 1999; 67: 1618–22.
28. Choi J, Costa ML, Mermelstein CS, et al. MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. Proc Natl Acad Sci USA. 1990; 87: 7988–92.
29. Anastasia L, Sampaolesi M, Papini N, et al. Reverse engineered fibroblasts acquire myogenic competence in vitro and in regenerating skeletal muscle. Cell Death Differ. 2006; 13: 2042–51.
30. Lattanzi L, Salvatori G, Coletta M, et al. High efficiency myogenic conversion of human fibroblasts by adenoviral vector-mediated MyoD gene transfer. An alternative strategy for ex vivo gene therapy of primary myopathies. J Clin Invest. 1998; 101: 2119–28.
31. Del Bo R, Torrente Y, Corti S, et al. In vitro and in vivo tetracycline-controlled myogenic conversion of NIH-3T3 cells: evidence of programmed cell death after muscle cell transplantation. Cell Transplant. 2001; 10: 209–21.
32. Meng J, Muntoni F, Morgan JE. Stem cells to treat muscular dystrophies – where are we? Neuromuscul Disord. 2011; 21: 4–12.
33. Tomaskovic-Crook E, Crook JM. Human embryonic stem cell therapies for neurodegenerative diseases. CNS Neurol Drug Disc Targets. 2011; 10: 440–8.
34. Lowry WE, Richter L, Yachechko R, et al. Generation of human induced pluripotent stem cells from dermal fibroblasts. Proc Natl Acad Sci. 2008; 105: 2883–8.
35. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol. 2007; 25: 1177–81.
36. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131: 861–72.
37. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. Nature. 2008; 451: 141–6.
38. Park IH, Lerou PH, Zhao R, et al. Generation of human-induced pluripotent stem cells. Nat Protoc. 2008; 3: 1180–6.
39. Pang ZP, Yang N, Vierbuchen T, et al. Induction of human neuronal cells by
defined transcription factors. Nature. 2011; doi:10.1038/nature10202.
40. Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell. 1987; 51: 987–1000.
41. Tapscott SJ, Davis RL, Thayer MJ, et al. MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. Science. 1988; 242: 405–11.
42. Weintraub H, Tapscott SJ, Davis RL, et al. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci USA. 1989; 86: 5434–38.
43. Sakurai H, Inami Y, Tamamura Y, et al. Bidirectional induction toward paraxial mesodermal derivatives from mouse ES cells in chemically defined medium. Stem Cell Res. 2009; 3: 157–69.
44. Johansson BM, Wiles MV. Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. Mol. Cell. Biol. 1995; 15: 141–51.
45. Ng ES, Azzola L, Sours K, et al. The primitive streak gene Mix1 is required for efficient haematopoiesis and BMP4-induced ventral mesoderm patterning in differentiating ES cells. Development. 2005; 132: 873–84.
46. Era T, Iizumi N, Hayashi M, et al. Multiple mesoderm subsets give rise to endothelial cells, whereas hematopoietic cells are differentiated only from a restricted subset in embryonic stem cell differentiation culture. Stem Cells. 2008; 26: 401–11.
47. Pick M, Azzola L, Mossman A, et al. Differentiation of human embryonic stem cells in serumfree medium reveals distinct roles for bone morphogenetic protein 4, vascular endothelial growth factor, stem cell factor, and fibroblast growth factor 2 in hematopoiesis. Stem Cells. 2007; 25: 2206–14.
48. Park C, Afrikanova I, Chung YS, et al. A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells. Development. 2004; 131: 2749–62.
49. Honda M, Kurisaki A, Ohnuma K, et al. N-cadherin is a useful marker for the progenitor of cardiomyocytes differentiated from mouse ES cells in serum-free condition. Biochem Biophys Res Commun. 2006; 351: 877–82.
50. Bruce SJ, Rea RW, Steptoe AL, et al. In vitro differentiation of murine embryonic stem cells toward a renal lineage. Differentiation. 2007; 75: 337–49.
51. Barberi T, Willis LM, Socci ND, et al. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. PLoS Med. 2006; 2: 161.
52. Barberi T, Bradbury M, Dincer Z, et al. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. Nat Med. 2007; 13: 642–6.
53. Firth AL, Yao W, Ogawa A, et al. Multipotent mesenchymal progenitor cells are present in endarterectomized tissues from patients with chronic thromboembolic pulmonary hypertension. Am J Physiol Cell Physiol. 2010; 298: 1217–25.
54. Van der Velden JL, Langen RC, Kelders MC, et al. Inhibition of glycogen synthase kinase-3beta activity is sufficient to stimulate myogenic differentiation. Am J Physiol Cell Physiol. 2006; 290: 453–62.
55. Schatteman GC, Morrison-Graham K, van Koppen A, et al. Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. Development. 1992; 115: 123–31.
56. Chargé SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiol Rev. 2004; 84: 209–38.
57. Walsh FS, Ritter MA. Surface antigen differentiation during human myogenesis in culture. Nature. 1981; 289: 60–4.
58. Wang X, Yang P. In vitro differentiation of mouse embryonic stem (mES) cells using the hanging drop method. J Vis Exp. 2008; doi:10.3791/825.
59. Guan K, Rohwedel J, Wobus AM. Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro. Cytotechnology. 1999; 30: 211–26.
60. Rohwedel J, Kleppisch T, Pich U, et al. Formation of postnaptically-like membranes during differentiation of embryonic stem cells in vitro. Exp Cell Res. 1998; 239: 214–25.
61. Zheng JK, Wang Y, Karandikar A, et al. Skeletal myogenesis by human embryonic stem cells. Cell Res. 2006; 16: 713–22.
62. Darabi R, Gehlback K, Bachoo RM, et al. Functional skeletal muscle regeneration from differentiating embryonic stem cells. Nat Med. 2008; 14: 134–43.
63. Belanto JJ, Diaz-Perez SV, Magyar CE, et al. Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation. Neuromuscul Disord. 2010; 20: 111–21.
64. Konieczny SF, Emerson CP Jr. 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell. 1984; 38: 791–800.
myogenic commitment of pericyte-derived iPSCs. J Pathol. 2011; 223: 593–603.
76. Nizzardo M, Simone C, Falcone M, et al. Human motor neuron generation from embryonic stem cells and induced pluripotent stem cells. Cell Mol Life Sci. 2010; 67: 3837–47.
77. Chidgey AP, Boyd RL. Immune privilege for stem cells: not as simple as it looked. Cell Stem Cell. 2008; 3: 357–8.
78. Swijnenburg RJ, Tanaka M, Vogel H, et al. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation. 2005; 112: 166–72.
79. Moretti A, Bellin M, Welling A, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. N Engl J Med. 2010; 363: 1397–409.
80. Maehr R, Chen S, Snitow M, et al. Generation of pluripotent stem cells from patients with type 1 diabetes. Proc Natl Acad Sci USA. 2009; 106: 15768–73.
81. Kazuki Y, Hiratsuka M, Takiguchi M, et al. Complete genetic correction of iPS cells from Duchenne muscular dystrophy. Mol Ther. 2010; 18: 386–93.