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Stem cell-based therapies for Duchenne muscular dystrophy

Congshan Sun[1,3], **Carlo Serra**[1,3], **Gabsang Lee**[2], **Kathryn Wagner**[1,3]

[1.]Departments of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland, 21205, USA.

[2.]Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, 21205, USA.

[3.]Center for Genetic Muscle Disorders, Hugo W. Moser Research Institute at Kennedy Krieger Institute, Baltimore, Maryland, 21205, USA.

Abstract

Muscular dystrophies are a group of genetic muscle disorders that cause progressive muscle weakness and degeneration. Within this group, Duchenne muscular dystrophy (DMD) is the most common and one of the most severe. DMD is an X chromosome linked disease that occurs to 1 in 3,500 to 1 in 5,000 boys. The cause of DMD is a mutation in the dystrophin gene, whose encoded protein provides both structural support and cell signaling capabilities. So far, there are very limited therapeutic options available and there is no cure for this disease. In this review, we discuss the existing cell therapy research, especially stem cell-based, which utilize myoblasts, satellite cells, bone marrow cells, mesoangioblasts and CD133+ cells. Finally, we focus on human pluripotent stem cells (hPSCs) which hold great potential in treating DMD. hPSCs can be used for autologous transplantation after being specified to a myogenic lineage. Over the last few years, there has been a rapid development of isolation, as well as differentiation, techniques in order to achieve effective transplantation results of myogenic cells specified from hPSCs. In this review, we summarize the current methods of hPSCs myogenic commitment/differentiation, and describe the current status of hPSC-derived myogenic cell transplantation.

Keywords

Duchenne muscular dystrophy (DMD); stem cell therapy; hiPSC; myogenic lineage specification

Muscular dystrophies (MDs) are a group of more than 50 heterogeneous genetic diseases, marked by degeneration of skeletal muscle and progressive weakness. The different MDs vary in terms of groups of muscles involved, age at disease onset, progression, and ultimate level of disability. Furthermore, several MDs show compromised physiology of other organs, such as the heart and brain in Duchenne muscular dystrophy (DMD) (Connuck et al., 2008; Rahimov and Kunkel, 2013; Yoshioka et al., 1980).

Corresponding Author: Congshan Sun, PhD, Center for Genetic Muscle Disorders, Kennedy Krieger Institute, Departments of Neurology and Neuroscience, The Johns Hopkins University, School of Medicine, csun23@jhmi.edu.

The most common form of MD is DMD, a fatal disease affecting around 1 in 3,500 to 1 in 5,000 live male births (Emery, 2002; Mendell et al., 2012). Boys with DMD usually lose the ability to walk in early teenage years, lose the ability to feed themselves in late teenage years, and die from respiratory insufficiency or cardiomyopathy in early adulthood (Emery, 2002; Mavrogeni et al., 2015).

Current standard of care includes the use of corticosteroids, cardioprotective treatment, ventilatory support, and physical therapy (Mah, 2016). However, these treatments have limitations and side effects, and are only able to delay the progression of the disease. No curative therapies are available for DMD.

In recent years, considerable research effort has been directed to developing new therapeutic options to treat DMD. Exon skipping, gene therapy and cell therapy have received considerable research attention. Antisense oligonucleotide (AON) mediated exon skipping that restores partial but functional dystrophin protein has advanced significantly during recent years with several AONs in clinical trials. Among them, Eteplirsen (for exon 51 skipping, affecting 13% of DMD patients) has been conditionally approved by the FDA in 2016 (Lim et al., 2017; Nakamura et al., 2017). Gene therapy that aims to produce a mini-dystrophin in muscle fibers is also in clinical trials with promising initial results, while gene editing using the CRISPR/Cas9 systems, to correct the dystrophin gene, may not be far behind. In this review, we present current stem cell-based therapies whose goal is to replenish the muscle stem cell pool with dystrophin-competent cells, with the focus on human pluripotent stem cells (hPSCs), which allows autologous cell therapies.

1. Pathological features of DMD

DMD is due to mutations in the dystrophin gene. The dystrophin gene is translated into a 427kDa protein, which is part of the Dystrophin Glycoprotein Complex (DGC) that provides a structural and signaling link between the cytoskeleton of the muscle fiber and the extracellular matrix (Ervasti, 2007). In healthy individuals, the dystrophin protein stabilizes the plasma membrane of the striated muscle fibers. However, in patients with DMD or the allelic Becker muscular dystrophy (BMD), mutations in the dystrophin gene cause the complete loss of the dystrophin protein (in DMD), or the production of a truncated, partially-functional, dystrophin protein (in BMD) (Rahimov and Kunkel, 2013).

In DMD patients, loss of a functional DGC leads to damage of the sarcolemma upon muscle contraction, which results in loss of sarcoplasmic proteins from the muscle fiber, and extensive damage of the muscle (Ervasti, 2007). As a consequence, DMD muscles are subject to chronic cycles of necrosis and regeneration, in the attempt to replenish the damaged fibers with new, functional fibers (Ervasti, 2007).

Muscle regeneration starts with the activation of the muscle stem cells, the satellite cells. These cells are embedded between the sarcolemma and the basal lamina, in a quiescent state (Charge and Rudnicki, 2004; Mauro, 1961). Upon activation, satellite cells enter the cell cycle, start to migrate toward the regenerating areas of the muscle, and give rise to more functionally committed cells, the myoblasts, which differentiate to generate new myofibers

(Charge and Rudnicki, 2004). Satellite cells usually generate their progeny by asymmetric cell division, when specific, mostly unknown fate determinants are segregated in a polarized manner between the two daughter cells, to generate a new satellite cell and a myoblast (Gurevich et al., 2016; Kuang et al., 2007; Rocheteau et al., 2012; Wang et al., 2019).

In DMD, muscle regeneration is compromised because the continuous rounds of muscle degeneration and regeneration deplete the pool of satellite cells (Lu et al., 2014). Moreover, because dystrophin is also expressed in satellite cells, its loss results in distorted polarity of the satellite cells, deficits in their asymmetric division, and precocious differentiation (Dumont et al., 2015; Wang et al., 2019). Consequently, the DMD muscle becomes progressively unable to build new muscle fibers, which further contributes to its wasting. Furthermore, DMD muscle fibers are gradually replaced by fat and fibrotic tissue, which further hampers the mechanical and physiological activity of the skeletal muscle (Alvarez et al., 2002; Rahimov and Kunkel, 2013; Serrano and Munoz-Canoves, 2010; Villalta et al., 2011).

In healthy muscle, degeneration and regeneration is orchestrated by a strong, localized, inflammatory response, in which T cells, macrophages, and neutrophils infiltrate the muscle after injury, and release elevated levels of pro-inflammatory cytokines and regulatory cues (Shin et al., 2013). However, in the DMD muscle, chronic inflammation leads to excessive levels of intramuscular reactive oxygen species, which further contribute to the muscle wasting, and hampers the regenerative power of the satellite cells (Shin et al., 2013).

Muscle regeneration also requires the action of the fibro-adipogenic progenitors (FAPs), a population of muscle interstitial cells (Joe et al., 2010; Uezumi et al., 2010). Inflammatory cells regulate activation and proliferation of the FAPs, which, in turn, coordinate the regenerative action of the satellite cells (Heredia et al., 2013; Joe et al., 2010; Lemos et al., 2015; Uezumi et al., 2010; Uezumi et al., 2011). However, in DMD muscles, the continuous rounds of muscle degeneration and regeneration increase the FAPs' differentiation into adipocytes or fibroblasts, the accumulation of which further compromises the mechanical features of the muscle (Lemos et al., 2015; Malecova et al., 2018; Mozzetta et al., 2013; Saccone et al., 2014).

2. Skeletal muscle determination in the vertebrate embryo

Muscle commitment and differentiation are mainly controlled by the regulated spatio-temporal expression of a set of four proteins (Myf5, MyoD1, Myogenin, and Mrf4) termed the muscle regulatory factors (MRFs) (Buckingham and Rigby, 2014). The MRFs are transcription factors that drive the expression of a multitude of genes regulating establishment and maintenance of the myogenic fate.

In embryonic development, myogenic cells originate from mesodermal precursors that colonize the paraxial mesoderm (PM), and that initially become part of the anterior area of the primitive streak (PS) (Buckingham and Rigby, 2014; Chang and Kioussi, 2018; Pourquie, 2004). The initial mesodermal differentiation is controlled by specific signaling proteins and molecules emanating from the anatomical regions surrounding the developing

PM (Cunningham et al., 2015; Hamade et al., 2006). As a result, these morphogenetic gradients mark the first distinction between the anterior and posterior muscle groups of the future body (Chang and Kioussi, 2018).

A population of cells termed neuromesodermal progenitors (NMPs) is considered the precursor of the PM. NMPs initially reside in the anterior PS, and give rise to the presomitic mesoderm (PSM) on both sides of the neural tube (Gouti et al., 2017; Tzouanacou et al., 2009). For their role as PSM precursors, NMPs are considered the source of the first muscle precursors during development (Pourquie et al., 2018).

Several signaling pathways govern the patterning of the PM towards a mesodermal or neural fate. In particular, signaling by the transforming growth factor β (TGF-β)/Nodal, and the bone morphogenetic proteins (BMPs), play an inductive role on the early specification of the PM in the PS (Robertson, 2014). Initially, NMPs express both the mesodermal marker T/Brachyury and the neural marker Sox2 (Gouti et al., 2017; Henrique et al., 2015; Olivera-Martinez et al., 2012; Takemoto et al., 2011; Tzouanacou et al., 2009; Wymeersch et al., 2016). Subsequently, proliferating NMPs downregulate Sox2, sequentially express the transcription factors Tbx6, Snai1, and Mesogenin 1 (Msgn1), and progressively downregulate T/Brachyury expression (Chalamalasetty et al., 2011; Chalamalasetty et al., 2014; Chapman et al., 1996; Gouti et al., 2017). These transcriptional changes, in both NMPs and PSM cells, seem to be regulated by an oscillatory activity of the Notch, FGF, and Wnt signaling (Hubaud and Pourquie, 2014). As a result, NMPs express the transcription factors Mesp1 and Mesp2, which in turn, activate a transcriptional program leading to the segmentation of the PSM into somites (Hubaud and Pourquie, 2014; Morimoto et al., 2005). At this stage, PSM cells downregulate the expression of Msgn1 and Tbx6, and begin to express the paired-box domain transcription factor Pax3, which marks the first transition toward a true muscle cell commitment (Aulehla et al., 2008).

Somitogenesis is the most important step towards the complete determination of the muscle progenitors. Similar to formation of the PM, the segmentation of the PSM, as the first step of somitogenesis, is controlled by extracellular gradients of specific signaling molecules and proteins. The morphological processes underlying the separation of each somite are regulated, among them, by FGF8 and Wnt3 emanating from the caudal portion of the embryo, retinoic acid (RA) released by the rostral region of the forming somites, and the notochord-released sonic hedgehog (Shh) (Chang and Kioussi, 2018).

Somites initially appear as spherical clusters of epithelial-like cells that differentiate into two main regions: (i) a ventro-medial, mesenchymal-like, sclerotome/syndetome, from which derive vertebral bones, ribs cartilage, and the tendons of the trunk, and (ii) a dorso-lateral, epithelial-like, dermomyotome (DM). From the DM derive the precursor cells of the skeletal muscle of trunk and limbs, of brown fat, of dermis, and part of the endothelial and smooth muscle cells of the blood vessels (Buckingham and Rigby, 2014). The DM further differentiates into three distinct domains: (i) a dorsomedial (epaxial) DM, close to the neural tube, (ii) a central DM (dermatome), and (iii) a ventromedial (hypaxial) DM (Chang and Kioussi, 2018).

During the above process, the descendants of the mesodermal precursors continue their progressive myogenic determination. The Pax3+ cells of the DM, which derive from the PSM, begin to express Pax7 (Buckingham and Relaix, 2015). The resulting double Pax3+/Pax7+ cells will generate the muscle precursors that will give rise to the fetal myogenesis, and to the perinatal satellite cells (Buckingham and Relaix, 2015; Gunther et al., 2013; Seale et al., 2000; von Maltzahn et al., 2013). The pro-myogenic action of Pax3 and Pax7 is finely tuned by the intracellular and extracellular stimuli of FGFs, Wnts and Shh (Buckingham and Rigby, 2014).

The initial expression of the MRFs can be traced in the epaxial DM, where Myf5 is detected at day E8.0 in the mouse embryo (Ott et al., 1991). Shortly after, Myf5 is also expressed in the hypaxial DM (Buckingham and Rigby, 2014). These two groups of Myf5+ cells then express MyoD1, to further support myogenic specification (Sassoon et al., 1989). Very soon, cells residing in the epaxial and lateral lips of the DM begin to migrate toward its ventral face to form the myotome, the first skeletal muscle of the body, and the source of the muscle precursor cells that will be incorporated in the trunk (Buckingham and Rigby, 2014). Around the same time, Pax3+ cells delaminate from the hypaxial DM to migrate into distinct anatomical fields where they will give rise to vertebral and abdominal muscles, the diaphragm, and limb muscles (Chang and Kioussi, 2018).

In addition to pro-myogenic inputs, the anatomical structures surrounding the somites release negative cues to restrain the potentially massive differentiation of the early muscle precursors, and to preserve their survival and proliferation for the future waves of muscle formation. For example, Bmp4 coming from the lateral mesoderm, and Bmp7 secreted by the neural tube, restrain the muscle differentiation in the somite (Amthor et al., 2002; Pourquie et al., 1996; Reshef et al., 1998; Wang et al., 2010). In a similar way, the local activation of Notch in some of the muscle precursors of the DM/myotome inhibits their differentiation (Hirsinger et al., 2001; Schuster-Gossler et al., 2007; Vasyutina et al., 2007).

In summary, the balanced combination of signaling molecules secreted from the anatomical structures surrounding the PM, the somites, and the migrating muscle precursors, orchestrates the setting of the muscle commitment, and its maintenance during the cell divisions.

3. Cell therapy for DMD

Cell therapy is based on the heterologous, or autologous, transplantation of cells, with the goal of regenerating the damaged tissue or organ of the patient, and replenishing specific stem cell populations. In the case of DMD, the main goal is to reconstitute the satellite cell pool with dystrophin competent cells, and thereby restore muscle function due to the presence of dystrophin expressing muscle fibers. The source of the therapeutic cells can be healthy, histocompatible donors, or genetically corrected autologous cells. Thus far, a number of different cell types have been applied in transplantation experiments in DMD animal models, and in DMD clinical trials.

3a Myoblasts and satellite cells

Experimental myoblast transplantation dates back to 1989, when research from groups led by Kunkel and Partridge pioneered the intramuscular (IM) transplantation of normal neonatal mouse myoblasts into mdx mice, a dystrophin deficient DMD mouse model (Morgan et al., 1990; Partridge et al., 1989). Subsequent experiments in humans and mice showed that IM-injected normal human or mouse myoblasts formed new dystrophin+ fibers, with the partial reconstitution of a normal muscle morphology (Gussoni et al., 1992; Huard et al., 1992; Kinoshita et al., 1994).

However, these early successful results in mice did not extend into the clinic. Following transplantation of muscle stem cells harvested from healthy immunocompatible donors, only small percentages of normal dystrophin (0–5% (Karpati et al., 1993); 0–10.3% (Mendell et al., 1995); 0–3.6% (Tremblay et al., 1993); 0% (Morandi et al., 1995); 0–80% (Huard et al., 1992)) were detected in patient's biopsies post transplantation. With the exception of one study (Huard et al., 1992), DMD patients receiving heterologous, partially immune-compatible, human myoblasts, did not show any functional improvements of the transplanted limb. These results can be explained by immune-rejection, the limited number and scarce migration of injected cells, and by massive cell death after transplantation (Skuk and Tremblay, 2003).

Satellite cells have a strong therapeutic advantage over myoblasts, because of their self-renewal capability, which maintains their stemness. Indeed, mouse muscles transplanted with a single mouse muscle fiber, containing around seven satellite cells, or even with a single mouse satellite cell, showed a much better muscle engraftment than was the case with transplanted myoblasts (Collins et al., 2005; Sacco et al., 2008). Similarly, human satellite cells isolated by fluorescence-activated cell sorting (FACS) from muscle biopsies, and transplanted into immune-compromised mice, also on a mdx background, led to stable engraftments, formation of mouse fibers expressing human markers, and colonization of the mouse satellite cell niche, the latter being a key feature for the regeneration of a chronically damaged muscle (Garcia et al., 2018; Xu et al., 2015).

However, many practical limitations and safety concerns still affect the use of human satellite cells in the clinic. Four issues are of particular note: (i) only a very small amount of satellite cells can be isolated from a biopsy, especially from the dystrophic muscle, thus only a very limited number of recipient muscles can be transplanted; (ii) cultured satellite cells show a reduced transplantation efficiency; (iii) most satellite cells die after transplantation, and their dissemination rate is negligible; and (iv) delivery of the satellite cells to muscles such as the diaphragm requires a systemic delivery. However, when injected into the bloodstream, satellite cells aggregate as micro-thrombi inside small vessels, and do not colonize the muscle. Thus, to be therapeutically viable, satellite cells must be given the ability to survive inside the blood, to extravasate from circulation to enter the muscles, and to migrate inside the muscle.

New protocols have been designed to overcome these hurdles, for example by using hydrogels or hypoxia conditioning (Gilbert et al., 2010; Liu et al., 2012), by expressing proteins regulating cell migration (Morgan et al., 2010), and by elongating the telomeres to

increase cell proliferation (Zhu et al., 2007). However, despite the promising potential of muscle cell transplantation and numerous efforts to optimize cell culture conditions in a lab setting, the use of myoblasts or satellite cells to treat DMD in the clinic has not been realized yet.

3b Bone marrow cells and mesoangioblasts

To overcome the therapeutic limitations of satellite cells, researchers sought, and found, other cell populations with myogenic capability, such as those inside the bone marrow (BM) (Ferrari et al., 1998; Gussoni et al., 1999). BM-derived myogenic cells can migrate into the regenerating muscle via the circulation, suggesting their potential use for DMD treatment. However, BM transplantation (BMT) in mdx mice, in canine models of DMD, and in a DMD patient, did not show improved dystrophin production, nor amelioration of muscle function (Dell'Agnola et al., 2004; Ferrari et al., 2001; Gussoni et al., 2002). These results indicate that the BM-derived myogenic cells either do not support muscle regeneration, or make only a negligible contribution.

The finding of circulating cells with myogenic potential increased the scientific interest in identifying new types of non-muscle cells with the potential to contribute to muscle regeneration. That led to the discovery of mesoangioblasts (De Angelis et al., 1999). Originally isolated from the embryonic dorsal aorta of the mouse embryo (De Angelis et al., 1999; Minasi et al., 2002), mesoangioblasts contribute to postnatal muscle development (De Angelis et al., 1999), and are considered the developmental precursors of pericytes, perivascular cells resident in the adult muscle (Dellavalle et al., 2007; Minasi et al., 2002). While mesoangioblasts derived from mouse embryo aorta express myogenic markers such as MyoD1, and endothelial markers such as VE-cadherin and CD31 (De Angelis et al., 1999), pericytes express neither, rather they express markers such as NG2, PDGFRβ and CD146 (Birbrair et al., 2013; Dellavalle et al., 2007). Nevertheless, pericytes can differentiate into muscle when exposed to low serum conditions, or when co-cultured with myoblasts (Dellavalle et al., 2007).

The potential plasticity of pericytes was also confirmed by their ability to leave the perivascular niche, and adopt the fate of the recipient local tissue (Minasi et al., 2002). This evidence further suggested their therapeutic use for DMD. Indeed, the intra-vascular injection of mouse mesoangioblasts, or of human pericytes, in both mouse and canine models of DMD and of limb-girdle muscular dystrophy, demonstrated their ability to colonize the muscle (Dellavalle et al., 2011; Dellavalle et al., 2007; Sampaolesi et al., 2006; Sampaolesi et al., 2003).

In a phase I/IIa clinical trial, five DMD individuals were injected intra-arterially with donor HLA-matched normal mesoangioblasts (Cossu et al., 2015). The trial showed in one individual a band corresponding to the full-length dystrophin by immunoblotting that could not be explained by revertant fibers, but no functional improvement in any of the patients (Cossu et al., 2015). All patients had undetectable or extremely low immunological responses against dystrophin protein domains.

3c CD133+ cells

CD133+ cells, first isolated from human peripheral blood, are multipotent stem cells with the capacity to repopulate the BM, and differentiate into endothelial cells (Torrente et al., 2004). CD133+ cells have also myogenic potential, as they express myogenic markers, and can give rise to satellite cells and to dystrophin positive myofibers after IM or intra-arterial transplantation into immunocompromised dystrophic mice (Meng et al., 2014; Negroni et al., 2009; Torrente et al., 2004). In 2007, a clinical trial of IM transplantation of DMD autologous CD133+ cells showed increased muscle vascularization, but no integration of the donor cells in the muscle fibers (Torrente et al., 2007).

4. Pluripotent stem cells

Vertebrate pluripotent stem cells (PSCs) retain their ability to differentiate into the three germ layers of the embryo: ectoderm, mesoderm, and endoderm. Typical PSCs are the embryonic stem cells (ESCs) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998), and the induced PSC (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006).

The generation of iPSCs opened up new avenues in stem cell therapy, and solved many problems associated with use of ESCs. For example, while human ESCs (hESCs) can only be isolated from the inner cell mass of an early embryo, which incurs numerous technical and ethical problems, human iPSCs (hiPSCs) can be generated from somatic cells, thereby allowing for the possibility of designing autologous, patient-specific, cell therapeutic strategies. This feature, along with the ability to be expanded indefinitely *in vitro*, and the plasticity to differentiate into any cell type, make hiPSCs a unique source for therapy, and for the study of the mechanisms of development and diseases.

4a iPSCs

Initially, iPSCs were generated by transducing mouse or human somatic fibroblasts with lentiviruses expressing the four “Yamanaka” factors (Oct3/4, Sox2, c-Myc, and Klf4) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Subsequently, to reduce the risk of DNA mutagenesis and to improve the rate of reprogramming, protocols were created to model the iPSCs for clinical purposes (Fusaki et al., 2009; Kim et al., 2009; Soldner et al., 2009; Warren et al., 2010).

For therapy of muscular dystrophies, hiPSCs hold great promise. Transplantation of therapeutic cells differentiated from hiPSCs generated from the patient’s own cells will not induce immune rejection as in heterologous transplantation. Furthermore, patient’s-derived iPSCs make it possible to model *in vitro* the etiology, and the pathophysiological progression, of different muscular dystrophies, to perform automated pre-clinical drugs screenings, and to set up *in vitro* protocols of gene editing before *in vivo* testing (Abujarour et al., 2014; Choi et al., 2016; Dick et al., 2013; Li et al., 2015; Long et al., 2018; Maffioletti et al., 2018; Mondragon-Gonzalez and Perlingeiro, 2018; Shoji et al., 2015; Uchimura et al., 2017; Young et al., 2016). With patient-specific hiPSCs, we should be able to identify new correlations between the established etiologic cause of each type of muscular dystrophy and

the presence of genetic and epigenetic modifiers in the human genome, information which is crucial for design more efficacious pharmacological therapies.

5. Muscle linage specification systems

One of the strategies to achieve a direct myogenic specification of PSCs is to replicate in the culture dish the inductive stimuli which underlie the muscle determination in the developing embryos. To accomplish this goal, one approach is for monolayer PSCs to be treated *in vitro* with the specific cytokines and growth/morphogenetic factors that orchestrate the specification of the mesoderm *in vivo*, the somitogenesis, and the commitment of the early muscle progenitors (Chal et al., 2015). A second, simpler approach to convert the PSCs into myogenic cells is through the over-expression of key myogenic transcription factors, to induce a pro-myogenic transcriptome, which in turn, can make the PSCs more sensitive to myogenic inductive stimuli.

5a Early attempts to achieve myogenic differentiation of the PSCs *in vitro*

The first attempt to direct ESCs into a myogenic fate was carried out by inducing the formation of the embryoid body (EB) *in vitro*. The EBs are clusters of PSCs that can spontaneously differentiate into precursor cells of the three germ layers of the embryo (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). For example, mouse ESCs (mESCs) were allowed to aggregate in EBs, that in turn, were cultivated in hanging drops for two days, then in suspension for three days. After five days, the EBs were plated, with the myotubes detected four days later, showing the capability of the ESCs to acquire the myogenic fate almost spontaneously (Rohwedel et al., 1998; Rohwedel et al., 1994). However, a large proportion of cells in these mouse EBs (mEBs) differentiated into neuronal cells (Bain et al., 1995; Rohwedel et al., 1998). Similar results were obtained using human EBs (hEBs) cultured in growth medium supplemented with dexamethasone, insulin-transferrin-selenium (ITS), glutamine, and epidermal growth factor (EGF) (Zheng et al., 2006). However, the myogenic precursors induced in this study differentiated properly only when transplanted in the regenerating muscle of a recipient mouse (Zheng et al., 2006).

These early experiments demonstrated that to obtain homogenous and consistent muscle commitment and differentiation *in vitro*, it was necessary to identify the proper combination of pro-myogenic cues. A seminal advancement in this direction was achieved by the Studer group, which, for the first time, reported the successful differentiation of hESCs into CD73+ mesenchymal progenitor cells, and then into myoblasts (Barberi et al., 2007; Barberi et al., 2005). In these studies, hESCs were cultured at low-density in serum-free medium supplemented with ITS (Barberi et al., 2007). Importantly, the progressive increase of serum concentration over the course of the cell culture increased the percentage of CD73+ cells, and allowed the mesenchymal precursors to progress toward different mesodermal fates, such as bone and muscle, through a transient endodermal/mesodermal stage (Barberi et al., 2007). Eventually, this process resulted in the generation of muscle-committed cells, positive for the neural cell adhesion molecule (NCAM+), an established marker of human myogenic cells. Sorted NCAM+ cells generated myotubes *in vitro*, and colonized the regenerating muscle of recipient, immunocompromised, mice (Barberi et al., 2007; Barberi et al., 2005).

Subsequently, Sakurai et al. were able to generate myogenic cells from mESCs, via the induction of an early mesodermal lineage, using serum-free, chemically-defined, culture media supplemented with the synchronized addition of Bmp4 and lithium chloride (LiCl) (Sakurai et al., 2009). These pioneer studies set the foundation for step-wise systems for the myogenic commitment *in vitro*.

5b Step-wise differentiation systems

According to the process of muscle commitment in the vertebrate embryo, the first logical step to differentiate the PSCs into the myogenic linage is to induce them to a PM-, NMPs-like fate. In this regard, several groups successfully generated PM cells by treating mouse and human PSCs with the inhibitor of the glycogen synthase kinase3-β (Gsk3-β) CHIR99021 (Chal et al., 2015; Mendjan et al., 2014). Since Gsk3-β inhibits the nuclear internalization of β-catenin, treating the cells with CHIR99021 mimics the addition of Wnt in the culture medium. Additional protocols showed that the induction of a PM fate can be further supported by supplementing the culture media of CHIR99021-treated PSCs with FGF (Gouti et al., 2014; Turner et al., 2014), or with activin, a TGF-β pathway activator (Loh et al., 2016). However, other reports showed FGF and activin to be dispensable for the acquisition of the NMP fate (Henrique et al., 2015; Pourquie et al., 2018), probably because the activation of the Wnt signaling in the PSCs results in the production of FGFs by the cells (Denham et al., 2015). These findings suggest that intracellular activation of Wnt signaling is sufficient for PSCs to acquire the PM/NMP fate (Pourquie et al., 2018).

The next step is to induce the differentiation of the NMP-like cells into PSM-like cells. As presented earlier (section 2), PSM cells express serially Tbx6 and Msgn1 (Chalamalasetty et al., 2014; Gouti et al., 2017), and several groups showed that activation of the Wnt signaling with CHIR99021 is sufficient to induce the expression of these two PSM markers in the PSCs. For example, Chal et al. (Chal et al., 2015) showed that by inhibiting BMP and activating Wnt, both mouse and human ESCs can differentiate into PSM progenitors. In contrast, Shelton et al. (Shelton et al., 2014) reported that the treatment of the same types of cells with CHIR99021 alone robustly induced the PSM in serum-free media. These two investigations, and similar work from other labs including our own, indicate that the initial activation of the Wnt pathway by CHIR99021 is sufficient to differentiate the PSCs to the PSM stage (Choi et al., 2016; Henrique et al., 2015; Loh et al., 2016; Sudheer et al., 2016; Xi et al., 2017).

From this step onward, the cell culture conditions used to induce the full myogenic commitment *in vitro* vary among the different protocols, including further treatments to increase the muscle programming efficiency, via the addition of hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1) and FGF2 to the culture medium (Chal et al., 2015; Shelton et al., 2014). When treated in such a way, mESCs generate Pax7+ myogenic cells, which give rise to Myogenin+ myoblasts and fuse into myosin heavy chain (MyHC)+ myotubes that show contractile activity *in vitro* (Chal et al., 2015).

A simplified protocol of muscle commitment has recently been devised in our lab by treating normal, and DMD-derived, hiPSCs (DMD-hiPSCs) with a Notch inhibitor (DAPT), after an initial treatment with CHIR99021 (Choi et al., 2016). In this study, we identified a defect in

myotube formation in the DMD-hiPSCs caused by the up-regulation of the BMP and TGF-β signaling in the DMD myoblasts. The addition of a TGF-β inhibitor into the medium significantly improved the fusion of the muscle programmed DMD-hiPSCs (Choi et al., 2016). Increased myogenic linage differentiation of the healthy hPSCs was also observed by using different TGF-β inhibitors on CHIR99021 pre-treated hiPSCs *in vitro*, or by treating mice transplanted with muscle programmed, genetically corrected DMD-hiPSCs (Hicks et al., 2018).

The protocols that involve human iPSC step-wise differentiation without EB formation are compared in Table 1 (Chal et al., 2016; Chal et al., 2015; Choi et al., 2016; Sakai-Takemura et al., 2018; Wu et al., 2018).

5c Direct-programming of mPSCs and hPSCs into the myogenic lineage

Muscle specification in PSCs can also be induced through the forced expression of specific myogenic transcription factors such as MyoD1, Pax3 and Pax7. As previously mentioned, these transcription factors play critical roles in myogenic specification during development, and in the adult. Initial attempts to induce myogenesis in somatic cells, as well as in ESCs, used the ectopic expression of MyoD1 by means of cell transfection or viral transduction (Davis et al., 1987; Ozasa et al., 2007). Subsequent studies were carried out by over-expressing MyoD1, Pax3, or Pax7 into mouse and human iPSCs (Abujarour et al., 2014; Darabi et al., 2012; Filareto et al., 2013; Goudenege et al., 2012). In particular, by using a doxycycline inducible system, Perlingeiro and colleagues over-expressed Pax3, or Pax7, in mouse and human ESCs and hiPSCs (iPax3, iPax7 cells), and successfully isolated myogenic progenitors by sorting the EB-derived cells for the platelet-derived growth factor receptor α (Pdgfrα), an early PM marker that discriminates between the skeletal and the cardiac muscle fate *in vitro* (Darabi et al., 2008; Darabi et al., 2011; Magli et al., 2014). Importantly, iPax3 and iPax7 cells can generate muscle fibers, and colonize the satellite cell niche upon transplantation in the mouse dystrophic muscle (Darabi et al., 2012; Magli et al., 2017).

5d hPSCs-derived myogenic cell transplantations

As an ideal autologous cell source for therapy of muscular dystrophies, hiPSCs can be generated from patient's somatic cells, processed for genetic correction, differentiated *in vitro*, and then transplanted back into the patient. The step-wise differentiation system has some advantages. For example, it is inherently transgene-free, thus avoiding mutagenic risks for the patient, and can be standardized according to good manufacturing practices. However, there are some limitations. One of the major issues of the step-wise differentiation systems is the generation *in vitro* of mixed cell populations, including terminally differentiated myotubes and other non-muscle cell types, such as neurons. Consequently, the presence of a potentially large percentage of contaminating, non-myogenic, cells *in vitro* strongly reduces the engraftment efficiency of the therapeutic cells *in vivo*, and results in low rates of satellite cell niche colonization and muscle regeneration (Kim et al., 2017). In comparison, the directly-programmed iPAX7/iPAX3 hiPSC-derived myogenic progenitors, which comprise mostly PAX7+/PAX3+ cells, resulted in far better muscle engraftment (Darabi et al., 2012; Darabi et al., 2011; Kim et al., 2017). The differences in engraftment

efficiency between the direct-programming and step-wise differentiation protocols can be explained by the fact that the iPAX7/iPAX3 hiPSCs may represent a purer and more homogeneous myogenic population than the myogenic cells generated through the step-wise differentiation systems. In addition, iPAX7/iPAX3 hiPSCs can have a cellular status more similar to muscle progenitors than myoblasts.

Evidence indicates that the Pax7+ myogenic progenitors, such as freshly isolated satellite cells, hold a better regeneration capacity than do the myoblasts, which allows the former cells to enter the muscle stem cell niche, a key prerequisite for long-term therapeutic effects. Indeed, both satellite cells and iPax7/iPax3 PSCs-derived myogenic cells show comparable engraftment rates in mouse recipient muscles, and contribution to serial cycles of muscle regeneration (Incitti et al., 2019; Magli et al., 2017; Sacco et al., 2008). In addition, it is now clear that the human myogenic progenitor cells derived from step-wise differentiation cultures are more similar to the muscle progenitors of the early fetal stages than they are to the adult satellite cells, as shown by assays of *in vitro* differentiation and transcriptomic analysis (Chal et al., 2015; Choi et al., 2016; Hicks et al., 2018; Shelton et al., 2014). Moreover, recent results show that the iPax7/iPax3 PSCs-derived myogenic progenitors increase their myogenic potential after the transplantation in the muscle of immunocompromised mice, and, once in the satellite cell niche, they show a molecular signature comparable to that of adult satellite cells (Incitti et al., 2019). The above evidence indicates that the muscle environment *in vivo* instructs the PSCs-derived myogenic cells to progress from a fetal/perinatal-like status into an adult-like myogenic status (Incitti et al., 2019). Nevertheless, the molecular basis of this maturing process is still unknown.

Recently, several groups have started to identify new surface markers characterizing the human muscle precursors, to improve the engraftment rates of the hiPSCs-derived myogenic precursors, with the goal of standardizing the *in vitro* procedures for clinical applications. For example, Hicks et al. purified PAX7+ myogenic progenitors from hPSCs-derived myogenic cells *in vitro*, based on the expression of the Erb-B2 receptor tyrosine kinase 3, and the nerve growth factor receptor (Hicks et al., 2018). Similarly, Magli et al. and Wu et al. identified CD10, CD24, CD54, Integrin α9β1, and Syndecan 2, as markers useful for purifying hiPSC-derived myogenic progenitors *in vitro*, by using MYF5 and PAX7 double reporter hESCs, or iPAX7-hPSCs (Magli et al., 2017; Wu et al., 2018). These studies reported better engraftment results using cells isolated with these markers than non-isolated cells.

6. Genetic engineering of hiPSCs to restore functional dystrophin expression

In order to generate dystrophin expressing muscle fibers, hiPSCs derived from DMD patients can be genetically corrected to express functional dystrophin for autologous cell replacement therapy. CRISPR-Cas9 mediated gene editing is currently being investigated as a tool to perform such correction. It involves two components: a single guide RNA (sgRNA) and the Cas9 endonuclease. Cas9 endonuclease associates with the sgRNA at the genomic target sequence to create DNA double strand breaks leading to homology-directed repair

(HDR), or nonhomologous end-joining (NHEJ) repair (Garcia-Doval and Jinek, 2017; Hochheiser et al., 2018). Thereafter, this technique could, theoretically, correct most of the DMD mutations including point mutations, deletions and insertion, re-establishing the correct sequence of the dystrophin gene, or at least its translational frame after RNA transcription (Amoasii et al., 2018; Bengtsson et al., 2017; Wong and Cohn, 2017). The clear advantage of CRISPR-Cas9 over other strategies, is that it affords permanent repair of the dystrophin gene (Amoasii et al., 2018; Bengtsson et al., 2017; Long et al., 2015; Nelson et al., 2016; Tabebordbar et al., 2015). To date, there have not been any clinical trials of genetically corrected DMD-hiPSCs for cell transplantation therapy. However, this concept has been proven feasible by successful restoration of dystrophin protein expression, and DGC formation, from engraftment of myotubes derived from genetically corrected DMD-hiPSCs in immunocompromised mdx mice (Young et al., 2016).

7. Potential limitations of using hiPSCs for DMD treatment

The use of hiPSCs for treatment of muscular dystrophies is very promising. However, before proceeding to clinical trials, four key limitations must be overcome, and potential safety issues addressed. (i) We have to identify the patients' best somatic cell type from which to generate the hiPSCs, and we have to improve the hiPSCs muscle commitment protocols, for example by generating the myogenic cells more quickly *in vitro*, and by using culture media free of animal factors. (ii) We have to optimize the delivery route of the therapeutic cells, to get the highest rate of muscle engraftment *in vivo*. Delivery could use innovative methods of systemic dissemination, thereby avoiding the dangerous accumulation of injected cells in highly vascularized organs such as liver and lungs. Furthermore, transplanted cells could be instructed to selectively cross the muscle-endothelial barrier, to fuse with the regenerating muscle fibers. (iii) For long-term benefits, we have to ensure that the therapeutic cells will stably colonize the satellite cell niche. (iv) The gene editing tools, which will be used to correct the causative genetic defect, must be without off-target effects.

Progress on overcoming these limitations is being made, including the development of DNA-free-based protocols for myogenic induction *in vitro* using a combination of chemical compounds and specific growth factors/morphogens, the identification of specific cell surface markers to separate the myogenic cells for transplantation from the other types of cells in the differentiation culture, and the use of new generation CRISPR-based systems (Fu et al., 2014; Hicks et al., 2018; Magli et al., 2017; Wu et al., 2018).

8. Future directions of the hiPSCs therapy development for the MDs

To develop clinically applicable hiPSCs-based therapies, researchers have focused on deriving cells that have high potency in terms of regenerating and self-renew, i.e. cells that have similar features to those of adult satellite cells (Incitti et al., 2019) (Figure 1). Based on this, progenitor cells can acquire a higher clinical potential. As discussed earlier, hiPSCs-derived myogenic progenitors have a molecular profile that is similar to fetal-stage myoblasts. Therefore, one of the most critical experiments to do is the induction, *in vitro*, of the progression of these cells toward more mature myogenic stages. We can achieve this goal by seeking compounds that can mature myogenic progenitors, or we can co-culture these

cells with primary myotubes to mimic the *in vivo* muscle environment. Moreover, it is important to consider whether the hiPSCs-derived myogenic cells could be delivered systemically. In this respect, Gerli et al. (Gerli et al., 2019) recently demonstrated that modulating NOTCH and PDGF pathways can endow satellite cells with the ability to migrate trans-endothelially. Based on this finding, we can predict that a proper combination of modulatory growth factors and cytokines *in vitro* can instruct the hiPSCs-derived myogenic cells to reach all the muscles of the body via the bloodstream.

9. Conclusion

Stem cells, due to their advantageous regeneration capability, bring the promise for cell transplantation therapy (Fig.1 summary of cells that can be used for stem cell-based therapies for muscular dystrophies). hiPSCs that can be derived from patients open the avenue for autologous cell therapy. With the rapid development of serum-free lineage specification protocols, expandable myogenic progenitor cells can be differentiated from hiPSCs. This population of cells has similar characteristics to stem cells and has superior muscle regeneration capability compared with myoblasts. In combination with gene editing techniques, hiPSC-derived myogenic progenitor cells hold potential as an efficacious therapeutic avenue for MDs.

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Abbreviations:

hPSCs	human pluripotent stem cells
hiPSCs	human induced pluripotent stem cells
PSC	pluripotent stem cells
AON	antisense oligonucleotide
FAPs	fibro-adipogenic progenitors
DGC	Dystrophin Glycoprotein Complex
PM	paraxial mesoderm
PS	primitive streak
NMPs	neuromesodermal progenitors
PSM	presomitic mesoderm
TGF-β	transforming growth factor β
BMPs	bone morphogenetic proteins
RA	retinoic acid

Shh	sonic hedgehog
DM	dermomyotome
EB	embryoid body
ITS	insulin-transferrin-selenium
NCAM+	neural cell adhesion molecule
Gsk3-β	glycogen synthase kinase3-β

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Highlights:

1. Myogenic progenitor cells could provide a source for cell therapy for Duchenne muscular dystrophy (DMD) to regenerate and replace the diseased tissue.
2. An orchestration of signaling molecules, including Notch, FGF, Wnt and TGF-β, directs the myogenic lineage determination during an embryo's development, and sets the basis for the hPSC (human pluripotent stem cell) myogenic linage specification *in vitro*.
3. There were several attempts of available cell-based therapies for DMD with limited success, therefore, there is a need to develop more cell therapy options (e.g. iPSC based).
4. This review describes the most recent studies of human induced pluripotent stem cells muscle lineage specification, and the potential of their clinical application to treat muscular dystrophies.

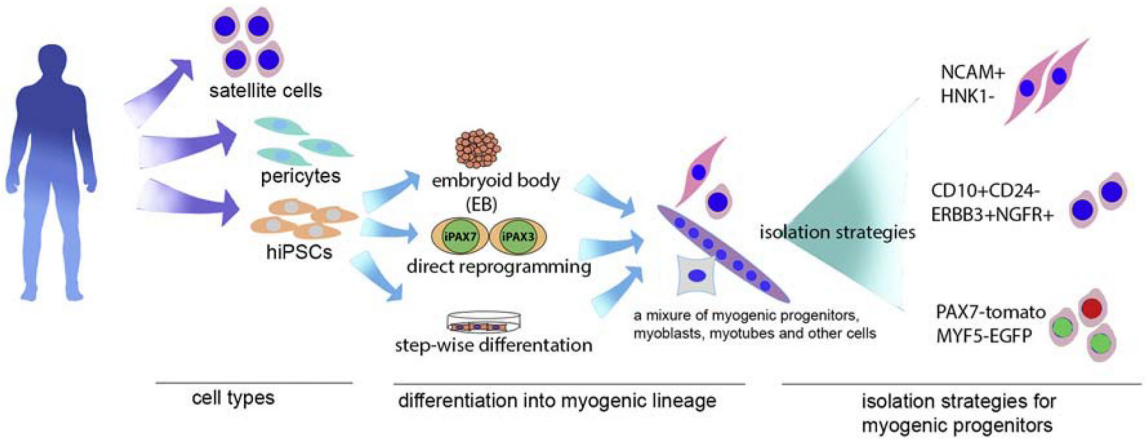

Figure 1.

Overview of potential stem cell therapy strategies for DMD that have been studied. Three type of cells, satellite cells, pericytes and hiPSCs have the highest potential for clinical application. Satellite cells and pericytes can be directly transplanted after isolation, hiPSCs require myogenic lineage differentiation, for which three methods are shown. The final cell product after myogenic commitment/differentiation can be isolated by FACS using the combination of surface markers or fluorescent reporter proteins.

Table 1

human iPSC step-wise differentiation

Study	Induction factor	Medium	Duration of muscle commitment and differentiation (days)	Cell composition	Purification	In vitro	In vivo
Chal et al., 2015	days 0–6 CHIR99021 and LDN193189, insulin-transferrin-selenium; days 3–6 addition of FGF-2; days 6–8 IGF-1, HGF, FGF2 and LDN193189; days 8–12 IGF-1; days 12–50 IGF-1 and HGF	DMEM based medium; days 0–6 serum free, thereafter + knock-out serum replacement (KSR)	20–30	~22% of nuclei were MYOG+, and 23% of nuclei were PAX7+	No purification	MyHC+, MYOG+ fibers, PAX7+ cells, organized sarcomeres, with the periodic distribution of the sarcomeric proteins Titin and fast MyHC	No
Chal et al., 2016	days 0–6 CHIR99021 and LDN193189, insulin-transferrin-selenium; days 3–6 addition of FGF-2; days 6–8 IGF-1, HGF, FGF2 and LDN193189; days 8–12 IGF-1; days 12–50 IGF-1 and HGF	DMEM based medium; days 0–6 serum free, thereafter + KSR	30	Most MYOG+ cells, MyHC+ myofibers. PAX7+ satellite-like cells.	No purification	Sub-culturing of human myo-progenitors by day 28. MyHC+, MYOG+ fibers, PAX7+ cells, organized sarcomeres. Fast MyHC+ and h-Dystrophin+ fibers in muscle construct.	No
Choi et al., 2016	days 0–4 CHIR99021; days 4–12 DAPT	Serum-free N2 medium	30	~15% NCAM+ HNK1-myoblasts, 61.5% MYOG+ and 63.6% MyHC+ cells	NCAM+ HNK1-	MyHC, TITIN, DES, DYSTROPHIN, α-ACTININ	PAX7+ cells, h-Dystrophin+ fibers
Hicks et al., 2018	days 0–2 CHIR99021; days 12–20 FGF2, TGF-β inhibitor when in differentiation condition	days 0–12, days 20–35 E6 medium, days 12–20 StemPro-34 medium, days 35–50 DMEM/F12 + 1% ITS medium	30–50	PAX7, MYF5, MYOD, MYOGENIN and spontaneously contracting myotubes	ERBB3+NGFR+	Enriched for PAX7 and MYF5	h-LaminA/C+, h-Dystrophin+ fibers. In vivo engraftment of ERBB3+ hiPSC-SMPCs restored dystrophin to levels approaching uncultured fetal muscle
Sakai-Takemura et al., 2018	days 0–12 Chal et al. 2016 method; days 12–42 floating culture;	days 0–12 DMEM, days 12–70 10% FBS/DMEM	70	MYOGENIN+ myotubes	CD57(−) CD108(−) CD271(+) ERBB3(+) cells	MYOGENIN, MyHC	h-LaminA/C+ (nuclear membrane) and h-Spectrin+

Study	Induction factor	Medium	Duration of muscle commitment and differentiation (days)	Cell composition	Purification	In vitro	In vivo
	days 42–70 adhesion culture						(sarcolemma) myofibers
Wu et al., 2018	days 1–4 CHIR99021, BMP inhibitor, TGF-β inhibitor; days 5–15 BMP inhibitor, TGF-β inhibitor	MDM-I medium days1–4, MDM-II medium day 5–15, MDM-III medium for terminal differentiation	15	at day 4 99.4% PAX7+, at day 15, 50–55% MYF5+	CD10+CD24-	MyHC+ myotubes	myofibers expressing human markers (maximum of 50–60 fibers positive for h-Dystrophin and h-Lamin A/C)