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Pluripotent Stem Cell-Based Therapeutics for Muscular Dystrophies

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Abstract

Pluripotent stem cells represent an attractive cell source for treating muscular dystrophies since they easily allow for the generation of large numbers of highly regenerative myogenic progenitors. Using reprogramming technology, patient-specific pluripotent stem cells have been derived for several types of muscular dystrophies, and genome editing has allowed correction of mutations, opening the opportunity for their therapeutic application in an autologous transplantation setting. However, there has been limited progress on preclinical studies that validate the therapeutic potential of these gene corrected pluripotent stem cell-derived myogenic progenitors. In this review, we highlight the major research advances, challenges and future prospects towards the development of pluripotent stem cell-based therapeutics for muscular dystrophies.

Keywords

pluripotent stem cells; gene correction; myogenic progenitors; cell therapy; muscular dystrophy

Muscle Degeneration and Pluripotent Stem Cells

Muscle degeneration is a condition that affects millions of people worldwide. It is primarily observed in patients suffering from 1 of the more than 30 types of muscular dystrophy (MD) identified so far. Among these, Duchenne Muscular Dystrophy (DMD), caused by mutations in the DMD gene, is the most common and characterized by relentless and catastrophic progression, usually culminating in death by the third decade of life. There is currently no cure for MD disorders, and therefore they represent a serious unmet medical need.

Current research focuses mostly on the development of gene- and cell-based therapeutic approaches to cure MD. Gene therapy involves delivering the missing gene in affected

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muscles to rescue protein function. Cell therapy (see Glossary) focuses on the replacement of the diseased muscle tissue with muscle stem/progenitor cells, which upon engraftment give rise to new healthy myofibers as well as fuse with regenerating muscle fibers, thus restoring muscle function. Both gene- and cell-based strategies hold great potential, but for the purposes of this review, we will focus on the latter.

Pluripotent stem cells (PSC) are an attractive source for cell-based therapeutics due to their unlimited proliferative potential and their ability to differentiate into all cell types of the body. Considering the development of methodologies that enable the efficient generation of PSC-derived myogenic progenitors endowed with *in vivo* regenerative potential, combined with the significant recent progress in genome editing technologies, it is plausible to envision the use of the MD patient's own cells for autologous cell transplantation upon gene correction. This is theoretically feasible since MD progression takes over a decade, allowing more than sufficient time to generate and test iPS cells, correct the mutation, and derive/characterize large numbers of transplantable skeletal muscle derivatives. This review will focus on recent developments in the areas of gene editing and iPS cells and their potential therapeutic applications for MDs.

Muscular Dystrophies and Skeletal Muscle Regeneration

Muscular dystrophy (MD) denotes a large group of heterogeneous genetic diseases characterized by progressive muscle wasting. In addition to the genetic heterogeneity, the age of onset, severity, and types of muscles affected vary significantly among different types of MD (Table 1). In the case of DMD, the most common and severe form of MD, patients are usually wheelchair-bound by their early teens, and rarely survive past their mid-twenties due to severe cardio-respiratory failure. To date, there is no cure for MDs, and current treatments mostly alleviate disease symptoms, which in some cases can slow down disease progression. Recently, exon-skipping with antisense oligonucleotides has been used to treat DMD patients carrying frameshift mutations. However, the clinical data have not shown clear efficacy therefore the conditional approval of the drug Exondys 51, which targets DMD exon 51 skipping [1], has generated controversy.

Many types of MDs are associated with genetic and biochemical defects of the dystrophin-glycoprotein complex (DGC) [2]. These alterations lead to cell membrane damage and apoptosis of muscle cells, resulting in chronic successive cycles of degeneration/regeneration, culminating in compromised regeneration overtime [3]. Nevertheless, the regenerative nature of skeletal muscle provides an opportunity for utilizing cell therapy through delivery of healthy myoblasts that can engraft, fuse to the regenerating muscle fibers and rescue the missing protein function in the dystrophic muscle, as shown by Partridge and colleagues in pioneering studies [4]. Unfortunately, results from early clinical trials did not show clinical benefit [5–8], which was attributed mostly to the poor survival and limited migratory ability of injected myoblasts [9, 10] (see Clinician's corner). Instead of myoblasts, skeletal muscle stem cells (also known as satellite cells) would be preferable for therapeutic application since these cells have the ability to self-renew and efficiently contribute to muscle regeneration. A major hurdle with muscle tissue is the impossibility of obtaining enough satellite cells without permanently damaging the muscle of the donor. Small muscle

biopsies allow for the *ex vivo* expansion of satellite cell progeny (myoblasts), and therefore cell preparations with reduced engraftment ability [11, 12].

There have been several studies demonstrating the regenerative potential of mesoangioblasts in mouse and dog models of muscular dystrophy [13, 14]. These encouraging findings prompted a phase I/II clinical trial, consisting of multiple intra-arterial infusions of mesoangioblasts in pediatric DMD patients (Eudract 2011–000176-33)[I]. Although the therapy was proven to be feasible and relatively safe, there was no demonstration of efficacy or clinical benefit [15]. Therefore, further studies and alternate sources of early skeletal muscle progenitors are still necessary for the development of an effective stem cell therapy for MDs.

Pluripotent Stem Cells

PSCs can differentiate into cells of all three germ layers, ectoderm, mesoderm and endoderm. In addition to this broad differentiation ability, they possess extraordinary self-renewal capacity, allowing for unlimited expansion *in vitro*. PSCs encompass embryonic and induced pluripotent stem cells (ESCs and iPSCs, respectively). ESCs are established from the inner cell mass of blastocyst stage embryos [16, 17], and were first derived from human embryos in 1998 by James Thomson and colleagues [18]. ESC derivation is inefficient and requires destruction of human embryos, which raises ethical concerns. About a decade later, these issues were overcome with the technology of reprogramming somatic cells to the pluripotent state (iPS cells). This strategy, pioneered by Shinya Yamanaka and colleagues, based on the transient expression of the pluripotency-associated transcription factors, brings PSC-based therapy much closer to reality since it allows for the derivation of patient-specific iPS cells, eliminating the ethical and practical constraints associated with ES cells [19, 20]. For therapeutic application to muscular dystrophies, one could envision the use of healthy human leukocyte antigen (HLA)-matched iPS cell-derived myogenic progenitors (allogeneic transplantation) or the patient's own iPS cell-derived myogenic progenitors (autologous transplantation). In allogeneic therapy, HLA matching reduces the risk of immune rejection of the transplanted cells and thus universal donor iPS cell banks referred to as the HLA haplobank model are being established [21–24]. The autologous approach requires *in vitro* genetic correction of dystrophic iPS cells prior to transplantation (Figure 1), allowing for personalized medicine. In any case, some aspects should be taken in to consideration for the development of a PSC-based therapy for muscular dystrophies, including i) the implementation of a controlled differentiation protocol to specifically derive skeletal myogenic progenitors, devoid of residual PSCs and other non-muscle lineages; ii) myogenic progenitors should be endowed with robust *in vivo* regenerative potential, and iii) for long-term efficacy, it is critical that transplanted cells also seed the satellite cell compartment, therefore allowing for sustained expression of the therapeutic protein in newly formed muscle fibers.

I. https://www.clinicaltrialsregister.eu/ctr-search/search?query=eudract_number:2011-000176-33

Derivation of PSC-Derived Myogenic Progenitors

Several protocols have been developed for the derivation of myogenic progenitors from PSCs, which may involve the use of transgenes or not. In this section, we will focus mostly on strategies that have documented the *in vivo* regenerative potential of resulting myogenic progenitors, but we also describe a few *in vitro*-only publications that were important for progress in the field (Table 2).

Transgene-dependent myogenic differentiation

The first description of somatic cell fate transdifferentiation was reported by Weintraub and colleagues [25, 26] when they observed that MYOD could reprogram fibroblasts into myoblasts. Accordingly, several investigators have used this strategy to generate myoblasts from human PS cells. One study utilized adenoviral vector-based delivery of MYOD to generate myoblasts from human ES and DMD iPS cells, which upon transplantation into *Rag/mdx* mice (an immunodeficient DMD mouse model) fused with existing myofibers [27]. Tedesco and colleagues applied conditional expression of MYOD using lentiviral vectors to promote the muscle differentiation of human iPSC-derived mesoangioblasts. Transplantation of these cells into α-Sgca-null immunodeficient mice led to myofiber engraftment and rescue of SGCA protein [28]. Despite this positive outcome, a caveat with the use of MYOD is the derivation of a more committed myogenic cell (myoblast), which possess limited proliferative capacity and may not contribute to the stem cell pool upon transplantation.

In the transcription factor hierarchy of skeletal myogenesis, the transcription factor PAX7, which is positioned upstream of MYOD, is critical for postnatal muscle regeneration [29, 30], being expressed in satellite cells during their specification, proliferation, and activation [31]. With the premise that PAX7 expression would target a more primitive cell (muscle stem/progenitor cell) within the muscle hierarchy, Darabi and colleagues used conditional expression of PAX7 (iPAX7) to promote the *in vitro* differentiation of human ES and iPS cells towards the myogenic lineage. Using this strategy, these authors generated a highly expandable population of myogenic progenitors, which upon intramuscular transplantation into *NSG/mdx* mice resulted in donor-derived myofibers expressing human dystrophin, improvement of muscle contractile parameters, and seeding of the satellite cell compartment, and therefore, contribution to long-term regeneration [32]. Through gene expression profiling of iPAX7 differentiating cells, Magli and colleagues recently identified CD54, integrin α9β1, and SYNDECAN-2 (SDC2) as surface markers to be used for the prospective isolation of human PS cell-derived myogenic progenitors using both fluorescent- and cGMP-compatible magnetic-based sorting technologies [33]. Therefore, the use of PAX7 transgene allows efficient derivation of therapeutically relevant myogenic progenitors from PSCs.

Transgene-free myogenic differentiation

Numerous transgene-free protocols have been published for the *in vitro* derivation of myogenic cells from PSCs. Two studies described the derivation of myogenic cells through a mesenchymal precursor [34, 35], but engraftment data overall was limited. Most recent

protocols make use of small molecules to direct the differentiation of PSCs towards the myogenic lineage. Since most skeletal muscles derive from paraxial mesoderm during development, a key aspect is to recapitulate this process during the *in vitro* differentiation of PSCs. Initial studies utilized small molecule treatment to induce WNT signaling activation through GSK3β inhibition to differentiate PSCs into paraxial mesoderm, and then fibroblast growth factor 2 (FGF2) to derive myogenic progenitors [36, 37], and transplantation of resulting myogenic progenitors into immunodeficient mice produced myofiber engraftment [38]. This protocol was further improved by Chal and colleagues as they applied inhibition of GSK3β and BMP signaling along with subsequent exposure to pro-myogenic growth factors, including FGF2, hepatocyte growth factor (HGF), and insulin-like growth factor 1 (IGF-1) to induce differentiation of human PSCs towards the myogenic lineage [39, 40]. Even though these improvements enhanced the generation of cells with *in vitro* myogenic differentiation potential, the heterogeneity within these cultures (presence of non-myogenic cells) and lack of *in vivo* regenerative potential [41] limit their use for therapeutic application. In 2017, Giacomazzi and colleagues reported that myogenic progenitors derived from mesoangioblast iPSCs engraft better than from fibroblast iPSCs, and that treatment with microRNA cocktail further enhances this engraftment potential [42, 43]. In 2018, Hicks and colleagues documented that myogenic progenitors can be purified from PSC-derived monolayer cultures [36, 37] using the cell surface proteins ERBB3 and NGFR [44], which upon transplantation into NSG/mdx mice, along with TGFβ signaling inhibitor, gave rise to dystrophin-expressing myofibers. Another group reported myofiber and satellite cell engraftment upon the utilization of GSK3β inhibition followed by NOTCH signaling inhibition to generate PSC-derived myoblasts [45]. Most recently, Wu and colleagues reported a transgene-free protocol utilizing a novel PAX7/MYF5 double reporter PSC line [46–48]. By applying inhibition of the GSK3β, TGFβ and BMP signaling pathways during the early stages of PSC, in combination with subsequent purification based on the expression of CD10 and absence of CD24, these authors showed the derivation of myogenic progenitors capable of giving rise to myofibers and satellite cells upon their transplantation into NSG/mdx mice [48]. Despite significant progress on the generation of transgene-free protocols that result in engraftable myogenic progenitors, the heterogeneity within these cultures remain a challenge for therapeutic applications.

Recent Progress in Genome Editing Technologies

The recent and rapid progress in the development of genome-editing technologies has allowed investigators to easily introduce sequence-specific modifications into the human genome. Genome editing exploits endogenous DNA repair mechanisms to induce these modifications. Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9 (CRISPR-Cas9) are the three sequence-specific nucleases commonly utilized for genome editing (Figure 2). ZFNs are artificial restriction enzymes, which consist of DNA binding zinc finger domains fused to a nuclease to achieve sequence specific DNA cleavage [49]. The rules underlying sequence specificity of ZFNs are imprecise, meaning design involves a large empirical component, therefore effective ZFNs generally require a long phase of trial and error optimization to obtain sufficient specificity. TALENs are fusions of a

nuclease to TAL effector DNA domains, whose sequence specificity is predetermined and therefore TALENs can be easily designed and quickly synthesized [50–52]. Their principal disadvantage is their large size. CRISPR-Cas9 was developed from a bacterial immune defense mechanism targeting DNA of viruses. Sequence specificity is defined by a short RNA that is homologous to and derived from viral sequences, and the RNA-protein complex targets a nuclease to destroy viral DNA. CRISPR-Cas9 nuclease has been adapted for genome editing wherein short RNA complementary to the DNA sequence of interest can direct the Cas9 to cleave genomic DNA [53–56]. CRISPR-Cas9 is much easier to design than TALENs and ZFN as it requires a short RNA as opposed to designing a protein, however its principal weakness is the complexity introduced by the need to employ both a protein and an RNA for sequence specificity. To induce genome editing using these sequence specific nucleases, endogenous DNA repair mechanisms, such as non-homologous end joining (NHEJ), homologous recombination (HR) and microhomology-mediated end joining (MMEJ) are exploited (Figure 3). NHEJ involves repair of the double-strand break (DSB) by ligation of the ends without the need for a homologous template [57]. This method of DNA repair can induce short insertions or deletions, which can change the reading frame – useful in cases where disease is due to a nonsense mutation that shifted reading frame. HR makes use of a homologous template to repair the DSB through exchange of sequence information, and thus it can introduce an exactly defined sequence at the site of interest. This strategy can also be used to insert large DNA sequences if a template containing left and right homology arms is provided [58, 59]. Alternatively, short single-stranded oligonucleotides can be used as a template. MMEJ repairs the DSB by fusing the ends of broken DNA based on micro-homology of about 2–25 base pair (bp), leading to deletion of sequence flanking the DSB [60].

Gene Correction of MD Patient-Specific iPS Cells

In the past decade, several reports have documented the gene correction of MD patient-specific iPS cells (Table 3). Kazuki et al delivered wild-type DMD gene to DMD patient-specific iPS cells using a human artificial chromosome (HAC) as a gene delivery vector. Because HAC delivery in the cell type of interest requires fusion with microcells carrying the HAC, there is potential for the transfer of other genetic information [61]. Studies that use sequence specific nucleases focus mostly on exon skipping approaches aimed at reverting mutations caused by frameshift, leading to rescue by expression of functional dystrophin protein bearing a small deletion. Among these, Li and colleagues applied TALENs and CRISPR-Cas9 to correct a deletion mutation of DMD exon 44. To correct this mutation, they applied three different approaches, i) NHEJ-based deletion of exon 45 splice acceptor, ii) the entire exon 45, and iii) HR-based knock-in of exon 44. Although no transplantation studies were performed, all three of these approaches showed rescue of protein expression upon the *in vitro* differentiation of gene corrected iPS cells into myotubes [62]. Young et al. reported a genome editing approach to correct DMD mutations between exons 45 to 55 (known as a “hot spot”), and thus applicable to around 60% of all DMD mutations in humans. This was achieved by designing a pair of CRISPR-Cas9 constructs able to delete everything from exon 45 to 55, which then resulted in restoration of the DMD reading frame. Utilizing this approach, the authors showed correction of DMD mutations in three patient-specific iPSCs,

and rescue of dystrophin protein expression both *in vitro* and *in vivo* [63]. Long and colleagues designed and validated guide RNAs for CRISPR-Cas9 to induce deletion of splice acceptor or donor sites that can lead to skipping of 12 different exons of DMD by NHEJ, thereby correcting frameshift mutations. This approach was shown to rescue DMD protein in gene edited DMD iPSC-derived cardiomyocytes [64].

Several types of limb-girdle MD (LGMD) have also been targeted for gene correction. In the case of LGMD type 2D (LGMD2D), investigators have engineered lentiviral vectors to deliver SGCA gene to LGMD2D iPS cells, and transplantation of muscle derivatives in a mouse model of LGMD2D led to rescue of α-sarcoglycan protein expression [28]. Turan and colleagues reported correction of LGMD2B and LGMD2D iPS cells using CRISPR-Cas9 and TALENs. They used two HR based approaches, one used short oligonucleotide donor to correct a point mutation and the second applied knock-in of the corrected cDNA in a safe harbor locus. Both approaches resulted in rescue of respective missing dysferlin and α-sarcoglycan proteins in corrected LGMD2B and LGMD2D iPSC-derived myotubes respectively *in vitro* [65]. Iyer et al. reported the gene correction of LGMD2G patient-specific iPS cells. The mutation was an 8-bp microduplication in the TCAP gene, which leads to frameshift. They utilized CRISPR-Cas9 induced MMEJ-based approach to delete the microduplication and rescue wild-type TCAP protein expression in iPSC-derived myotubes *in vitro* [66].

Wang et al. reported knock-in of polyA signal sequence in the 3'UTR, directly upstream of the DNA repeats as a gene correction approach in DM1 patient-specific iPS cells. They used CRISPR-Cas9 and HR for this knock-in to prevent the transcription of the repeats but retain the transcription of full-length DMPK gene. This gene correction led to the elimination of nuclear foci in the corrected DM1 patient-specific iPSC-derived cardiomyocytes, neural stem cells and myotubes [67] [68]. Another group reported an approach for complete deletion of the DNA repeats in the DM1 patient-specific iPS cells. They used a pair of CRISPR-Cas9 to induce NHEJ based deletion of the repeats. This correction led to elimination of nuclear foci and rescue of splicing defects in corrected iPSC derived myotubes [69]. van der Waal and colleagues have shown the gene correction of Pompe disease patient-specific iPS cells. They used CRISPR-Cas9 and HR to knock-in GAA cDNA to the AAVS1 safe harbor locus [38]. This led to the rescue of the GAA protein expression in myotubes derived from gene corrected iPSC. Therefore, as discussed here and outlined in Table 3, gene correction of several and different types of MD patient-specific iPS cells have been reported in recent years, providing the scope for future autologous cell therapy applications.

Preclinical Studies and the Current Challenges

Although several studies have shown engraftment of PSC-derived myogenic progenitors, very few studies show significant myofiber engraftment, and importantly, functional recovery of transplanted dystrophic muscles. Validation of functional improvement is critical for confirming the therapeutic efficacy of the PSC-derived myogenic progenitors. Furthermore, those studies that have tested cells *in vivo* have utilized intramuscular transplantation in mice. However, it will be quite challenging to perform intramuscular

injections in humans, as this would require an enormous number of injections due to the size and number of muscles affected. Ideally, systemic transplantation will be the method of choice to deliver cells to all the affected muscles. However, so far there is no proof of concept for efficient systemic delivery of human PSC derived myogenic progenitors. The other critical aspect to validate is survivability of the transplanted muscle progenitors in a dystrophic environment, which is quite hostile to the incoming cells. This was a major issue in clinical trials with myoblasts [70, 71].

Safety of PSC-based cell therapeutics is another aspect to consider. Delivery of transgenes by lentiviral vector poses a risk of mutagenesis due to random genomic integrations, but the third-generation lentiviral vector is significantly safer than the previous versions [72, 73], and are currently being used in gene therapy clinical trials (NCT01745120[II], NCT01896102[III], NCT01515462[IV], NCT01560182[V]) [74–77]. Another critical issue is the potential for formation of teratoma from contaminating undifferentiated pluripotent stem cells [78].

To translate the PSC-based cell therapeutics from small animal studies to human, scaling up and preparation of clinically compatible cell preparation are important aspects. Although PSCs possess unlimited proliferative potential, their differentiation from pluripotent cells into myogenic progenitors is complex and somewhat variable. It would therefore be ideal if the derived myogenic progeny were expandable without sacrificing engraftment potential as this would allow scale up post-differentiation batches of cells. The procedure for derivation of myogenic progenitors from PSCs must be GMP optimized for clinical compatibility [79]. Thus, many preclinical studies are required to address these important aspects before PSC-based cell therapeutics for MD are realized. Importantly, planned/ongoing iPSC-based clinical trials for macular degeneration (UMIN000011929)[VI] [80], Parkinson's disease (UMIN000033564)[VII] [81], spinal cord injury [82] and ischemic heart disease [83] will provide key lessons for the future PSC-based cell therapeutics.

Concluding Remarks

Proof-of-concept transplantation studies in mouse models of MD have shown that human PSC-derived myogenic progenitors possess a certain capacity for *in vivo* regenerative potential. The significant progress in the fields of iPSC differentiation and genome editing technologies in the past decade has brought the concept of autologous stem cell therapy closer to reality for MD patients. Nevertheless, many challenges remain (see Outstanding Questions). In addition to the scientific challenges summarized in this review, it is important to consider the high cost of manufacturing a cell preparation that is suitable for only one patient. In addition, despite significant progress, the amount of time it takes to generate, genetically correct, screen, as well as characterize clones can be lengthy, which further significantly increases the cost. The development of universal gene correction strategies to

II. https://clinicaltrials.gov/ct2/show/NCT01745120
III. https://clinicaltrials.gov/ct2/show/NCT01896102
IV. https://clinicaltrials.gov/ct2/show/NCT01515462
V. https://clinicaltrials.gov/ct2/show/NCT01560182
VI. https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000013279
VII. https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000038278

correct all mutations of a gene of interest for each type of MD will make it more amenable to develop autologous cell therapy. Thus, we believe that translation of allogeneic cell therapy may be more feasible in the near future, as it would allow a single cell line to be used in many patients with different types of MD. Although many challenges exist, the past decade has provided grounds for great optimism that PSC based cell therapeutics for MD will eventually be translated to the clinic.

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Glossary

Allogeneic cell transplantation
The use of cells from a healthy donor for cell therapy usually from an individual genetically non-identical to the patient.

Autologous cell transplantation
The use of the patient's own cells for transplantation.

Cell therapy
Transfer of live cells into a patient for the purpose of mitigating or curing a disease.

Engraftment
The ability of transplanted cells to reside and function as part of the host environment. If the transplanted cells become part of the muscle fiber and seed the satellite cell pool, it is denoted by the terms myofiber and satellite cell engraftment, respectively.

Gene correction
The technique of altering a genetic mutation, either by exactly reversing it or by introducing a sequence that will result in equivalent-to-normal function, in order to cure the disease in the patient derived cells.

Genome editing
The technique used to modify the genome of the cells in order to fix or create a genetic defect.

Mesoangioblasts
also known as pericytes, reside in association with blood vessels and are multipotent cells can give rise to different mesodermal cell types.

Myoblasts
Proliferating muscle progenitors that derive from activated muscle stem cells and give rise to multinucleated myotubes *in vitro* and muscle fibers *in vivo*.

Myogenic progenitors
Precursor cells which can differentiate to form muscle *in vitro* and *in vivo*.

Pluripotent stem cells

Undifferentiated cells that can be differentiated into any cell type of the body.

Satellite cells

Adult muscle stem cells whose nomenclature is based on their location beneath the basal lamina of the muscle fiber. Satellite cells regenerate muscle in response to injury or disease.

Transgene

An exogenous DNA sequence introduced into the genome generally for the purpose of expressing a protein of interest.

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BOX 1,

Clinician's Corner

Immunosuppression is considered to be crucial for the success of cell therapies. Lack of proper immunosuppression is proposed to be one of the reasons for the poor engraftment of myoblasts in clinical trials for muscular dystrophy (MD) [84]. Pluripotent stem cells (PSCs) could be used in the future for either allogeneic or autologous cell transplantation for MD. Allogeneic cell transplantation involves the use of cells from a genetically non-identical healthy individual for therapy, which may invoke an immune response in the absence of continual immune suppression. Autologous cell transplantation involves the use of the patient's own cells for therapy, after the correction of genetic defect in the context of genetic disease. However, restoration of the missing protein's expression due to gene correction could still elicit an immune response, since it will introduce new epitopes to the patient's immune system. Therefore, whether by autologous or allogeneic cell transplantation, immune considerations are relevant and the use of immunosuppression to some extent may be necessary even in the autologous setting for some patients. The current proof-of-concept studies test the therapeutic potential of human PSCs in immunodeficient mouse models, as human cells will not engraft in immunocompetent mice, thus they cannot address these important issues. Future research should focus on optimizing the use of immunosuppression in relevant preclinical models for PS- based-cell therapy, for example in non-human primates. The choice of the method, dosage and timing for immunosuppression will need to be determined on a case by case basis. Immunosuppression poses a not insignificant risk of side effects for cell transplant recipients. To avoid the use of immunosuppression, a method to generate hypoimmunogenic iPSCs that cannot be recognized by the immune system has recently been reported [85]. Although these immune evading cells could provide a universal donor cell population for therapeutic applications, they could pose a safety issue in the case of potential tumorigenesis.

Cardio-respiratory failure is the major cause of fatality in the case of DMD, for which there is no cure. Therefore, future research should explore the possibility of iPSC-based cell therapy for the treatment of cardiac failure as well. Differentiation of PSCs into cardiac progenitors that could regenerate the dystrophic hearts will be critical. Recent studies reporting on the use of hESC-derived cardiomyocytes restoring the function in the macaque monkey model of myocardial infarction are encouraging [86]. The possibility of using a similar approach for DMD is yet to be tested.

Outstanding Questions

- Do PSC-derived myogenic progenitors mature *in vivo* upon engraftment?
- Will there be limitations in the survival and migratory ability of PSC-derived myogenic progenitors upon transplantation in the dystrophic muscle environment?
- Will the transplanted PSC-derived myogenic progenitors contribute sufficiently to the satellite cell pool to maintain the long-term efficacy of potential cell therapy applications?
- Will it be possible to develop universal gene correction methods applicable for all mutations in a given gene to develop efficient autologous cell therapy with iPSC?
- Is it possible to perform systemic transplantation of human PSC-derived myogenic progenitors and have them engraft in the affected skeletal muscles?

Highlights

- Several methodologies have been reported for the derivation of myogenic progenitors from human pluripotent stem cells
- Proof-of-concept studies have shown the therapeutic potential of pluripotent derived myogenic progenitors in mouse models of muscular dystrophy
- Induced pluripotent stem cell technology allows for the generation of muscular dystrophy patient-specific pluripotent stem cells
- Progress in genome editing techniques has enabled gene correction of mutations in muscular dystrophy patient-specific induced pluripotent stem cells allowing for their potential therapeutic application in autologous cell transplantation settings.

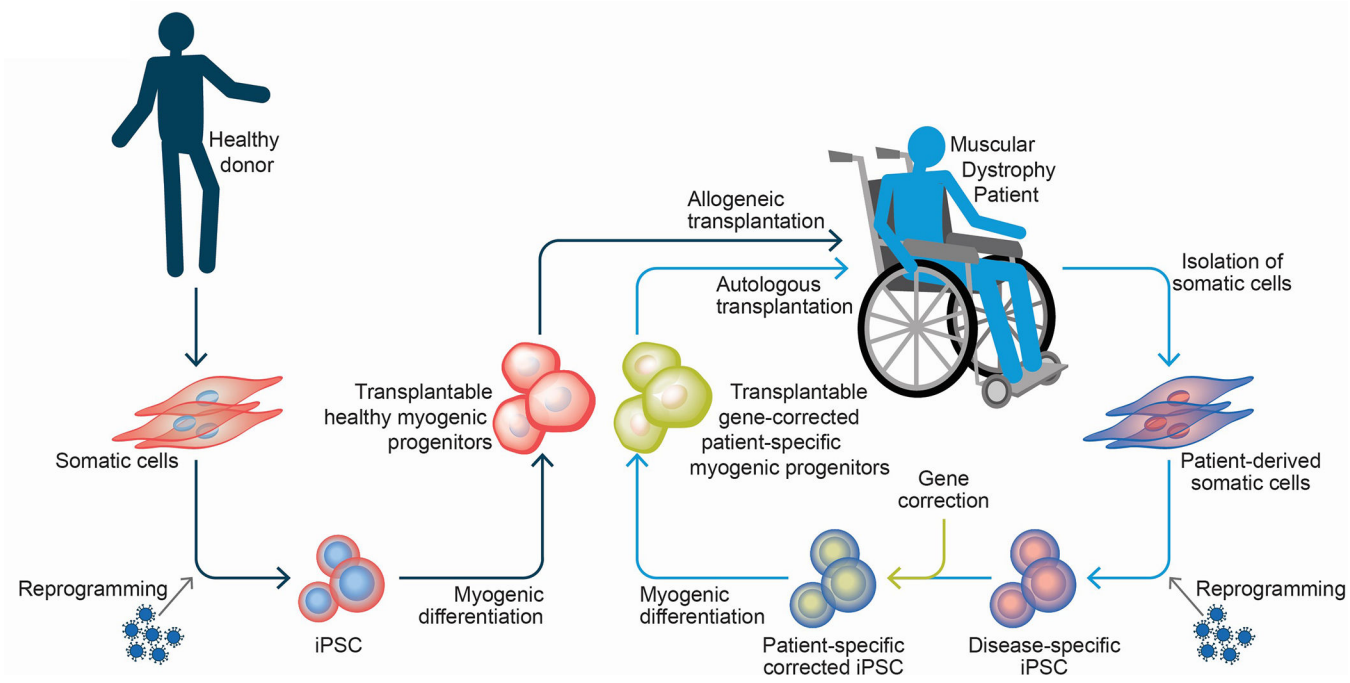

Figure 1: Pluripotent stem cell-based therapeutics for muscular dystrophy.

PSC-derived myogenic progenitors could be used for the potential treatment of MD patients through either autologous or allogeneic cell transplantation. For allogeneic transplantation, iPSCs would be derived from somatic cells of a healthy individual. Upon inducing myogenic differentiation, healthy myogenic progenitors obtained from these iPSCs would be transplanted in the MD patient. In the autologous transplantation setting, iPSCs would be derived from the MD patient's own cells. These patient-specific iPSCs would be corrected for the genetic defect using genome editing techniques to derive gene corrected iPSCs, which would then be differentiated into transplantable gene corrected myogenic progenitors. These myogenic progenitors would then be used for autologous cell transplantation.

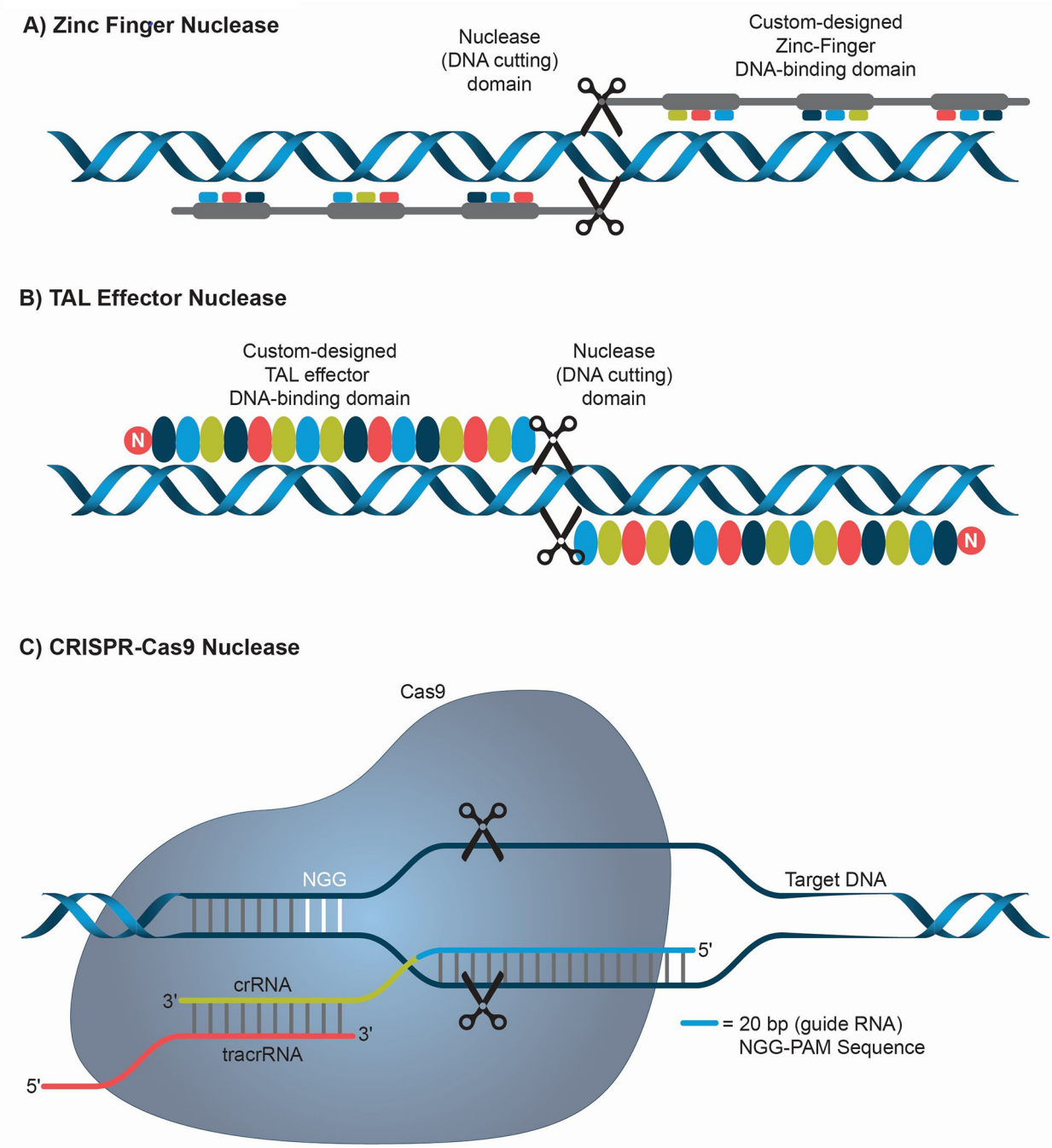

Figure 2: Sequence-specific nucleases used for genome editing.

(A) Zinc Finger Nucleases (ZFN) are artificial restriction enzymes that possess a DNA binding protein domain fused with a nuclease domain to achieve sequence-specific cleavage. ZFNs are designed in pairs to produce double stranded breaks (DSB). (B) TAL Effector Nucleases (TALEN) are restriction enzymes that possess TAL effector DNA binding domains fused to a nuclease domain to achieve sequence-specific cleavage. TALENs are also designed in pairs to enable a highly specific DSB. (C) CRISPR-Cas9 (Clustered Regularly Interspaced Palindromic Repeats-CRISPR associated protein 9) are sequence

specific nucleases discovered as part of a bacterial antiviral immune mechanism. A short RNA (guide RNA, usually 20 bp) complementary to the target DNA sequence is utilized to direct the Cas9 protein to a specific genomic region to create DSB. The system requires the presence of a short sequence in the genomic DNA adjacent to the guide RNA target sequence called the protospacer adjacent motif (PAM) which in the case of Streptococcus pyogenes Cas9 is NGG (N can be any nucleotide). crRNA and tracrRNA associate with the Cas9 to help in recognizing and cleaving target DNA.

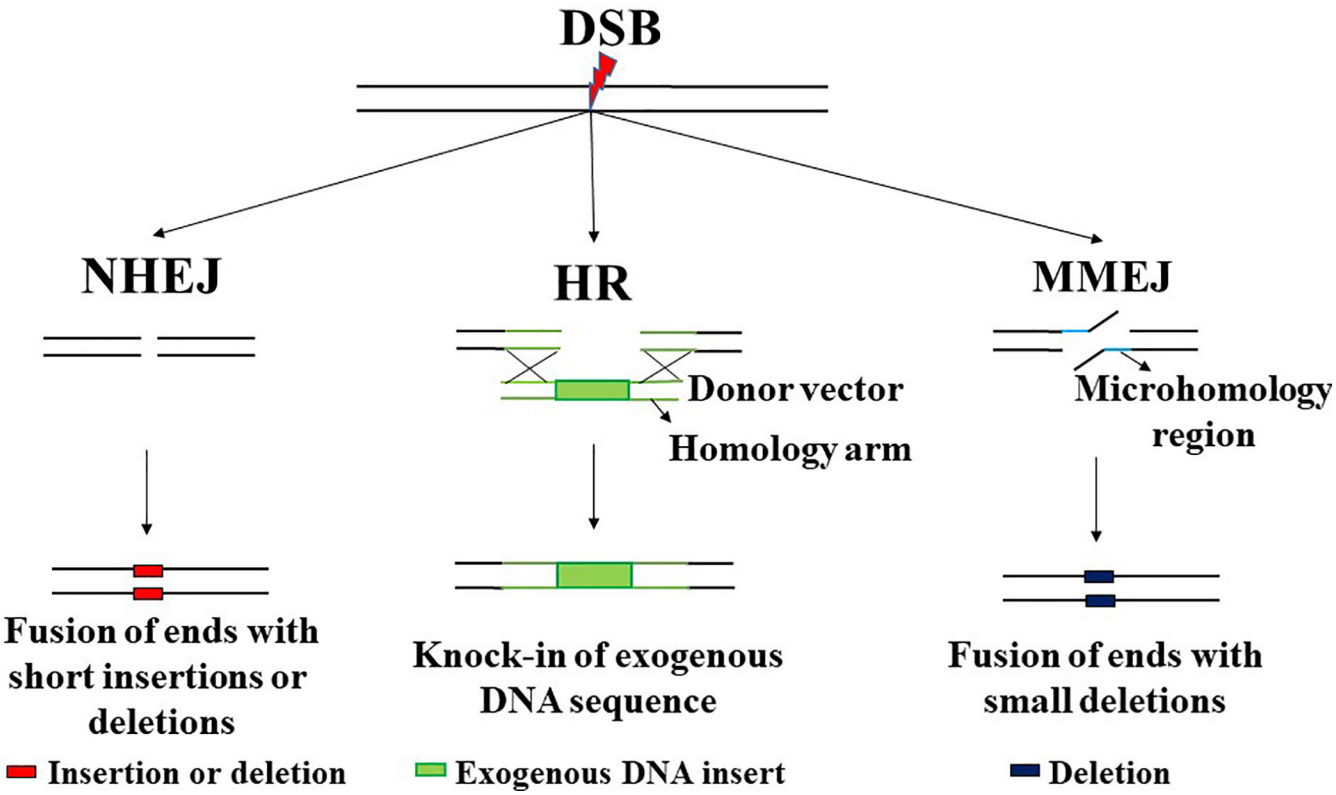

Figure 3: DNA repair mechanisms utilized for genome editing.

(A) NHEJ (Non-Homologous End Joining) is a double stranded break repair (DSB) pathway which causes fusion of the two ends directly without requiring a template for repair. NHEJ could be error prone as it usually creates short insertions or deletions (in red). For genome editing, NHEJ is used to create large deletions (2 DSBs) or short deletions (1 DSB) usually for correcting frameshift mutations and thereby restoring the reading frame in a subset of targeted cells. (B) HR (Homologous recombination) is a DSB repair pathway that utilizes a homologous template to exchange sequence information and is thus usually error free. For genome editing, HR is used for knocking in an exogenous DNA sequence by providing exogenous donor vector with the insert (green box) flanked by homology arms (green line). Single stranded oligonucleotides can also be used as a template for HR. (C) MMEJ (Microhomology Mediated End Joining) is an error prone DSB repair pathway that utilizes the microhomology region (2–25 bp) to repair and thereby cause deletion of the flanking sequence. For genome editing, MMEJ can be utilized to create small deletions (blue box) for correcting frame shift mutations caused by microduplication. Alternatively, MMEJ can also be used for knock-in of exogenous DNA sequence.

Table 1:

Types of muscular dystrophies, their origin and phenotype

Type	Gene associated	Mutation	Inheritance pattern	Age of onset	Muscles affected	Phenotype
Duchenne muscular dystrophy (DMD)	DMD	Loss of function	X-linked (recessive)	3–5 years	Several muscles of the body and cardiac muscle	Progressive disease, which is fatal due to severe respiratory failure around 20–30 years of age. Patients are wheel chair bound in their early teenage
Becker muscular dystrophy (BMD)	DMD	Partial loss of function	X-linked (recessive)	Late childhood	Several muscles of the body and cardiac muscle	Milder than DMD but patients may die after forties if there are respiratory issues
Facioscapulo humeral muscular dystrophy (FSHD1 and 2)	DUX4 (type 1 and 2) SMCHD1 (type 2)	Contraction of DNA repeats in chromosome 4 and mutations in SMCHD1	Autosomal dominant (type 1) Digenic (type 2)	Variable but mostly in 20s	Muscles of the face, shoulder, upper arm and lower legs	Rarely fatal but severely affects the quality of life
Myotonic dystrophy (DM1 and DM2)	DMPK (type 1) CNBP (type 2)	Expansion of DNA repeats	Autosomal dominant	Variable for DM1 (birth to 40 years), adult onset for DM2	Muscles of face, shoulder, lower arms and legs (type 1), Muscles of the neck, shoulders, elbows and hips (type 2)	Difficulty in muscle relaxation and muscle weakness. Congenital onset can be fatal if there are respiratory issues
Limb girdle muscular dystrophy (LGMD1 and LGMD2)	MYOT (LGMD1A), (LMNA) LGMD1B, CAV3 (LGMD1C), DNAJB6, (LGMD1D), DES (LGMD1E), CAPN3 (LGMD2A), DYSF (LGMD 2B), SGCG (LGMD2C), SGCA (LGMD2D), SGCB (LGMD2E), SGCD (LGMD2F), TCAP (LGMD2G), TRIM32 (LGMD2H), FKRP (LGMD2I), TTN (LGMD2J),	Usually loss of function mutations	Autosomal dominant (Type 1) or recessive (Type 2)	Variable	Typically muscles around the should and pelvic girdles but some types can affect cardiac muscle	Phenotype is variable. Affects the quality of life. Rarely fatal if there is weakness of cardiac and respiratory muscles
Pompe disease	GAA	Usually loss of function mutations	Autosomal recessive	Variable, from birth to adulthood	Respiratory muscles, muscles of hip, upper arms, legs and shoulder	Phenotype is variable. Early onset forms can be fatal

Table 2:

Methods for the differentiation of hPSC into myogenic progenitors and in vivo engraftment

Method	Purification	*In vivo* experiment	Engraftment and function	Transgene	Reference
Adenoviral based delivery of MYOD1; monolayer cultures	N/A	Transplantation of 0.5×10^6 cells per TA muscle of immunodeficient Rag/mdx mice	150–175 fibers positive for human SPECTRIN per section	MYOD	[27]
Lentiviral delivery of MYOD1 in PSC-derived mesoangioblasts	N/A	Transplantation of 10^6 cells per TA muscle of immunodeficient *Sgca*-null/ scid/beige mice	50 fibers positive for α-sarcoglycan per section	MYOD	[28]
Lentiviral delivery of doxycycline- inducible PAX7; embryoid body cultures	Purification of PAX7+ myogenic progenitors based on GFP transgene	Transplantation of 0.3×10^6 cells per TA muscle of immunodeficient NSG-mdx4^{Cv} mice	100 fibers positive for human dystrophin per section. Also showed satellite cell engraftment and functional improvement of transplanted muscles in comparison with untreated muscles.	PAX7	[32]
Similar to [32]	Positive selection for surface markers CD54, integrin α.9β1, and Syndecan2 for the purification of PAX7+ myogenic progenitors	Transplantation of 0.5×10^6 cells per in TA muscles of immunodeficient NSG and NSGmdx4^{Cv} mice	50 fibers positive for human dystrophin per section. Also showed satellite cell engraftment	PAX7	[33]
hESC differentiated into mesenchymal precursor and then into myogenic progenitors	Positive selection for CD73 and NCAM	Transplantation of 0.5×10^6 cells per TA muscle of immunodeficient SCID/Beige mice	Few fibers positive for human nuclear antigen and human laminin positive fibers were detected	Transgene-free	[34]
hPSC differentiated into embryoid bodies and then into myogenic mesenchymal progenitor cells.	N/A	Transplantation of 0.5×10^6 cells per TA muscle of immunodeficient NOG mice	10–20% fibers containing human nuclei. Also showed satellite cell engraftment	Transgene-free	[35]
Monolayer culture based on the use of GSK3β inhibitor and FGF2 growth factor treatment	Positive selection for surface markers CXCR4 and CMET, and negative selection for ACHR and HNK1	N/A	N/A	Transgene-free	[36]
Similar to [36]	Positive selection for C-MET and negative selection for HNK1	Transplantation of 0.5×10^6 cells per TA muscle of immunodeficient NSG mice	50 fibers positive for human spectrin per section	Transgene-free	[38]
Similar to [36]	N/A	N/A	N/A	Transgene-free	[37]
Monolayer culture based on the use of GSK3β and BMP inhibition, and treatment with FGF2, HGF and IGF1	N/A	N/A	N/A	Transgene-free	[39, 40]
Embryoid body cultures and microRNA cocktail treatment	Positive selection for surface markers CD140a, CD140b, and CD44	Transplantation of 0.5×10^6 cells in the femoral artery of immunodeficient *Rag2/Il2rg* null Sgcb null mice	15–20% fibers positive for human dystrophin. Functional improvement shown in the Extensor Digitorum Longus (EDL) muscle	Transgene-free	[42, 43]

Method	**Purification**	***In vivo* experiment**	**Engraftment and function**	**Transgene**	**Reference**
Similar to protocols in [37, 39]	Positive selection for surface markers ERBB3 and NGFR	Transplantation of 10^6 cells per TA muscles of immunodeficient mdx-NSG mice	150 fibers positive for human dystrophin/spectrin upon co-injection of cells with TGFβ inhibitor	Transgene-free	[44]
Monolayer culture based on the use of GSK3β and NOTCH inhibition	Positive selection for NCAM, and negative selection for HNK1	Transplantation of 1–3 × 10^6 cells per TA muscles of immunodeficient NOD-*Rag1*^{null} *IL2rg*^{null} and NSG-mdx^{4cv} mice	Approximately 100 fibers positive for human laminin and lamin A/C per section in NSGmdx^{4cv}, and 200300 fibers in NOD-*Rag1*^{null} *IL2rg*^{null} mice. Also showed satellite cell engraftment	Transgene-free	[45]
Monolayer culture based on the use of GSK3β, BMP and TGFβ inhibition	Positive selection for CD10, and negative selection for CD24	Transplantation of 0.25 × 10^6 cells per TA muscles of in immunodeficient NSG-mdx^{4cv} mice	Approximately 50 fibers positive for human dystrophin/lamin A/C per section. Also showed satellite cell engraftment	Transgene-free	[46–48]

Table 3:

Gene correction studies in MD patient-specific iPS cells

Gene correction tool	DNA repair mechanism	Type of MD and mutation	Validation of gene correction	Reference
HAC	N/A	DMD	Dystrophin protein rescue was detected in teratoma formed from the gene corrected iPS cells in immunodeficient mice.	[61]
TALEN, CRISPR-Cas9	NHEJ for deletion of either splice acceptor or the entire sequence of exon 45 or HR for rescue of fulllength exon 44.	DMD (exon 44 deletion)	Dystrophin protein rescue was detected in the myotubes derived from the gene corrected iPS cells.	[62]
CRISPR-Cas9	NHEJ for deletion of exons 45 to 55 to restore reading frame	DMD (any frameshift mutation between exon 45–55)	Dystrophin protein rescue was detected in vitro in myotubes derived from gene corrected iPS cells and in vivo upon transplantation of the gene corrected iPSC derived myogenic progenitors in immunodeficient mdx mice.	[63]
CRISPR-Cas9	NHEJ for deletion of splice donor or acceptor sites to restore the reading frame	DMD (mutations in 12 different exons causing frameshift)	Dystrophin protein rescue was shown in vitro in gene corrected iPSC-derived cardiomyocytes.	[64]
Lentiviral vector for SGCA delivery	N/A	LGMD2D (SGCA mutations)	α-sarcoglycan protein expression was detected in vitro in myotubes derived from corrected iPSC and in vivo upon transplantation into SGCA null immunodeficient mice.	[28]
TALEN, CRISPR-Cas9	HR with ssODN donor for correction or HR for knock-in of DYSF cDNA in H11 safe harbor locus	LGMD2B (DYSF, nonsense mutation), LGMD2D (SGCA, missense mutation)	Dysferlin and α-sarcoglycan protein rescue was shown in gene corrected iPSC- derived myotubes	[65]
CRISPR-Cas9	MMEJ on the microduplication site to restore the reading frame	LGMD2G (TCAP microduplication causing frameshift)	TCAP protein rescue was shown in gene corrected iPSC derived myotubes	[66]
TALEN	HR for knock-in of polyA signal sequence in intron 9 of DMPK to prevent transcription of the repeats	DM1 (expansion of CTG repeats in the 3’ UTR of DMPK gene)	Elimination of nuclear foci and rescue of splicing defects was shown in gene corrected iPSC derived cardiomyocytes and neural stem cells.	[67]
CRISPR-Cas9	HR for knock-in of polyA signal sequence in the 3’UTR upstream of the repeats to prevent their transcription	DM1 (expansion of CTG repeats in the 3’ UTR of DMPK gene)	Elimination of nuclear foci in gene corrected iPSC derived cardiomyocytes, neural stem cells and myotubes.	[68]
CRISPR-Cas9	NHEJ for deletion of CTG repeats	DM1 (expansion of CTG repeats in the 3’UTR of DMPK gene)	Elimination of nuclear foci and splicing defects in gene corrected iPSC derived myotubes.	[69]
CRISPR-Cas9	HR from exogenous donor vector for knockin of GAA cDNA in the AAVS1 safe harbor locus	Pompe disease (GAA mutation)	GAA protein and activity rescue in gene corrected iPSC derived myotubes.	[38]