Pluripotent Stem Cells for Gene Therapy of Degenerative Muscle Diseases

Mariana Loperfido[1,2,#], Heather B. Steele-Stallard[3,#], Francesco Saverio Tedesco[3,]* and Thierry VandenDriessche[1,2,]*

[1]Department of Gene Therapy & Regenerative Medicine, Free University of Brussels (VUB), Brussels, 1090, Belgium

[2]Center for Molecular & Vascular Biology, Department of Cardiovascular Sciences, University of Leuven, Leuven, 3000, Belgium

[3]Department of Cell and Developmental Biology, University College London, London, WC1E 6DE, United Kingdom

[#]The authors declare joint first authorship.

*Address correspondence to TV (Thierry.Vandendriessche@vub.ac.be) or FST (f.s.tedesco@ucl.ac.uk)

RUNNING TITLE

Pluripotent Stem Cells For Muscle Gene Therapy

Abstract (max 250 words)

Human pluripotent stem cells represent a unique source for cell-based therapies and regenerative medicine. The intrinsic features of these cells such as their easy accessibility and their capacity to be expanded indefinitely overcome some limitations of conventional adult stem cells. Furthermore, the possibility to derive patient-specific induced pluripotent stem (iPS) cells in combination with the current development of gene modification methods could be used for autologous cell therapies of some genetic diseases. In particular, muscular dystrophies are considered to be a good candidate due to the lack of efficacious therapeutic treatments for patients to date, and in view of the encouraging results arising from recent preclinical studies. Some hurdles, including possible genetic instability and their efficient differentiation into muscle progenitors through vector-/transgene-free methods have still to be overcome or need further optimization. Additionally, engraftment and functional contribution to muscle regeneration in pre-clinical models needs to be carefully assessed before clinical translation. This review offers a summary of the advanced methods recently developed to derive muscle progenitors from pluripotent stem cells, as well as gene therapy by gene addition and gene editing methods using ZFNs, TALENs or CRISPR/Cas9. We also discuss the main issues that need to be addressed for successful clinical translation of genetically corrected patient-specific pluripotent stem cells in autologous transplantation trials for skeletal muscle disorders.

KEYWORDS (6-8):

Cell therapy, designer nuclease, embryonic stem cells, gene therapy, induced pluripotent stem (iPS) cells, muscle stem cells, muscular dystrophies, regenerative medicine

Introduction

Skeletal muscle is a dynamic tissue capable of responding to exercise, injury or disease with a remarkable regenerative response. The ability of this tissue in providing a complete regeneration is primarily due to a pool of resident stem cells named satellite cells [1]. In addition to these cells, other stem/progenitor cells have been shown to participate in muscle regeneration. Subsequently, there has been much interest in the use of adult stem cells capable of myogenesis as a cellular therapy for degenerative muscle disorders (reviewed in [2] and in this issue by Sampaolesi et al.). However, the regenerative potential of these adult stem cells is not unlimited and exhaustion/dysfunction of muscle stem/progenitor cells has been reported in several muscular dystrophies and/or after expansion *in vitro* [3–9]. Recent studies have suggested the possibility of using embryonic stem (ES) cells [10,11] or induced pluripotent stem (iPS) cells [12,13] as a source for generating myogenic cells to use in cellular therapies for muscle regeneration. ES cells and iPS cells can be expanded indefinitely, overcoming the limitations of conventional adult muscle stem/progenitor cells. This is of particular importance for cell-based therapies of skeletal muscle disorders, since muscle is the most abundant tissue of the human body, which means that large numbers of cells would be required [14]. However, for this potential to be fulfilled, safe and efficient differentiation protocols need to be established to derive transplantable skeletal muscle progenitors from these pluripotent cells. In addition, the need to produce myogenic cells capable of regenerating skeletal muscles of the whole body of patients affected by muscular dystrophy upon systemic delivery, needs to be taken into consideration. Local intramuscular cell delivery is not a viable option to treat systemic muscle disorders since it would require a prohibitively large number of injections. In this article, we review current literature and discuss the recent challenges and future perspectives of gene and cell therapy strategies for muscular dystrophy based upon pluripotent stem cells, focusing specifically on the potential of genetically corrected myogenic cells derived from patient-specific iPS cells for autologous transplantation. To put this into context, we will also summarize the most salient features of normal skeletal muscle development and muscle regeneration.

Skeletal muscle development

Skeletal muscle embryogenesis and regenerative myogenesis share a combination of regulatory pathways and transcription factors that coax myogenic differentiation [15]. An overview of muscle

development during embryogenesis helps identify the key elements that regulate the myogenic program, and that serve as a basis for the development of protocols aimed at deriving myogenic stem/progenitor cells from pluripotent stem cells.

In vertebrates, the skeletal muscles of the trunk and limbs originate from somites, spheres of paraxial mesoderm distributed in pairs on either side of the neural tube [16–19]. Cells in the somites acquire lineage specification from signalling molecules emanating from their surrounding micro-environmental niche [20–22]. As somites mature they generate the dermomyotome, which contains multipotent cells that give rise to the skeletal muscles of the trunk and limbs. Interestingly, the head muscles are derived from a distinct site of origin during embryogenesis, involving distinct regulatory genes [23,24]. In the context of hereditary myopathies, these findings might be relevant to better understand the distribution of the affected muscles compared to those that escape the disease [25,15]. A fraction of cells of the dermomyotome will mature into the myotome, a primitive muscle structure containing progenitors expressing the regulatory factors myogenic differentiation 1 (MyoD) and myogenic factor 5 (Myf5). The remaining fraction consists of committed but undifferentiated muscle progenitors expressing the transcription factors paired box 3 ($Pax3^{+}$) and paired box 7 ($Pax7^{+}$). These cells will continue to proliferate without expressing differentiation markers [26–29] and will give rise to embryonic, fetal myoblasts and satellite cells residing in postnatal skeletal muscle [30,31].

The balance between cell renewal and proliferation vs. myogenic commitment and differentiation in vertebrate development is regulated by a hierarchical cascade of gene expression, as revealed by cell lineage and gene disruption studies [32]. Pax3 and Pax7 are paired-homeobox transcription factors that dominate myogenesis. Although Pax3 plays its role primarily during embryonic development and Pax7 during the later stages, the double mutant results in the loss of body muscles from mid-embryonic stages (E12.5) [29,28,33]. The next level in the genetic hierarchy controlling myogenesis is dominated by the myogenic regulatory factors MyoD, Myf5, and Myf6 (myogenic factor 6). These factors are all important for the specification of the skeletal muscle lineage, as triple-mutant embryos completely lack myoblasts and skeletal muscle fibres [34]. Studies further support a regulatory network where Pax3 is genetically upstream of the MyoD family members [35,36]. Collectively, these transcriptional regulators govern skeletal myogenesis and indeed some of them have been successfully used in recent studies to derive myogenic progenitor cells from pluripotent stem cells (e.g. Pax3/7 and MyoD).

Adult stem cells in muscle regeneration

Muscular dystrophies are degenerative skeletal muscle diseases characterized by repeated rounds of contraction-induced fibre damage [37]. These degraded fibres are replenished mainly by satellite cells; however, this has been shown to eventually lead to exhaustion of the resident stem cell populations [8]. Due to their malfunction in muscular dystrophies, and regenerative potential, therapeutic strategies aimed at replenishing muscle-derived adult stem cells represent a promising therapeutic avenue.

Adult skeletal muscle regenerates after exercise, injury or disease. Satellite cells are thought to be the primary drivers of this regenerative capacity. Ablation of Pax7-positive satellite cells has been shown to lead to the progressive loss of the satellite cell lineage in skeletal muscle and to impede the repair of injured muscle [38–41]. However, a recent study also demonstrated efficient regeneration of skeletal muscle after more than 90% reduction of satellite cells following inactivation of *Pax7* [42]. Satellite cells are mitotically quiescent in mature muscle [43] and are found in a distinct anatomical niche within the muscle, located next to the muscle fibre plasma membrane and underneath the basal lamina [1]. They express a range of characteristic, but not unique markers (reviewed in [44]). Amongst these markers is Pax7, which has been shown to be essential for satellite cell survival [45,46], and Pax3 and CXCR4 (C-X-C chemokine receptor type 4), which are expressed in a subset of quiescent cells [33,47]. Pax7 and Pax3 transcription factors have both been shown to activate the myogenic regulatory factors MyoD and Myf5 [48–50]. As highlighted in the previous section, Pax3 is essential for embryonic development of muscle [51] but is down regulated post-natally in most muscle progenitors [52]. Conversely, Pax7 is not required for embryonic myogenesis but is essential for the formation of functional adult satellite cells [45]; although a study indicated that Pax7 was entirely dispensable in adult life [53], more recent evidence shows that Pax7 is critical for satellite cell function in adult skeletal muscle [54]. Upon muscle injury quiescent satellite cells are activated, consistent with the expression of Myf5 and MyoD [55]. Once satellite cells have been activated they undergo asymmetric division to expand a pool of committed progenitors (i.e. the myoblasts) while preserving the compartment of undifferentiated stem cells [56–61]. In terms of molecular markers, both quiescent and activated satellite cells can express Pax7, Myf5, Syndecan-3 and -4, VCAM-1 (vascular cell adhesion molecule 1), c-Met, Foxk1 (forkhead box protein k1), CD34, M-cadherin, caveolin-1, α_7 and l_1 integrin, CD56 and nestin. It is worth noting that some of these markers identified in mouse models do

not fully correspond to those in human (i.e. CD34 is a marker of satellite cells in mice, but not in human muscle). Similarly, M-cadherin is not as consistent a marker of satellite cells in humans as it is in mice [62]. One of the more reliable markers of satellite cells/myoblasts in human muscle is CD56 [63].

Due to the central role of satellite cells in muscle regeneration, their use for cellular therapy has been thoroughly investigated. This has been outlined in detail in this issue by Sampaolesi and co-workers, and only their most salient features will be highlighted here. Despite promising results in the mdx dystrophic mouse, clinical trials based on intra-muscular injection of allogeneic satellite cell-derived myoblasts demonstrated safety but lack of efficacy [44,64]. This could possibly be ascribed to immune rejection, poor engraftment, low survival, inefficient migration and/or differentiation. However, these studies pioneered muscle stem cell transplantation in humans and the evidence of dystrophin production, though restricted to the injection site, is an important milestone [65]. Despite this setback, myoblast transplantation may be feasible for disorders that only affect a few isolated muscles. Indeed after promising preclinical work, a clinical trial using autologous transplantation of myoblasts isolated from non-affected muscles has shown promising results for oculopharingeal muscular dystrophy [66,67]. This opens new perspectives for localized myoblast delivery for at least some specific types of muscular dystrophy that manifest themselves predominantly in specific anatomical locations.

To overcome the limitations of satellite cells for cell therapy, other myogenic adult stem/progenitor cells have been investigated (reviewed in [2]). Briefly, $CD133^{+}$ cells have been shown to contribute to muscle regeneration in the severe combined immunodeficient (SCID)/X chromosome-linked muscular dystrophy (mdx) mouse model for Duchenne muscular dystrophy (DMD), and pre-clinical and preliminary clinical studies have been conducted for the treatment of DMD [68,69]. Recently, these cells were shown to contribute to satellite cell formation upon intramuscular injection into immunodeficient mice [70]. $PW1^{+}$ expressing interstitial cells have been identified in the mouse as a subset of cells capable of contributing to new muscle fibre formation and the satellite cell pool, although lineage tracing experiments have shown that these cells do not share the same origin as satellite cells [71]. Muscle-derived stem cells (MDSC) have been shown to lead to an improved therapeutic outcome when administered systemically to dystrophic dogs [72].

A subpopulation of skeletal muscle pericytes normally associated with capillaries has been shown to have myogenic potential. They are typically adjacent to endothelial cells and have been identified as the human adult tissue counterpart of mesoangioblasts [73,74]. They are a heterogeneous population and differ depending on their tissue of origin [75]. Lineage tracing experiments in mice have shown that pericytes also contribute to postnatal muscle development [73]. Importantly, pericytes/mesoangioblasts have been shown to contribute to muscle regeneration upon intra-arterial transplantation into different animal models of muscular dystrophy [76–81]. These encouraging findings have lead to a recently completed first-in-human phase I/II trial based upon intra-arterial delivery of allogeneic mesoangioblasts in five patients suffering from DMD (EudraCT no. 2011-000176-33). A number of other myogenic progenitors have been shown to be capable of engrafting and differentiating into muscle fibres but a detailed description of their properties falls outside the scope of this review and has been described elsewhere ([2]; Sampaolesi et al., submitted).

Differentiation of ES cells into muscle

ES cells are pluripotent stem cells isolated from early embryos. They were first isolated from the inner cell mass of blastocyst stage mouse embryos, and their human counterpart was subsequently isolated in the 1990s [10,11,82]. These ground-breaking studies led to much interest in the use of these cells as a potential therapy. In the case of muscle disorders, this required the establishment of protocols for the derivation of myogenic cells, which have mostly relied on exposing the ES cells to the same factors that lead to myogenic commitment in the developing embryo (see above). The desired differentiated myogenic cells could subsequently be enriched based on the expression of specific cell surface markers. The main methods investigated for deriving myogenic cells from ES cells are summarised in Table 1. By virtue of the unlimited proliferative potential, ES cells can be expanded virtually indefinitely, overcoming the limitation of adult stem/progenitor cells that have a more limited proliferative capacity and typically undergo senescence after extensive *in vitro* passaging.

Myosin heavy chain (MyHC)-expressing multinucleated myotubes were first derived from ES cells via the formation of embryoid bodies (EB) [83,84]. These are three-dimensional floating embryo-like structures obtained from growing ES cells in the absence of a murine feeder layer. In order to improve myogenic differentiation, Bhagavati and Xu co-cultured EBs derived from mouse ES cells with a preparation made from mouse muscle that was enriched for myogenic stem and precursor cells. Subsequent injection of the ES derived co-cultured cells into mdx mice, resulted occasionally in the formation of normal, vascularized skeletal muscle of transplanted cell origin [85]. However engraftment was relatively inefficient, and dystrophin positive fibres were observed in only 2 out of 8 injected mice. As an alternative route, Barberi *et al*. 2007 generated multipotent mesenchymal precursors from human ES cells which were then enriched for the $CD73^+$ fraction to obtain cells capable of forming bone, fat, cartilage and skeletal muscle [86]. However as the subset of $CD73^+$ cells that could form skeletal muscle was small (only 2-10%) this protocol required an additional sorting step for NCAM (neural cell adhesion molecule, alternative alias for CD56) and relatively long *in vitro* culture times. The ES cell-derived myogenic stem/progenitor cells obtained in these two studies had a lower proliferative and regenerative capacity than adult myoblasts [85,86]. This could be due at least in part to inefficient myogenic differentiation that resulted in the injection of a heterogeneous population of cells, incomplete expression of MyoD in transplanted cells or high cell death upon transplantation.

Hence, there was a need to increase the robustness of myogenic differentiation of ES cell-derived myogenic cells. During normal embryogenesis and muscle regeneration, expression of the regulatory factor MyoD is associated with the induction of myogenic differentiation program [26,55]. Initial work showed that expression of MyoD was sufficient to induce myogenic differentiation in fibroblasts [87]. Consequently, this work justified using MyoD to also coax the myogenic differentiation of ES cells. Transfection of murine ES cells with MyoD cDNA led to the formation of skeletal muscle-like cells capable of forming contracting myotubes *in vitro* after EB differentiation [88,89]. However not all cells were converted to skeletal muscle upon MyoD expression, and the *in vivo* potential of these cells to engraft in skeletal muscle was not assessed.

Building upon these pioneering studies, Ozasa and colleagues engineered mouse ES cells by including an inducible, tetracycline-regulated MyoD switch [90]. Consequently, simply culturing the ES cells in the absence of tetracycline can turn on the myogenic differentiation program. These ES cell-derived differentiated cells were transplanted into mdx mice where they formed dystrophin-positive

fibres at the injection site. However, this study did not assess long-term engraftment, and tumour formation was noted upon injection into immunodeficient mdx mice due to the presence of undifferentiated cells. In an attempt to further improve the efficiency of myogenic derivation from ES cells, they were genetically modified with an alternative genetic switch based on doxycycline-inducible expression of either Pax3 or Pax7 [91,92]. Myogenic cells were obtained by transient induction of either Pax3 or Pax7 expression during EB growth. Initial injection of these cells resulted in teratoma formation (tumours with cell derivatives originating from more than one germ layer) due to the presence of non-myogenic committed cells. Subsequently the authors used fluorescence activated cell sorting (FACS) to isolate platelet-derived growth factor-α receptor positive (PDGF-αR^{+}) fetal liver kinase 1 negative (Flk-1^{-}) cells. PDGF-αR and Flk-1 are expressed in unpatterned embryonic mesoderm, with subsequent Flk-1 down regulation specifying paraxial mesoderm, hence the authors hypothesised that by sorting for these markers they would isolate a population of myogenic precursor cells. These ES-derived myogenic cells exhibited relatively robust engraftment, differentiation and significant improvement in muscle force generation [91–93]. This method demonstrated improved contractile properties when mouse cells were injected systemically into dystrophic mdx mice [91,92] and was also adapted using other myogenic regulators downstream of Pax3/7 such as Myf5 [94].

In a similar study, Sakurai and colleagues generated ES-derived muscle-like cells after FACS enrichment of PDGF-αR^{+} cells but this time without relying on genetic modification with myogenic regulatory genes [95]. However whether these cells are capable of leading to a functional improvement similar to the results seen with the Pax3/Pax7 induced cells, or if they are capable of muscle engraftment following systemic delivery, needs to be further investigated. Alternatively, satellite-like myogenic cells could be derived from ES cells using an EB-based protocol followed by FACS enrichment of SM/C-2.6^{+} cells [96]. SM/C-2.6 is an antibody that detects murine quiescent satellite cells, however the exact antigen that this antibody is recognising is unknown [97]. Intramuscular injection into cardiotoxin-injured mice contributed to repair of muscle damage after primary and secondary injury, and after serial transplantation. However, whether this muscle repair is sufficient to lead to functional improvement remains to be assessed. Additionally, although these results are promising, it is worth noting that the SM/C-2.6 antibody protocol is not directly applicable to human ES/iPS cells, as this antibody has only been shown to be specific for murine satellite cells. Finally, the conditioning regimen based upon cardiotoxin is clinically not relevant and only serves as a model to

facilitate engraftment of the myogenic progenitors.

Recent promising work has developed further transgene free methods to drive myogenic differentiation, this time using small molecules to act on key signalling pathways. Shelton and colleagues used treatment with a WNT agonist, glycogen synthase kinase 3 (GSK-3) inhibitor, to obtain paraxial mesoderm and $Pax3^+$ premyogenic mesoderm in human and mouse ES cells [98]. Subsequent treatment with basic fibroblast growth factor (bFGF) was used to expand myogenic progenitor cells, followed by N2 treatment to induce terminal differentiation. This protocol used transgene-free, serum-free and chemically-defined methods; however, it requires relatively long in vitro culture times (40- 50 days) to obtain myocytes and myotubes. Additionally, the in vivo potential of these cells to engraft in muscle remains to be assessed. Although commitment to the myogenic lineage was efficient, it did not occur in the entire population of cells, so further cell sorting steps maybe required before in vivo transplantation.

Recently, a few laboratories have focused their efforts to induce direct MyoD-mediated myogenic conversion of pluripotent cells without intermediate commitment steps (e.g. mesodermal transition). In this context Puri and co-workers elegantly showed that absence of the SWI/SNF component BAF60C induces human ES cell resistance to direct MyoD-mediated activation of skeletal myogenesis. Expression of BAF60C activated skeletal myogenesis in human ES cells by instructing MyoD recruitment and chromatin remodeling at target genes [99]. This finding supports the model that MyoD-mediated myogenic conversion is indeed a complex process normally requiring cells in a permissive state in order to respond efficiently (i.e. differentiate), rather than a non-specific process whereby a dominant factor converts all cells to the muscle lineage irrespective of their origin or proximity to the mesoderm and myogenic program. Initial evidence supporting this model, such as very low frequency (or absence) of myogenic conversion in response to MyoD expression in some cell lines/lineages can indeed be traced back to seminal papers in the field of myogenic regulators and trans-differentiation [87,100,101].

Though these studies indicate the potential of ES cells to obtain myogenic precursor/stem cells that could be used for muscle regeneration *in vivo*, there are still some limitations that preclude their potential clinical use. In particular, there are ethical concerns over the use of ES cells as they are derived from, and result in the destruction of, human embryos. Nevertheless, there is an ethical

framework that potentially allows for the use embryos resulting from *in vitro* fertilization programs that will not be re-implanted. Furthermore, recent work has shown that it maybe possible to derive human ES lines from a single cell biopsy of an eight cell in vitro fertilised embryo without its subsequent loss [102]. However, despite this promising work certain intrinsic ethical concerns over the cloning of cells from human embryos will still remain for many individuals [103]. Additionally, as these cells are indeed derived from human embryos they cannot by definition be patient-specific, and therefore autologous transplantation is not possible raising immune concerns (as detailed in the section *iPS versus ES derived myogenic stem cells*). Moreover, there are important safety concerns regarding the persistence of residual undifferentiated ES cells that could eventually give rise to teratomas and/or the intrinsic genetic instability of ES cells, that may perturb cellular proliferation, survival and/or differentiation. In general, there is still a need to increase the robustness of system-wide phenotypic correction in the afflicted muscle groups in dystrophic animal models by intra-vascular administration of ES-derived myogenic progenitors. This is compounded by the clearance of the ES-derived myogenic progenitors by the reticulo-endothelial system. Ultimately, it would be reassuring to have safety and efficacy data in large animal models but this is far more challenging than the initial proof of concept studies in mdx mouse models described above. Despite these caveats, these pioneering studies in ES cell research have laid the groundwork for the use of iPS cells in regenerative medicine application for degenerative muscle disorders.

Differentiation of iPS cells into muscle

The discovery of protocols to reprogram adult cells into a pluripotent embryonic-like state revolutionized stem cell biology and regenerative medicine [104]. Reprogramming was achieved by the integration of four reprogramming factors: octamer-binding transcription factor 4 (Oct4), kruppel-like factor 4 (Klf4), sex determining region Y-box 2 (Sox2) and c-Myc. These reprogramming genes need to be transiently expressed during a critical window, after which their transcription is repressed by epigenetic mechanisms. The maintenance of the pluripotency of these cells *in vitro* is carefully controlled by exposure to specific culture parameters, with the recent formulation of xenofree, chemically defined media and substrate [105], which will facilitate the clinical translation of these cells and the establishment of GMP/GLP protocols. Pluripotency is lost when the cells are exposed to different culture conditions and signalling molecules, and when injected into immunocompromised

mice undifferentiated iPS cells undergo spontaneous uncontrolled differentiation giving rise to teratomas [106]. Recently these results have also shown to be true for autologous iPS cells in non human primate models, however iPS cell derivatives did not demonstrate any tumour formation [107].

To ensure that ES/iPS cell-derived myogenic progenitor/stem cells function normally *in vivo* after transplantation into the host, they would need to optimally integrate with the skeletal myofibres and, ideally, within the satellite cell niche. This is a defined anatomical niche along the surface of muscle fibres under the basal lamina that plays an important role in controlling muscle satellite cell self-renewal and differentiation (reviewed in [108–110]). Consequently, this skeletal muscle niche will be equally important for controlling the self-renewal and differentiation of the myogenic ES/iPS cell progeny. Based largely on studies with satellite cells, there are several components of the skeletal muscle niche that would need to be considered to optimally regulate the function of ES/iPS cell derivatives: (i) the host muscle fibre greatly influences the function of muscle stem/progenitor cells through mechanical, electrical, and chemical signals; (ii) the basal lamina, a layer of extracellular matrix (ECM) that consists mainly of laminin, proteoglycans and collagen. The maintenance of stem cell identity likely requires anchoring to this basal lamina; (iii) the microvasculature to ensure optimal blood supply, and interstitial cells (e.g. macrophages, fibroblasts). These distinctive features of the skeletal muscle niche therefore suggest that a combination of signals from the host muscle fibre, circulation system and ECM will likely be important control the quiescence, activation, and proliferation of both satellite cells and ES/iPS cell derivatives.

Transplantation of human ES/iPS-derived myogenic stem cells in immunodeficient mice has its merits and permits assessment of their safety and efficacy profiles. Nevertheless, murine and human muscle stem/progenitor cells are intrinsically different, consistent with their differential expression pattern of specific muscle markers (e.g. SM/C 2.6) [97]. Consequently, preclinical studies based on murine muscle stem/progenitor cells -or their cognate myogenic ES/iPS cell derivatives- may not necessarily replicate all of the features of their human counterparts in a clinical setting. This may reflect species-specific differences in survival, migration and differentiation that depend largely on the interaction of the transplanted cells with the host micro-environment [111]. To overcome these limitations, three-dimensional biomimetic models of human skeletal muscle are being generated *in vitro* to resemble as

much as possible the *in vivo* environment [112–114]. Synthetic scaffolds and decellularised devices from large animal models could ultimately be used to optimise stem cell maturation, differentiation and engraftment of ES/iPS-derived myogenic cells towards possible clinical applications.

iPS cells circumvent the ethical concerns of using ES cells since they can be derived from virtually any somatic cells, without the involvement of human embryos. Moreover, patient-specific iPS cell derivation opens new perspectives for autologous transplantation, as it should avoid immune rejection. However, in the case of using iPS cell derivatives to treat inherited disorders, such as muscular dystrophies, the underlying genetic defect would need to be corrected first by *ex vivo* gene therapy prior to transplantation. iPS cells are also well suited as an *in vitro* cellular platform for disease modelling and drug screening [115]. Another advantage of using iPS cell-derived cells over adult myogenic stem/progenitor cells is their unlimited proliferation capacity, as in the case of ES cells. Consequently, the generation of iPS cells requires only a limited number of patient cells, avoiding invasive procedure as in the case of adult stem/progenitor cells. Typically, this has been achieved through the reprogramming of fibroblasts isolated from minimally invasive skin punch biopsies and recent reports have successfully achieved similar results using cells isolated from blood or urine [116,117]. However, there is evidence that the origin of the cells used to generate iPS cells might bias the differentiation efficiency, in favour of the lineage of the donor somatic cell type (reviewed in [118,119]). This likely reflects an 'epigenetic memory' of the cell type of origin that is not fully reset during the reprogramming process. Indeed, this has been shown to be the case for iPS cells derived from mouse myogenic cells versus fibroblasts [120] and human cardiac versus non-cardiac cells [121]. Though the long-term consequences of this epigenetic memory on pluripotency, safety, cell survival and differentiation are not fully understood, it may facilitate coaxed differentiation of iPS cells into the desired cell type. Nevertheless, robust skeletal myogenic differentiation protocols may potentially over-ride the effects of epigenetic memory on differentiation bias [9]. For ultimate clinical translation it might be worthwhile standardising the tissue of origin of the reprogrammed cell type, to minimize variation in myogenic differentiation efficiencies and safety profiles.

iPS cells have initially been derived by retroviral vector-mediated integration of the reprogramming cassettes. Though very effective, this approach carries an intrinsic risk of insertional

oncogenesis [122]. Furthermore, some of the reprogramming factors are *de facto* oncogenic and their re-expression from the integrated reprogramming cassette cannot be excluded. However, potentially safer alternative reprogramming paradigms have been developed that rely on non-integrating vectors, protein transduction or 'transgene-free' reprogramming during which the reprogramming cassette is episomal or subsequently excised from the iPS genome [123,124]. Recent studies in non-human primates have laid the foundation for the first-in-man iPS pilot study to assess the safety of using patient-derived iPS cells for the treatment of an eye disorder, age-related macular degeneration [107,125–127]. This pilot clinical study is likely to generate critically important information towards extending iPS cell-based clinical protocols to other tissues and diseases.

As with ES cells, the use of iPS cells for the treatment of degenerative muscle disorders requires the establishment of protocols for their efficient directed differentiation into myogenic cells. Coaxed differentiation of iPS cells into muscle-like cells has mainly been achieved by applying similar techniques that were initially validated in ES cells. These methods are summarised in Table 2, and are discussed in more depth below.

Inducing myogenic differentiation of iPS cells using a Pax3 or Pax7 genetic switch

To coax myogenic differentiation of murine or human iPS cells, they have been genetically modified to contain a genetic switch that uses doxycycline-inducible expression of Pax7 during EB differentiation, followed by FACS enrichment for PDGF-α R^{+}Flk-1^{-} cells, or for co-expression of the green fluorescent protein GFP (where GFP is co-transfected/expressed with Pax7) [93,128], as described above in ES cells [91,92]. As with the previous studies, no tumour formation was observed in transplanted mice. Functional improvement as measured by isometric tetanic force, long-term engraftment and restoration of dystrophin expression was noted upon intramuscular transplantation of these cells into cardiotoxin injured dystrophic mice. Our studies were consistent with these findings, and showed that transposon-mediated Pax3 gene transfer in murine iPS cells coaxed their differentiation into multinucleated $MyoD^{+}MyHC^{+}$ myotubes [129]. Similarly, a doxycycline-inducible Pax3 genetic switch was used to derive murine iPS cells from mice who were double knockouts for dystrophin and utrophin [130]. These iPS cell-derived myogenic cells were also able to engraft skeletal muscle of double knockout mice for dystrophin and utrophin upon systemic intravenous (IV) delivery. However, significant pre-clinical toxicity studies would be necessary to support possible clinical translation of this strategy, as it is not clear what mechanism would prevent the accumulation of IV-delivered cells in filter organs, including the lungs.

Inducing myogenic differentiation of iPS cells using a MyoD switch

MyoD expression is thought to generate a pool of transient-amplifying progenitors at the expense of undifferentiated stem cells; this is a consequence of its role in myogenic commitment. However, it was also reported that a significant number of MyoD-negative satellite cells derive from MyoD-positive parents, supporting the idea that MyoD expression does not necessarily result in terminal differentiation and implying a mechanism to bring cells back to a stem/precursor state even after MyoD expression [61]. Furthermore, we have also reported the generation of donor-derived sublaminar Pax7-positive cells in SCID/mdx muscles transplanted with genetically-corrected mouse mesoangioblasts that also expressed a MyoD transgene [80]. Based upon this evidence and to overcome the limited availability of muscle pericytes in patients with limb-girdle muscular dystrophy 2D (LGMD2D), we developed a protocol to differentiate human iPS cells into pericyte/mesoangioblast-like progenitors that can be expanded in culture (designated as HIDEMs: human iPS-derived mesoangioblast-like cells). To

achieve this, a stepwise mesodermal differentiation protocol was employed that does not rely on EB formation and requires no additional FACS purification steps [9,131]. HIDEMs were genetically corrected (as detailed in the section *Ex vivo gene therapy using genetically corrected iPS cells*) and then transduced with a lentiviral vector encoding a chimeric, tamoxifen-inducible MyoD-ER(T) (estrogen receptor). Specifically, in the presence of tamoxifen MyoD-ER is translocated to the nucleus where it initiates the myogenic differentiation program. Consequently, myogenic differentiation of the HIDEMs could be accomplished *in vitro* or *in vivo* after transplantation into immune-deficient dystrophic mice in a tamoxifen-dependent manner [132]. In support of the safety of this differentiation protocol, we showed that HIDEMs were not tumorigenic in vivo.

An alternative MyoD-based approach was used to differentiate DMD iPS cells [133]. Cells were cultured in myogenic medium to induce mesenchymal differentiation, after which they were transduced with an adenoviral vector expressing MyoD. The iPS cell-derived progenitors were capable of generating multinucleated myotubes *in vitro*, and upon transplantation into the immunodeficient and dystrophic RAG/mdx mice participated in muscle regeneration. However, potential immunogenicity issues related to the use of adenoviral vectors might limit the application of this strategy.

A MyoD inducible system was also used for myogenic differentiation of human iPS cells from patients with Miyoshi myopathy (MM), an inherited muscular dystrophy caused by dysferlin mutations, and for carnitine palmitoyltransferase II deficiency, an inherited myopathy caused by mutations in *CPT2* [134,135]. This inducible MyoD method differs from those used by us and by Goudenege et al., in that the doxycycline inducible MyoD is transposed directly into the undifferentiated iPS cells. Resulting human MyoD-iPS cells were able to undergo direct myogenic differentiation without a mesodermal transition step (10 days), however the *in vivo* regenerative potential of the myogenic cells derived using this method remains to be determined. Finally, Abujarour and colleagues also reported direct MyoD-mediated differentiation of iPS cells into myocytes *in vitro* without the need for an intermediate step or cell sorting [136]. Although relatively faster than other methods, these strategies appear to be more suitable for drug screening than for gene therapy applications. Indeed genetic correction of pluripotent colonies is technically challenging and the absence of an expandable myogenic cell might limit banking and standardization of a possible cellular medicinal product.

Inducing myogenic differentiation of iPS cells without genetic modification

Satellite-like myogenic cells were derived from murine iPS cells using an EB-based protocol followed by FACS enrichment of SM/C-2.6+ cells that was initially developed for ES cells [96,137]. A similar step-wise EB-based protocol was used to obtain myogenic cells from human ES and iPS cells, which included EB formation followed by dissociation and culture on collagen type-I coated plates with serum containing medium [138]. In this case, there was no enrichment step based on the use of anti-SM/C-2.6 antibodies since they do not bind on human cells. An advantage of these differentiation protocols is that they did not require genetic modification with genes encoding myogenic differentiation factors. However, it required relatively prolonged *in vitro* culture times, 49 days to obtain myogenic precursors used in transplantation studies and 63 days for the *in vitro* formation of mature myotubes, and myogenic induction was not very efficient.

Recently Borchin *et al* 2013 developed an alternative method that does not rely on genetic modification to generate muscle precursors from iPS cells [139]. Paraxial mesoderm was derived from human ES cells and iPS cells by treating cells with a GSK-3β inhibitor, and myogenic cells were subsequently expanded by the addition of bFGF. In order to obtain a pure $Pax3^{+}/Pax7^{+}$ population, myogenic cells were subsequently sorted for the chemokine receptor CXCR4 and hepatocyte growth factor receptor C-MET/HGF, where as sorting for the muscle-specific acetylcholine receptor was used to obtain mature skeletal myocytes. However the therapeutic potential of these cells has not yet been tested *in vivo*.

To identify small molecules capable of promoting myogenesis, Xu and colleagues performed a high-throughput screening to test the ability of 2400 compounds to promote myogenesis in zebrafish embryos [140]. As a result of this screening, the authors identified a ‘triple cocktail’ of bFGF, the adenylyl cyclase activator forskolin, and the GSK-3 inhibitor BIO that could promote skeletal muscle differentiation when applied to EBs derived from human iPS cells. The resulting cells were also transplanted by intramuscular injection into immunodeficient mice, where they engrafted skeletal muscle. No tumour formation was observed. Although this does not require transgene insertion, the authors did not provide evidence of genetic manipulation/correction of the cells, engraftment in dystrophic muscle or test systemic delivery of these cells.

iPS versus ES derived myogenic stem cells

A head-to-head comparison between human ES and iPS-derived myogenic stem cells [93] did not reveal any major differences in terms of phenotypic markers or functional attributes, including their regenerative potential, engraftment efficiency, functional recovery and contributions to the satellite cell pool. Similarly, using a distinct differentiation protocol, both human iPS and human ES-derived mesoangioblast-like cells could be obtained, with similar properties [131]. It would therefore appear that under the same differentiation conditions, the myogenic progenies derived from either ES or iPS cells show similar myogenic potential *in vitro* and regenerative capability *in vivo*. This further supports the use of iPS cell-derived muscle stem cells as an adequate alternative to ES cell derivatives for future clinical applications. Similarly, other human ES and iPS cell-derived cell types (i.e. neural, hepatic, and mesenchymal lineages) are nearly equivalent transcriptionally [141]. Though transcriptome analysis of iPS and ES cells revealed some differences [142], this type of comparisons has never been made between cells obtained from the same individual. Consequently, inter-individual variations cannot be excluded.

Another aspect to consider in order to use ES/iPS cell-derived myogenic cells in a clinical setting, concerns their possible immune-modulatory properties. ES cells are de facto allogeneic. Consequently, any ES cell-based cell therapy would require immune suppression to minimize the risk of inadvertent immune responses that would reject the ES-derived cells after transplantation. This is compounded by the increase in immunogenicity during differentiation of ES cells [143,144]. In another study, it was shown that proliferating myogenic progenitors derived from mouse ES (or iPS) cells express major histocompatibility complex (MHC) class I molecules [93]. Elevated MHC class I expression is known to increase their sensitivity towards cytotoxic T cells (CTL) but also suppress natural killer (NK) cell-mediated immune response [145].

Since iPS cells are typically obtained from autologous somatic cells, it was assumed that this would obviate immune concerns after transplantation of its differentiated iPS cell-derived progeny. However, since syngeneic mouse iPS cells have been rejected following transplantation *in vivo* into mice, this indicates that even autologous iPS cells can be recognised by the immune system [146]. Hence, the immunogenicity of iPS cells and their derivatives would need to be carefully examined. Nevertheless, analysis of the interaction between human iPS cell-derived mesoangioblasts (HIDEMs) and immune

cells suggests a reduced risk of evoking potential immune responses [147]. Indeed, HIDEMs suppress T cell proliferation through IDO and PGE-2 dependent pathways, consistent with the results from tissue-derived mesoangioblasts [148].

The remarkable similarity between HIDEMs and their tissue-derived counterpart, together with the recent completion of a clinical trial based upon the transplantation of human pericyte-derived mesoangioblasts, could facilitate future clinical translation of this cell type. Ideally, an autologous, personalized cell therapy could be envisaged, where patient-specific iPS cells or myogenic derivatives are genetically corrected first and subsequently transplanted into the same patient. Nevertheless, an allogeneic cell transplant could also be considered, whereby muscle-like cells derived from iPS cells of a healthy donor would be transplanted into an HLA-matched recipient [149]. This would require immune suppression, as in the case where tissue-derived allogeneic mesoangioblasts or other muscle stem/progenitor cells were employed. Regardless of the immune response against the transplanted cells, it cannot be excluded that the therapeutic protein itself may provoke an immune reaction.

The use of regulatory T cells (Treg), capable of suppressing or "regulating" the activation of alloreactive lymphocytes [150] represents an alternative strategy that could potentially be exploited to limit alloreactive and/or transgene-specific immune reactions in the context of cell/gene therapy with iPS or ES-derived muscle cells. Interestingly, a particular Treg subtype was enriched in muscle upon acute or chronic injury, and promotes tissue repair *in vivo* [151]. The possibility to reinforce immune tolerance induction by potentiating Treg activity is an attractive possibility to foster transplant graft acceptance, including that of ES/iPS cell-derived cells [152]. Further studies are needed to investigate the immune consequences of ES/iPS cell-derived myogenic cells *in vivo* to ensure stable cell engraftment in inflamed muscles.

Ex vivo gene therapy using genetically corrected iPS cells

The development of iPS cell technology in combination with advanced gene therapy methods offers a unique scenario for the treatment of genetic diseases by *ex vivo* gene therapy, moving closer to the prospect of an autologous and personalized cell therapy. This approach consists of isolating cells from the patient, modifying them in the laboratory, and then transplanting them back into the very same

patient, an overview of this potential approach is summarised in Figure 1. The genetic modification is based either on the introduction *de novo* of a functional copy of the therapeutic gene (i.e. ‘gene addition’) or on *in situ* targeted correction of the defective gene (i.e. ‘genome editing’) in the iPS cell colonies or derivatives. Several gene transfer technologies have been developed that were used to genetically modify iPS cells and/or their differentiated progeny. Most importantly, to be effective, the genetic modification needs to be stably transmitted to the iPS cell progeny. Consequently, gene addition has typically been achieved using integrating vector platforms (i.e. transposon, retroviral or lentiviral vectors) or stably persisting episomes such as human artificial chromosomes (HACs). In contrast, specific targeted gene correction by genome editing requires the use of engineered designer nucleases that enhance homology-directed gene repair 10^4 to 10^5-fold by inducing a double-strand DNA break at the genomic target locus. The most commonly used designer nucleases are based on the zinc finger nuclease (ZFN) transcription-activator like effector nuclease (TALEN) and, more recently, clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) platforms. Whereas targeted gene correction by gene editing is specifically tailored towards specific mutations in the target gene and thus specific patient sub-populations harbouring these mutations, gene addition is widely applicable irrespective of the underlying genotype.

Gene addition

In 2012 we provided the first evidence of successful *ex vivo* gene therapy using patient-specific differentiated iPS cells for a muscle disorder. Specifically, limb-girdle muscular dystrophy 2D (LGMD2D) iPS cell-derived inducible myogenic cells (HIDEMs) were genetically corrected with a lentiviral vector encoding the human α-sarcoglycan gene (whose mutations cause LGMD2D) under a muscle specific-promoter and then also transduced with another lentiviral vector encoding a MyoD-ER(T) transgene as a myogenic differentiation switch, as described above. Genetically corrected LGMD2D HIDEMs were successfully transplanted intramuscularly and intra-arterially into α-sarcoglycan-null immunodeficient (Sgca-null/scid/beige) mice, a preclinical model of LGDM2D, and produced α-sarcoglycan-positive muscle fibres that were detectable one month post transplantation [9]. Consequently, *de novo* expression of the missing α -sarcoglycan led to the reconstitution of the dystrophin-associated protein complex in host myofibres. Similarly, species-specific transplantation of mouse iPS cell-derived progenitors in Sgca-null/scid/beige mice led to robust engraftment of large areas of host muscle, re-establishment of muscle pericytes *in vivo* and functional amelioration of the dystrophic phenotype. This proof of concept study established the validity of an autologous gene therapy approach for the treatment of LGMD2D, and possibly other forms of muscular dystrophy, based upon transplantable iPS cell-derived myogenic cells. Indeed, the same protocol allowed the generation of HIDEMs also from DMD iPS cells, which were genetically corrected with a HAC containing the entire dystrophin genetic locus (DYS-HAC) [9,153]. This stably maintained, episomal DYS-HAC accommodates the entire genomic dystrophin locus (2.4 Mb) avoiding integration-associated risks. Furthermore, such delivery of the entire gene is mutation independent, thus ensuring applicability to the variety of mutations/duplications/deletions that cause DMD.

A different approach for the treatment of DMD has been proposed by Filareto *et al.*, where the dystrophic phenotype of dystrophin/utrophin null mouse iPS cells was corrected by providing micro-utrophin (μUTRN) with a non viral vector Sleeping Beauty transposon [130]. Utrophin is a protein closely related to dystrophin, whose over expression has been shown to ameliorate the dystrophic phenotype in mdx mice [154]. In order to achieve myogenic differentiation Filareto *et al.* used Pax3 induction and sorting for PDGFαR^{+}/Flk1^{-} expression. Subsequently, the genetically corrected myogenic cells were transplanted in dystrophin/utrophin null mice where contribution to muscle regeneration and improvement in contractility were achieved. This study does not represent

dytrophin/DMD gene correction *sensu stricto*, as the defective dystrophin is replaced by utrophin; importantly, this strategy is so far limited to mouse iPS cells and data supporting its validity using DMD iPS cells will be necessary in order to consider potential clinical translation.

By using a similar gene transfer approach, in the work of Tanaka *et al.* correction of human iPS cells derived from patients affected by Myoshi myopathy (MM) was achieved by providing the full-length dysferlin (DYSF) transgene using the piggyBac transposon system [134]. Restoration of expression of the missing protein has been detected *in vitro* on the membrane of genetically corrected MM human iPS cell derived myotubes. Moreover the transgenic expression of full-length DYSF rescued the MM phenotype, as demonstrated by an improvement in the defective membrane repair phenotype of MM myotubes during *in vitro* functional tests such as two-photon laser-induced injury of the sarcolemma. Transplantation in MM animal models is expected to move this strategy forward. Despite the previously mentioned studies that have demonstrated the validity of using transposons as a tool to genetically correct iPS cells derived from patients with muscular dystrophies, their use has yet to be explored for the delivery to patient iPS cells of larger transgene such as the full-length dystrophin. Furthermore, extensive analysis must be conducted to delineate the integration profile of transposons in iPS cells in order to exclude the risk of insertional mutagenesis and move towards clinical applications.

Targeted gene editing

The use of designer nucleases such as ZFNs, TALENs and CRISPR/Cas9 allows for efficient targeted gene correction of the dystrophin locus in iPS cells and/or myogenic progenitor cells and therefore provides an attractive alternative to the aforementioned gene addition strategies. These powerful genome engineering tools have shown great effectiveness for the correction of disease mutations in iPS cells for several diseases such as ß-thalassemia [155], α1-antitrypsin deficiency [156] and epidermolysis bullosa [157]. In a recent work, neural stem cells (NSCs) generated from iPS cells of myotonic dystrophy type 1 (DM1) patients were corrected *in vitro* by TALEN-mediated homologous recombination (HR) [158]. These results provide the first proof-of-principle evidence that TALENs may be used to generate genetically modified DM1 progenitor cells as a first step toward autologous cell transfer therapy for DM1. An extension of this approach to revert a possible muscle phenotype of DM1 iPS cell-derived myogenic progenitors might be considered as a promising area of investigation. In the case of DMD, proof-of-concept had been established by Gersbach and colleagues, who

demonstrated correction of the dystrophin open reading frame using TALENs specifically design to target and edit the dystrophin exon 51 locus [159]. This led to restoration of dystrophin protein expression in DMD skeletal myoblasts and dermal fibroblasts that were reprogrammed to the myogenic lineage by forced expression of MyoD. There was no evidence of off-target effects based on exome sequencing of *in silico* predicted target sites, suggesting that TALEN-mediated genome editing was highly specific for the target locus. An alternative approach was based on ZFNs that were specifically designed to permanently remove essential splicing sequences in exon 51 of the dystrophin gene and thereby exclude exon 51 from the resulting dystrophin transcript [160]. DMD myoblasts engineered using these designer ZFNs contained the expected deletion of exon 51 resulting in concomitant restoration of dystrophin protein expression. Furthermore, transplantation of the ZFN-corrected myoblasts into immunodeficient mice resulted in human dystrophin expression *in vivo*. This provides an attractive alternative to oligonucleotide-induced exon skipping to permanently and irreversibly restore the dystrophin reading frame and protein production, obviating the need for continuous drug administration. One caveat of these proof-of-concept studies is that they are based on DMD myoblasts, which are not ideally suited for cell therapy in DMD patients based on their limited proliferation potential and inability to efficiently migrate out of the blood stream following their systemic intravascular administration.

In a recent study, a multiplex CRISPR/Cas9-based gene editing system has been designed to restore the dystrophin reading frame in DMD myoblasts by targeting the mutational hotspot at exons 45–55 [161]. Although this approach has the advantage to correct potentially more than 60% of DMD patient mutations with a single genome-editing strategy, the final product is a shorter version of the human dystrophin. In another study, instead, the specificity of the TALEN/CRISPR gene editing system for mutations/deletions in the dystrophin gene that lead to a lack of exon 44 has been explored to restore the expression of the full-length human dystrophin in DMD patient-derived iPS cells [162]. The high specificity of the nuclease design was done after a comprehensive genome-wide mutation analyses to assess the risk of off-target mutagenesis in DMD iPS cell clones treated according to the TALEN or CRISPR approach. By using three different methods, they demonstrated the genetic correction of the dystrophin gene in patient-derived iPS cells. The first of these methods restored the reading frame via disruption of the splice sites leading skipping of exon 45, the second introduced small indels (insertions or deletions); and the third used 'knock-in' of the missing exon 44 in front of

exon 45 to restore the full protein coding region. Although restoration of dystrophin expression was detected in all of the three different genetically corrected iPS cells differentiated into myogenic cells, only the exon knock-in approach restored the full-length rather than truncated (i.e. lacking exon 44 or 44-45) dystrophin protein. These encouraging results pave the way towards an *ex vivo* designer nuclease-based gene therapy approach using patient-specific human iPS cells. Efficient functional correction of the dystrophic phenotypic *in vivo* would still need to be demonstrated. One caveat of these gene editing approaches is that their overall efficiency is not as high as with more conventional gene addition strategies but incremental changes in technology may eventually overcome this.

TRANSLATIONAL CHALLENGES AND CONCLUDING REMARKS

The clinical translation of iPS cells for the autologous gene and cell therapy of genetic diseases represents perhaps one of the greatest challenges of modern molecular medicine. Even though this process may benefit from the knowledge acquired to translate viral vectors into new therapeutics [163,164], several hurdles are being (or will need to be) addressed to successfully complete this process, including: possible genome instability, residual undifferentiated tumorigenic cells, large scale production under GMP/GLP grade regulations, preservation of self-renewing potential, epigenetic memory of the cells of origin and re-expression of potentially oncogenic reprogramming factors (e.g. Myc). In the case of skeletal muscle and its diseases, this also needs to be considered in the context of other tissue-specific obstacles, including the need to target the diaphragm and the heart in order to counteract the cardiac and respiratory failure that underline several forms of muscular dystrophy (e.g. DMD).

Pluripotent stem cells offer the invaluable prospect of obtaining an unlimited number of cells for regenerative therapies, which is vital for the treatment of muscular dystrophies (skeletal muscle being the most abundant human tissue). Indeed recent clinical studies highlight the need to use large number of cells to treat dystrophic patients, the amount of cells and their delivery depending primarily on the type of cells and disorder. Local transplantation of autologous myoblasts in the pharyngeal muscles of OPMD patients showed that improvements in both swallowing and quality of life were observed in patients who had been injected with more than 178 million cells [67]. On the other hand, to test the safety of allogeneic transplantation of mesoangioblasts in DMD children the average amount of

transplanted cells was in the order of 10^9 per patient. Then, to reach clinical efficacy, it is expected that a dose of cells significantly higher than this would be required. Transplanted cell engraftment and contribution to host myofibres are key issues to face in cell therapy. Satellite cell long-lasting self-renewal has been showed for up to 7 rounds of transplantation with as little as 16 cells [58]. These findings support the idea that for a successful cell therapy, the self-renewal potential of the donor cells might be as important as their capability to be expanded extensively.

Further studies are needed to optimise genome-integration- and animal-component- free protocols that can be used for the large-scale production of iPS cells, and for the derivation/purification of myogenic cells from iPS cells under GMP/GLP conditions. Moreover, new chemically-defined methods should also focus on obtaining myogenic progenitors able to migrate and engraft into muscles upon loco-regional/systemic delivery, thus improving the likelihood of efficacy of such therapies.

Different vectors have been exploited for the *ex vivo* correction of patient/disease-specific iPS cells. Theoretically the most valuable of the studied gene therapy approaches are those able to provide functional expression of the missing gene irrespective of the underlying mutation, and will thus be applicable to a larger number of patients. However, the recent introduction of fast and cost-effective CRISPR/Cas9-based gene editing platform might also shift this paradigm toward a more "*a la carte*" personalised approach. Furthermore, for safe *ex vivo* genetic correction the risk of vector-induced immune responses needs to be thoroughly investigated, and the danger of insertional mutagenesis avoided either through the use of site-specific integration or non-integrating systems that are stably maintained.

In conclusion, emerging methods for the generation and genetic correction of pluripotent stem cells and their myogenic derivatives have an extraordinary potential for the cell therapy of muscle disorders, and many encouraging initial results have been described in this review. We believe that the recent effort to translate iPS cells into medicinal products for cell therapies [125–127] may provide invaluable insights for the future challenge of autologous iPS cell-based therapies for muscle diseases.

LIST OF ABBREVIATIONS

bFGF = basic fibroblast growth factor

Cas9 = clustered regularly interspaced short palindromic repeat associated 9

C-MET/HGF = hepatocyte growth factor c MET receptor

CRISPR = clustered regularly interspaced short palindromic repeat

CTL = cytotoxic T cells

CXCR4 = C-X-C chemokine receptor type 4

DM1 = myotonic dystrophy type 1

DMD = Duchenne muscular dystrophy

DYS = dystrophin

EB = embryoid bodies

ECM = extracellular matrix

ES = embryonic stem

FACS = fluorescence activated cell sorting

Flk-1 = fetal liver kinase 1

Foxk1 = forkhead box protein k1

GFP = green fluorescent protein

GSK-3 = glycogen synthase kinase 3

DYS-HAC = human artificial chromosome containing the entire dystrophin locus

HAC = human artificial chromosome

HIDEMs = human iPS-derived mesoangioblast-like cells

HLA = human leukocyte antigen

HR = homologous recombination

iPS = induced pluripotent stem

IV = systemic intravenous delivery

Klf4 = kruppel-like factor 4

LGMD2D = limb-girdle muscular dystrophy 2D

µUTRN = micro-utrophin

MDSC = muscle-derived stem cells

mdx = X chromosome-linked muscular dystrophy

MHC = major histocompatibility complex

MM = Miyoshi myopathy

Myf5 = myogenic factor 5

Myf6 = myogenic factor 6

MyHC = myosin heavy chain

MyoD = myogenic differentiation 1

NCAM = neural cell adhesion molecule (otherwise known as CD56)

NK = natural killer

NSCs = neural stem cells

Oct-4 = octamer-binding transcription factor 4

Pax3 = paired box 3

Pax7 = paired box 7

PDGF-αR = platelet-derived growth factor-α receptor

SCID = severe combined immunodeficient

Sgca = α-sarcoglycan

Sox2 = sex determining region Y-box 2

TALENs = transcription activator-like effector nucleases

VCAM-1 = vascular cell adhesion molecule 1

ZFNs = zinc finger nucleases

CONFLICT OF INTEREST

FST is inventor of a patent application filed by his institution describing the HIDEM protocol (no. PCT/GB2013/050112; publication no. WO2013108039 A1) He is also the principal investigator of a grant funded by Takeda New Frontier Science programme and provided speaking and consulting services to Takeda Pharmaceuticals International Inc. via UCL Consultants.

ACKNOWLEDGEMENTS

Work in the VandenDriessche laboratory is supported by grants form the Flanders Fund for Scientific Research (FWO), Association Française contre les Myopathies (AFM); Walter Pyleman Fund (Koning Boudewijn Stichting). M. Loperfido is supported by a PhD studentship of the Flanders Fund for Scientific Research (FWO) and the Willy Gepts Fund (Wetenschappelijk Fonds Willy Gepts – Vrije Universiteit Brussel). Work in the Tedesco laboratory is funded by the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant

agreement no. 602423 (PluriMes), Takeda New Frontier Science, the UK Biotechnology and Biological Sciences Research Council (BBSRC; PhD studentship to H. B. Steele-Stallard) and Medical Research Council (MRC), Duchenne Parent Project Onlus, Muscular Dystrophy UK, Duchenne Children's Trust and the Duchenne Research Fund. Authors declare having no potential competing financial interests.

REFERENCES

[1] Mauro A. Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 1961;9:493–5.
[2] Benedetti S, Hoshiya H, Tedesco FS. Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies. FEBS J 2013;280:4263–80. doi:10.1111/febs.12178.
[3] Blau HM, Webster C, Pavlath GK. Defective myoblasts identified in Duchenne muscular dystrophy. Proc Natl Acad Sci U S A 1983;80:4856–60.
[4] Cassano M, Dellavalle A, Tedesco FS, Quattrocelli M, Crippa S, Ronzoni F, et al. Alpha sarcoglycan is required for FGF-dependent myogenic progenitor cell proliferation in vitro and in vivo. Dev Camb Engl 2011;138:4523–33. doi:10.1242/dev.070706.
[5] Cohn RD, Henry MD, Michele DE, Barresi R, Saito F, Moore SA, et al. Disruption of DAG1 in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. Cell 2002;110:639–48.
[6] Kudryashova E, Kramerova I, Spencer MJ. Satellite cell senescence underlies myopathy in a mouse model of limb-girdle muscular dystrophy 2H. J Clin Invest 2012;122:1764–76. doi:10.1172/JCI59581.
[7] Montarras D, Morgan J, Collins C, Relaix F, Zaffran S, Cumano A, et al. Direct isolation of satellite cells for skeletal muscle regeneration. Science 2005;309:2064–7. doi:10.1126/science.1114758.
[8] Sacco A, Mourkioti F, Tran R, Choi J, Llewellyn M, Kraft P, et al. Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. Cell 2010;143:1059–71. doi:10.1016/j.cell.2010.11.039.
[9] Tedesco FS, Gerli MFM, Perani L, Benedetti S, Ungaro F, Cassano M, et al. Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. Sci Transl Med 2012;4:140ra89. doi:10.1126/scitranslmed.3003541.
[10] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981;292:154–6.
[11] Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–7.
[12] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663–76. doi:10.1016/j.cell.2006.07.024.
[13] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–72. doi:10.1016/j.cell.2007.11.019.
[14] Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. J Appl Physiol Bethesda Md 1985 2000;89:81–8.
[15] Tajbakhsh S. Skeletal muscle stem cells in developmental versus regenerative myogenesis. J Intern Med 2009;266:372–89. doi:10.1111/j.1365-2796.2009.02158.x.
[16] Cossu G, Tajbakhsh S, Buckingham M. How is myogenesis initiated in the embryo? Trends Genet TIG 1996;12:218–23.
[17] Miller JB, Schaefer L, Dominov JA. Seeking muscle stem cells. Curr Top Dev Biol 1999;43:191–219.
[18] Pirskanen A, Kiefer JC, Hauschka SD. IGFs, insulin, Shh, bFGF, and TGF-beta1 interact synergistically to promote somite myogenesis in vitro. Dev Biol 2000;224:189–203. doi:10.1006/dbio.2000.9784.
[19] Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meilhac S, et al. The formation of skeletal muscle: from somite to limb. J Anat 2003;202:59–68.

[20] Tajbakhsh S, Buckingham M. The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. Curr Top Dev Biol 2000;48:225–68.
[21] Borycki AG, Emerson CP. Multiple tissue interactions and signal transduction pathways control somite myogenesis. Curr Top Dev Biol 2000;48:165–224.
[22] Bryson-Richardson RJ, Currie PD. The genetics of vertebrate myogenesis. Nat Rev Genet 2008;9:632–46. doi:10.1038/nrg2369.
[23] Tzahor E. Heart and craniofacial muscle development: a new developmental theme of distinct myogenic fields. Dev Biol 2009;327:273–9. doi:10.1016/j.ydbio.2008.12.035.
[24] Harel I, Nathan E, Tirosh-Finkel L, Zigdon H, Guimarães-Camboa N, Evans SM, et al. Distinct origins and genetic programs of head muscle satellite cells. Dev Cell 2009;16:822–32. doi:10.1016/j.devcel.2009.05.007.
[25] Emery AEH. The muscular dystrophies. Lancet 2002;359:687–95. doi:10.1016/S0140-6736(02)07815-7.
[26] Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R. MyoD or Myf-5 is required for the formation of skeletal muscle. Cell 1993;75:1351–9.
[27] Kassar-Duchossoy L, Gayraud-Morel B, Gomès D, Rocancourt D, Buckingham M, Shinin V, et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature 2004;431:466–71. doi:10.1038/nature02876.
[28] Kassar-Duchossoy L, Giacone E, Gayraud-Morel B, Jory A, Gomès D, Tajbakhsh S. Pax3/Pax7 mark a novel population of primitive myogenic cells during development. Genes Dev 2005;19:1426–31. doi:10.1101/gad.345505.
[29] Relaix F, Rocancourt D, Mansouri A, Buckingham M. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 2005;435:948–53. doi:10.1038/nature03594.
[30] Gros J, Manceau M, Thomé V, Marcelle C. A common somitic origin for embryonic muscle progenitors and satellite cells. Nature 2005;435:954–8. doi:10.1038/nature03572.
[31] Schienda J, Engleka KA, Jun S, Hansen MS, Epstein JA, Tabin CJ, et al. Somitic origin of limb muscle satellite and side population cells. Proc Natl Acad Sci U S A 2006;103:945–50. doi:10.1073/pnas.0510164103.
[32] Mikkers H, Frisén J. Deconstructing stemness. EMBO J 2005;24:2715–9. doi:10.1038/sj.emboj.7600749.
[33] Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, et al. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol 2006;172:91–102. doi:10.1083/jcb.200508044.
[34] Valdez MR, Richardson JA, Klein WH, Olson EN. Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. Dev Biol 2000;219:287–98. doi:10.1006/dbio.2000.9621.
[35] Maroto M, Reshef R, Münsterberg AE, Koester S, Goulding M, Lassar AB. Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell 1997;89:139–48.
[36] Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 1997;89:127–38.
[37] Mercuri E, Muntoni F. Muscular dystrophies. Lancet 2013;381:845–60. doi:10.1016/S0140-6736(12)61897-2.
[38] Lepper C, Partridge TA, Fan C-M. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Dev Camb Engl 2011;138:3639–46. doi:10.1242/dev.067595.
[39] Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Dev Camb Engl 2011;138:3625–37. doi:10.1242/dev.064162.
[40] Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Dev Camb Engl 2012;139:2845–56. doi:10.1242/dev.069088.
[41] Sambasivan R, Yao R, Kissenpfennig A, Van Wittenberghe L, Paldi A, Gayraud-Morel B, et al. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. Dev Camb Engl 2011;138:3647–56. doi:10.1242/dev.067587.
[42] Günther S, Kim J, Kostin S, Lepper C, Fan C-M, Braun T. Myf5-positive satellite cells contribute to Pax7-dependent long-term maintenance of adult muscle stem cells. Cell Stem Cell 2013;13:590–601. doi:10.1016/j.stem.2013.07.016.

[43] Schultz E, Gibson MC, Champion T. Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. J Exp Zool 1978;206:451–6. doi:10.1002/jez.1402060314.
[44] Tedesco FS, Dellavalle A, Diaz-Manera J, Messina G, Cossu G. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. J Clin Invest 2010;120:11–9. doi:10.1172/JCI40373.
[45] Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. Cell 2000;102:777–86.
[46] Zammit PS, Relaix F, Nagata Y, Ruiz AP, Collins CA, Partridge TA, et al. Pax7 and myogenic progression in skeletal muscle satellite cells. J Cell Sci 2006;119:1824–32. doi:10.1242/jcs.02908.
[47] Sherwood RI, Christensen JL, Conboy IM, Conboy MJ, Rando TA, Weissman IL, et al. Isolation of adult mouse myogenic progenitors: functional heterogeneity of cells within and engrafting skeletal muscle. Cell 2004;119:543–54. doi:10.1016/j.cell.2004.10.021.
[48] Bajard L, Relaix F, Lagha M, Rocancourt D, Daubas P, Buckingham ME. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev 2006;20:2450–64. doi:10.1101/gad.382806.
[49] Hu P, Geles KG, Paik J-H, DePinho RA, Tjian R. Codependent activators direct myoblast-specific MyoD transcription. Dev Cell 2008;15:534–46. doi:10.1016/j.devcel.2008.08.018.
[50] McKinnell IW, Ishibashi J, Le Grand F, Punch VGJ, Addicks GC, Greenblatt JF, et al. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. Nat Cell Biol 2008;10:77–84. doi:10.1038/ncb1671.
[51] Bober E, Franz T, Arnold HH, Gruss P, Tremblay P. Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. Dev Camb Engl 1994;120:603–12.
[52] Relaix F, Rocancourt D, Mansouri A, Buckingham M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev 2004;18:1088–105. doi:10.1101/gad.301004.
[53] Lepper C, Conway SJ, Fan C-M. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. Nature 2009;460:627–31. doi:10.1038/nature08209.
[54] Von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc Natl Acad Sci U S A 2013;110:16474–9. doi:10.1073/pnas.1307680110.
[55] Punch VG, Jones AE, Rudnicki MA. Transcriptional networks that regulate muscle stem cell function. Wiley Interdiscip Rev Syst Biol Med 2009;1:128–40. doi:10.1002/wsbm.11.
[56] Le Grand F, Jones AE, Seale V, Scimè A, Rudnicki MA. Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. Cell Stem Cell 2009;4:535–47. doi:10.1016/j.stem.2009.03.013.
[57] Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2008;2:22–31. doi:10.1016/j.stem.2007.12.012.
[58] Rocheteau P, Gayraud-Morel B, Siegl-Cachedenier I, Blasco MA, Tajbakhsh S. A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell 2012;148:112–25. doi:10.1016/j.cell.2011.11.049.
[59] Shinin V, Gayraud-Morel B, Gomès D, Tajbakhsh S. Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. Nat Cell Biol 2006;8:677–87. doi:10.1038/ncb1425.
[60] Wang YX, Bentzinger CF, Rudnicki MA. Molecular regulation of determination in asymmetrically dividing muscle stem cells. Cell Cycle Georget Tex 2013;12:3–4. doi:10.4161/cc.23068.
[61] Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, Beauchamp JR. Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol 2004;166:347–57. doi:10.1083/jcb.200312007.
[62] Péault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, Partridge T, et al. Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. Mol Ther J Am Soc Gene Ther 2007;15:867–77. doi:10.1038/mt.sj.6300145.
[63] Illa I, Leon-Monzon M, Dalakas MC. Regenerating and denervated human muscle fibers and satellite cells express neural cell adhesion molecule recognized by monoclonal antibodies to natural killer cells. Ann Neurol 1992;31:46–52. doi:10.1002/ana.410310109.
[64] Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. Nature 1989;337:176–9. doi:10.1038/337176a0.

[65] Skuk D, Tremblay JP. Clarifying misconceptions about myoblast transplantation in myology. Mol Ther J Am Soc Gene Ther 2014;22:897–8. doi:10.1038/mt.2014.57.
[66] Périé S, Mamchaoui K, Mouly V, Blot S, Bouazza B, Thornell L-E, et al. Premature proliferative arrest of cricopharyngeal myoblasts in oculo-pharyngeal muscular dystrophy: Therapeutic perspectives of autologous myoblast transplantation. Neuromuscul Disord NMD 2006;16:770–81. doi:10.1016/j.nmd.2006.07.022.
[67] Périé S, Trollet C, Mouly V, Vanneaux V, Mamchaoui K, Bouazza B, et al. Autologous myoblast transplantation for oculopharyngeal muscular dystrophy: a phase I/IIa clinical study. Mol Ther J Am Soc Gene Ther 2014;22:219–25. doi:10.1038/mt.2013.155.
[68] Benchaouir R, Meregalli M, Farini A, D'Antona G, Belicchi M, Goyenvalle A, et al. Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Cell Stem Cell 2007;1:646–57. doi:10.1016/j.stem.2007.09.016.
[69] Torrente Y, Belicchi M, Marchesi C, D'Antona G, Cogiamanian F, Pisati F, et al. Autologous transplantation of muscle-derived CD133+ stem cells in Duchenne muscle patients. Cell Transplant 2007;16:563–77.
[70] Meng J, Chun S, Asfahani R, Lochmüller H, Muntoni F, Morgan J. Human skeletal muscle-derived CD133(+) cells form functional satellite cells after intramuscular transplantation in immunodeficient host mice. Mol Ther J Am Soc Gene Ther 2014;22:1008–17. doi:10.1038/mt.2014.26.
[71] Mitchell KJ, Pannérec A, Cadot B, Parlakian A, Besson V, Gomes ER, et al. Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol 2010;12:257–66. doi:10.1038/ncb2025.
[72] Rouger K, Larcher T, Dubreil L, Deschamps J-Y, Le Guiner C, Jouvion G, et al. Systemic delivery of allogenic muscle stem cells induces long-term muscle repair and clinical efficacy in duchenne muscular dystrophy dogs. Am J Pathol 2011;179:2501–18. doi:10.1016/j.ajpath.2011.07.022.
[73] Dellavalle A, Maroli G, Covarello D, Azzoni E, Innocenzi A, Perani L, et al. Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat Commun 2011;2:499. doi:10.1038/ncomms1508.
[74] Minasi MG, Riminucci M, De Angelis L, Borello U, Berarducci B, Innocenzi A, et al. The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Dev Camb Engl 2002;129:2773–83.
[75] Cappellari O, Cossu G. Pericytes in development and pathology of skeletal muscle. Circ Res 2013;113:341–7. doi:10.1161/CIRCRESAHA.113.300203.
[76] Sampaolesi M, Torrente Y, Innocenzi A, Tonlorenzi R, D'Antona G, Pellegrino MA, et al. Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. Science 2003;301:487–92. doi:10.1126/science.1082254.
[77] Sampaolesi M, Blot S, D'Antona G, Granger N, Tonlorenzi R, Innocenzi A, et al. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature 2006;444:574–9. doi:10.1038/nature05282.
[78] Gargioli C, Coletta M, De Grandis F, Cannata SM, Cossu G. PlGF-MMP-9-expressing cells restore microcirculation and efficacy of cell therapy in aged dystrophic muscle. Nat Med 2008;14:973–8. doi:10.1038/nm.1852.
[79] Díaz-Manera J, Touvier T, Dellavalle A, Tonlorenzi R, Tedesco FS, Messina G, et al. Partial dysferlin reconstitution by adult murine mesoangioblasts is sufficient for full functional recovery in a murine model of dysferlinopathy. Cell Death Dis 2010;1:e61. doi:10.1038/cddis.2010.35.
[80] Tedesco FS, Hoshiya H, D'Antona G, Gerli MFM, Messina G, Antonini S, et al. Stem cell-mediated transfer of a human artificial chromosome ameliorates muscular dystrophy. Sci Transl Med 2011;3:96ra78. doi:10.1126/scitranslmed.3002342.
[81] Giannotta M, Benedetti S, Tedesco FS, Corada M, Trani M, D'Antuono R, et al. Targeting endothelial junctional adhesion molecule-A/ EPAC/ Rap-1 axis as a novel strategy to increase stem cell engraftment in dystrophic muscles. EMBO Mol Med 2014;6:239–58. doi:10.1002/emmm.201302520.
[82] Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 1981;78:7634–8.
[83] Robbins J, Gulick J, Sanchez A, Howles P, Doetschman T. Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development in vitro. J Biol Chem 1990;265:11905–9.

[84] Sánchez A, Jones WK, Gulick J, Doetschman T, Robbins J. Myosin heavy chain gene expression in mouse embryoid bodies. An in vitro developmental study. J Biol Chem 1991;266:22419–26.
[85] Bhagavati S, Xu W. Generation of skeletal muscle from transplanted embryonic stem cells in dystrophic mice. Biochem Biophys Res Commun 2005;333:644–9. doi:10.1016/j.bbrc.2005.05.135.
[86] Barberi T, Bradbury M, Dincer Z, Panagiotakos G, Socci ND, Studer L. Derivation of engraftable skeletal myoblasts from human embryonic stem cells. Nat Med 2007;13:642–8. doi:10.1038/nm1533.
[87] Lassar AB, Paterson BM, Weintraub H. Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. Cell 1986;47:649–56.
[88] Dekel I, Magal Y, Pearson-White S, Emerson CP, Shani M. Conditional conversion of ES cells to skeletal muscle by an exogenous MyoD1 gene. New Biol 1992;4:217–24.
[89] Shani M, Faerman A, Emerson CP, Pearson-White S, Dekel I, Magal Y. The consequences of a constitutive expression of MyoD1 in ES cells and mouse embryos. Symp Soc Exp Biol 1992;46:19–36.
[90] Ozasa S, Kimura S, Ito K, Ueno H, Ikezawa M, Matsukura M, et al. Efficient conversion of ES cells into myogenic lineage using the gene-inducible system. Biochem Biophys Res Commun 2007;357:957–63. doi:10.1016/j.bbrc.2007.04.032.
[91] Darabi R, Gehlbach K, Bachoo RM, Kamath S, Osawa M, Kamm KE, et al. Functional skeletal muscle regeneration from differentiating embryonic stem cells. Nat Med 2008;14:134–43. doi:10.1038/nm1705.
[92] Darabi R, Santos FNC, Filareto A, Pan W, Koene R, Rudnicki MA, et al. Assessment of the myogenic stem cell compartment following transplantation of Pax3/Pax7-induced embryonic stem cell-derived progenitors. Stem Cells Dayt Ohio 2011;29:777–90. doi:10.1002/stem.625.
[93] Darabi R, Arpke RW, Irion S, Dimos JT, Grskovic M, Kyba M, et al. Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. Cell Stem Cell 2012;10:610–9. doi:10.1016/j.stem.2012.02.015.
[94] Iacovino M, Bosnakovski D, Fey H, Rux D, Bajwa G, Mahen E, et al. Inducible cassette exchange: a rapid and efficient system enabling conditional gene expression in embryonic stem and primary cells. Stem Cells Dayt Ohio 2011;29:1580–8.
[95] Sakurai H, Okawa Y, Inami Y, Nishio N, Isobe K. Paraxial mesodermal progenitors derived from mouse embryonic stem cells contribute to muscle regeneration via differentiation into muscle satellite cells. Stem Cells Dayt Ohio 2008;26:1865–73. doi:10.1634/stemcells.2008-0173.
[96] Chang H, Yoshimoto M, Umeda K, Iwasa T, Mizuno Y, Fukada S, et al. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. FASEB J Off Publ Fed Am Soc Exp Biol 2009;23:1907–19. doi:10.1096/fj.08-123661.
[97] Fukada S, Higuchi S, Segawa M, Koda K, Yamamoto Y, Tsujikawa K, et al. Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. Exp Cell Res 2004;296:245–55. doi:10.1016/j.yexcr.2004.02.018.
[98] Shelton M, Metz J, Liu J, Carpenedo RL, Demers S-P, Stanford WL, et al. Derivation and expansion of PAX7-positive muscle progenitors from human and mouse embryonic stem cells. Stem Cell Rep 2014;3:516–29. doi:10.1016/j.stemcr.2014.07.001.
[99] Albini S, Coutinho P, Malecova B, Giordani L, Savchenko A, Forcales SV, et al. Epigenetic reprogramming of human embryonic stem cells into skeletal muscle cells and generation of contractile myospheres. Cell Rep 2013;3:661–70. doi:10.1016/j.celrep.2013.02.012.
[100] Davis RL, Weintraub H, Lassar AB. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 1987;51:987–1000.
[101] Schäfer BW, Blakely BT, Darlington GJ, Blau HM. Effect of cell history on response to helix-loop-helix family of myogenic regulators. Nature 1990;344:454–8. doi:10.1038/344454a0.
[102] Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, et al. Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat Commun 2014;5. doi:10.1038/ncomms4195.
[103] De Miguel-Beriain I. The ethics of stem cells revisited. Adv Drug Deliv Rev 2015;82-83:176–80. doi:10.1016/j.addr.2014.11.011.
[104] Yamanaka S. Induced pluripotent stem cells: past, present, and future. Cell Stem Cell 2012;10:678–84. doi:10.1016/j.stem.2012.05.005.

[105] Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8:424–9. doi:10.1038/nmeth.1593.
[106] Liu Z, Tang Y, Lü S, Zhou J, Du Z, Duan C, et al. The tumourigenicity of iPS cells and their differentiated derivates. J Cell Mol Med 2013;17:782–91. doi:10.1111/jcmm.12062.
[107] Hong SG, Winkler T, Wu C, Guo V, Pittaluga S, Nicolae A, et al. Path to the clinic: assessment of iPSC-based cell therapies in vivo in a nonhuman primate model. Cell Rep 2014;7:1298–309. doi:10.1016/j.celrep.2014.04.019.
[108] Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2008;2:22–31. doi:10.1016/j.stem.2007.12.012.
[109] Pannérec A, Marazzi G, Sassoon D. Stem cells in the hood: the skeletal muscle niche. Trends Mol Med 2012;18:599–606. doi:10.1016/j.molmed.2012.07.004.
[110] Wang YX, Rudnicki MA. Satellite cells, the engines of muscle repair. Nat Rev Mol Cell Biol 2012;13:127–33. doi:10.1038/nrm3265.
[111] Maffioletti SM, Noviello M, English K, Tedesco FS. Stem cell transplantation for muscular dystrophy: the challenge of immune response. BioMed Res Int 2014;2014:964010. doi:10.1155/2014/964010.
[112] Sicari BM, Agrawal V, Siu BF, Medberry CJ, Dearth CL, Turner NJ, et al. A murine model of volumetric muscle loss and a regenerative medicine approach for tissue replacement. Tissue Eng Part A 2012;18:1941–8. doi:10.1089/ten.TEA.2012.0475.
[113] Criswell TL, Corona BT, Wang Z, Zhou Y, Niu G, Xu Y, et al. The role of endothelial cells in myofiber differentiation and the vascularization and innervation of bioengineered muscle tissue in vivo. Biomaterials 2013;34:140–9. doi:10.1016/j.biomaterials.2012.09.045.
[114] Fuoco C, Rizzi R, Biondo A, Longa E, Mascaro A, Shapira-Schweitzer K, et al. In vivo generation of a mature and functional artificial skeletal muscle. EMBO Mol Med 2015;7:411–22. doi:10.15252/emmm.201404062.
[115] Inoue H, Nagata N, Kurokawa H, Yamanaka S. iPS cells: a game changer for future medicine. EMBO J 2014;33:409–17. doi:10.1002/embj.201387098.
[116] Ye L, Muench MO, Fusaki N, Beyer AI, Wang J, Qi Z, et al. Blood cell-derived induced pluripotent stem cells free of reprogramming factors generated by Sendai viral vectors. Stem Cells Transl Med 2013;2:558–66. doi:10.5966/sctm.2013-0006.
[117] Zhou T, Benda C, Dunzinger S, Huang Y, Ho JC, Yang J, et al. Generation of human induced pluripotent stem cells from urine samples. Nat Protoc 2012;7:2080–9. doi:10.1038/nprot.2012.115.
[118] Vaskova EA, Stekleneva AE, Medvedev SP, Zakian SM. “Epigenetic memory” phenomenon in induced pluripotent stem cells. Acta Naturae 2013;5:15–21.
[119] Shtrichman R, Germanguz I, Itskovitz-Eldor J. Induced pluripotent stem cells (iPSCs) derived from different cell sources and their potential for regenerative and personalized medicine. Curr Mol Med 2013;13:792–805.
[120] Quattrocelli M, Palazzolo G, Floris G, Schöffski P, Anastasia L, Orlacchio A, et al. Intrinsic cell memory reinforces myogenic commitment of pericyte-derived iPSCs. J Pathol 2011;223:593–603. doi:10.1002/path.2845.
[121] Sanchez-Freire V, Lee AS, Hu S, Abilez OJ, Liang P, Lan F, et al. Effect of human donor cell source on differentiation and function of cardiac induced pluripotent stem cells. J Am Coll Cardiol 2014;64:436–48. doi:10.1016/j.jacc.2014.04.056.
[122] Biasco L, Baricordi C, Aiuti A. Retroviral integrations in gene therapy trials. Mol Ther J Am Soc Gene Ther 2012;20:709–16. doi:10.1038/mt.2011.289.
[123] Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 2009;458:771–5. doi:10.1038/nature07864.
[124] Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. Science 2008;322:945–9. doi:10.1126/science.1162494.
[125] Cyranoski D. Japanese woman is first recipient of next-generation stem cells. Nature 2014. doi:10.1038/nature.2014.15915.
[126] Kamao H, Mandai M, Okamoto S, Sakai N, Suga A, Sugita S, et al. Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. Stem Cell Rep 2014;2:205–18. doi:10.1016/j.stemcr.2013.12.007.
[127] Nakano-Okuno M, Borah BR, Nakano I. Ethics of iPSC-Based Clinical Research for Age-Related Macular Degeneration: Patient-Centered Risk-Benefit Analysis. Stem Cell Rev 2014;10:743–52. doi:10.1007/s12015-014-9536-x.

[128] Darabi R, Pan W, Bosnakovski D, Baik J, Kyba M, Perlingeiro RCR. Functional myogenic engraftment from mouse iPS cells. Stem Cell Rev 2011;7:948–57. doi:10.1007/s12015-011-9258-2.
[129] Belay E, Mátrai J, Acosta-Sanchez A, Ma L, Quattrocelli M, Mátés L, et al. Novel hyperactive transposons for genetic modification of induced pluripotent and adult stem cells: a nonviral paradigm for coaxed differentiation. Stem Cells Dayt Ohio 2010;28:1760–71. doi:10.1002/stem.501.
[130] Filareto A, Parker S, Darabi R, Borges L, Iacovino M, Schaaf T, et al. An ex vivo gene therapy approach to treat muscular dystrophy using inducible pluripotent stem cells. Nat Commun 2013;4:1549. doi:10.1038/ncomms2550.
[131] Maffioletti SM, Gerli MFM, Ragazzi M, Dastidar S, Benedetti S, Loperfido M, et al. Efficient derivation and inducible differentiation of expandable skeletal myogenic cells from human ES and patient-specific iPS cells. Nat Protoc 2015;10:941–58. doi:10.1038/nprot.2015.057.
[132] Gerli MFM, Maffioletti SM, Millet Q, Tedesco FS. Transplantation of induced pluripotent stem cell-derived mesoangioblast-like myogenic progenitors in mouse models of muscle regeneration. J Vis Exp JoVE 2014:e50532. doi:10.3791/50532.
[133] Goudenege S, Lebel C, Huot NB, Dufour C, Fujii I, Gekas J, et al. Myoblasts derived from normal hESCs and dystrophic hiPSCs efficiently fuse with existing muscle fibers following transplantation. Mol Ther J Am Soc Gene Ther 2012;20:2153–67. doi:10.1038/mt.2012.188.
[134] Tanaka A, Woltjen K, Miyake K, Hotta A, Ikeya M, Yamamoto T, et al. Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling Miyoshi Myopathy in vitro. PloS One 2013;8:e61540. doi:10.1371/journal.pone.0061540.
[135] Yasuno T, Osafune K, Sakurai H, Asaka I, Tanaka A, Yamaguchi S, et al. Functional analysis of iPSC-derived myocytes from a patient with carnitine palmitoyltransferase II deficiency. Biochem Biophys Res Commun 2014;448:175–81. doi:10.1016/j.bbrc.2014.04.084.
[136] Abujarour R, Bennett M, Valamehr B, Lee TT, Robinson M, Robbins D, et al. Myogenic differentiation of muscular dystrophy-specific induced pluripotent stem cells for use in drug discovery. Stem Cells Transl Med 2014;3:149–60. doi:10.5966/sctm.2013-0095.
[137] Mizuno Y, Chang H, Umeda K, Niwa A, Iwasa T, Awaya T, et al. Generation of skeletal muscle stem/progenitor cells from murine induced pluripotent stem cells. FASEB J Off Publ Fed Am Soc Exp Biol 2010;24:2245–53. doi:10.1096/fj.09-137174.
[138] Awaya T, Kato T, Mizuno Y, Chang H, Niwa A, Umeda K, et al. Selective Development of Myogenic Mesenchymal Cells from Human Embryonic and Induced Pluripotent Stem Cells. PLoS ONE 2012;7:e51638. doi:10.1371/journal.pone.0051638.
[139] Borchin B, Chen J, Barberi T. Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. Stem Cell Rep 2013;1:620–31. doi:10.1016/j.stemcr.2013.10.007.
[140] Xu C, Tabebordbar M, Iovino S, Ciarlo C, Liu J, Castiglioni A, et al. A Zebrafish Embryo Culture System Defines Factors that Promote Vertebrate Myogenesis across Species. Cell 2013;155:909–21. doi:10.1016/j.cell.2013.10.023.
[141] Patterson M, Chan DN, Ha I, Case D, Cui Y, Van Handel B, et al. Defining the nature of human pluripotent stem cell progeny. Cell Res 2012;22:178–93. doi:10.1038/cr.2011.133.
[142] Chin MH, Mason MJ, Xie W, Volinia S, Singer M, Peterson C, et al. Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell 2009;5:111–23. doi:10.1016/j.stem.2009.06.008.
[143] Drukker M, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, et al. Characterization of the expression of MHC proteins in human embryonic stem cells. Proc Natl Acad Sci U S A 2002;99:9864–9. doi:10.1073/pnas.142298299.
[144] Swijnenburg R-J, Tanaka M, Vogel H, Baker J, Kofidis T, Gunawan F, et al. Embryonic stem cell immunogenicity increases upon differentiation after transplantation into ischemic myocardium. Circulation 2005;112:I166–72. doi:10.1161/CIRCULATIONAHA.104.525824.
[145] Tabayoyong WB, Zavazava N. Meet the inlaws: embryonic stem cell derivatives meet the immune system. Cell Res 2009;19:397–8. doi:10.1038/cr.2009.38.
[146] Zhao T, Zhang Z-N, Rong Z, Xu Y. Immunogenicity of induced pluripotent stem cells. Nature 2011;474:212–5. doi:10.1038/nature10135.
[147] Li O, English K, Tonlorenzi R, Cossu G, Saverio Tedesco F, Wood KJ. Human iPSC-derived mesoangioblasts, like their tissue-derived counterparts, suppress T cell proliferation through IDO- and PGE-2-dependent pathways. F1000Research 2013;2:24. doi:10.12688/f1000research.2-24.v1.

[148] English K, Tonlorenzi R, Cossu G, Wood KJ. Mesoangioblasts suppress T cell proliferation through IDO and PGE-2-dependent pathways. Stem Cells Dev 2013;22:512–23. doi:10.1089/scd.2012.0386.
[149] Taylor CJ, Peacock S, Chaudhry AN, Bradley JA, Bolton EM. Generating an iPSC bank for HLA-matched tissue transplantation based on known donor and recipient HLA types. Cell Stem Cell 2012;11:147–52. doi:10.1016/j.stem.2012.07.014.
[150] Roncarolo M-G, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol 2007;7:585–98. doi:10.1038/nri2138.
[151] Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, et al. A special population of regulatory T cells potentiates muscle repair. Cell 2013;155:1282–95. doi:10.1016/j.cell.2013.10.054.
[152] Lui KO, Boyd AS, Cobbold SP, Waldmann H, Fairchild PJ. A role for regulatory T cells in acceptance of ESC-derived tissues transplanted across an major histocompatibility complex barrier. Stem Cells Dayt Ohio 2010;28:1905–14. doi:10.1002/stem.506.
[153] Kazuki Y, Hiratsuka M, Takiguchi M, Osaki M, Kajitani N, Hoshiya H, et al. Complete genetic correction of ips cells from Duchenne muscular dystrophy. Mol Ther J Am Soc Gene Ther 2010;18:386–93. doi:10.1038/mt.2009.274.
[154] Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM, et al. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nat Med 1998;4:1441–4. doi:10.1038/4033.
[155] Ma N, Liao B, Zhang H, Wang L, Shan Y, Xue Y, et al. Transcription activator-like effector nuclease (TALEN)-mediated gene correction in integration-free β-thalassemia induced pluripotent stem cells. J Biol Chem 2013;288:34671–9. doi:10.1074/jbc.M113.496174.
[156] Choi SM, Kim Y, Shim JS, Park JT, Wang R-H, Leach SD, et al. Efficient drug screening and gene correction for treating liver disease using patient-specific stem cells. Hepatol Baltim Md 2013;57:2458–68. doi:10.1002/hep.26237.
[157] Osborn MJ, Starker CG, McElroy AN, Webber BR, Riddle MJ, Xia L, et al. TALEN-based gene correction for epidermolysis bullosa. Mol Ther J Am Soc Gene Ther 2013;21:1151–9. doi:10.1038/mt.2013.56.
[158] Xia G, Gao Y, Jin S, Subramony S, Terada N, Ranum LPW, et al. Genome Modification Leads to Phenotype Reversal in Human Myotonic Dystrophy type 1 iPS-cell Derived Neural Stem Cells. Stem Cells Dayt Ohio 2015. doi:10.1002/stem.1970.
[159] Ousterout DG, Perez-Pinera P, Thakore PI, Kabadi AM, Brown MT, Qin X, et al. Reading frame correction by targeted genome editing restores dystrophin expression in cells from Duchenne muscular dystrophy patients. Mol Ther J Am Soc Gene Ther 2013;21:1718–26. doi:10.1038/mt.2013.111.
[160] Ousterout DG, Kabadi AM, Thakore PI, Perez-Pinera P, Brown MT, Majoros WH, et al. Correction of Dystrophin Expression in Cells from Duchenne Muscular Dystrophy Patients through Genomic Excision of Exon 51 by Zinc Finger Nucleases. Mol Ther J Am Soc Gene Ther 2014. doi:10.1038/mt.2014.234.
[161] Ousterout DG, Kabadi AM, Thakore PI, Majoros WH, Reddy TE, Gersbach CA. Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. Nat Commun 2015;6:6244. doi:10.1038/ncomms7244.
[162] Li HL, Fujimoto N, Sasakawa N, Shirai S, Ohkame T, Sakuma T, et al. Precise Correction of the Dystrophin Gene in Duchenne Muscular Dystrophy Patient Induced Pluripotent Stem Cells by TALEN and CRISPR-Cas9. Stem Cell Rep 2014. doi:10.1016/j.stemcr.2014.10.013.
[163] Aiuti A, Biasco L, Scaramuzza S, Ferrua F, Cicalese MP, Baricordi C, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. Science 2013;341:1233151. doi:10.1126/science.1233151.
[164] Biffi A, Montini E, Lorioli L, Cesani M, Fumagalli F, Plati T, et al. Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 2013;341:1233158. doi:10.1126/science.1233158.

Pluripotent stem cell	Origin	Myogenic differentiation method	Mouse model & genetic correction	*In vivo* results			Reference
				Engraftment/ Differentiation	Systemic delivery	Functional test	
ES cells	mouse	EB cocultured with primary muscle cells	WT cells into mdx mouse, no genetic correction	✔	✗	✗	[85]
		MyoD integration (IND)	WT cells into mdx mouse, no genetic correction	✔	✗	✗	[90]
		Pax3 integration (IND), EB, PDGF- αR^+ Flk-1^- cell sorting	WT cells into mdx mouse, no genetic correction	✔	✔	✔	[91]
		Pax3 or Pax7 integration (IND), EB, PDGF- αR^+ Flk-1^- cell sorting	WT cells into mdx mouse, no genetic correction	✔	✔	✔	[92]
		mesodermal commitment, PDGF- αR^+ cell sorting	WT cells into immunodeficient mouse, no genetic correction	✔	✗	✗	[95]
		EB and SM/C-2.6 cell sorting	WT cells into mdx mouse, no genetic correction	✔	✗	✗	[96]
		EB, GSK-3 inhibitor (CHIR99021), bFGF, N2	NA	✗	✗	✗	[98]
		Myf5 integration (IND), EB	NA	✗	✗	✗	[94]
	human	mesenchymal commitment, $CD73^+$ and $NCAM^+$ cell sorting	HD cells into immunodeficient mouse, no genetic correction	✔	✗	✗	[86]
		Pax7 LV integration (IND), EB, purification by cell sorting	HD cells into immunodeficient/mdx mouse, no genetic correction	✔	✗	✔	[93]
		EB, mesenchymal commitment	HD cells into immunodeficient mouse, no genetic correction	✔	✗	✗	[138]
		myogenic medium, MyoD AAV	HD cells into immunodeficient/mdx mouse, no genetic correction	✔	✗	✗	[133]
		BAF60C and MyoD LV integration	NA	✗	✗	✗	[99]
		GSK-3 inhibitor (CHIR99021), bFGF, N2	NA	✗	✗	✗	[98]
		Myf5 integration (IND), EB	NA	✗	✗	✗	[94]
		mesodermal commitment, MyoD-ER LV integration (IND)	NA	✗	✗	✗	[131]

Table **1**. Summary of muscle differentiation and genetic correction in ES cells.
AAV = Adeno associated viral vector, BAF60C also called SMARCD3 = SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3, bFGF = Basic fibroblast growth factor, DMD = Duchenne muscular dystrophy, EB = Embryoid bodies, Flk-1 = fetal liver kinase 1, GSK-3 inhibitor = Glycogen synthase kinase-3 inhibitor, HAC = Human artificial chromosome, HD = Healthy donor, IND = Inducible differentiation, LV = Lentiviral vector, mdx = X chromosome-linked muscular dystrophy, Myf5 = myogenic factor 5, NA = Not assessed (no disease, no genetic correction, in vitro only), MyoD = myogenic differentiation 1, NCAM = neural cell adhesion molecule, N2 = N2 supplemented media, Pax3 = paired box 3, Pax7 = paired box 7, PDGF-αR = platelet-derived growth factor-α receptor, WT = Wild type.

Pluripotent stem cell	Origin	Myogenic differentiation method	Mouse model & genetic correction	*In vivo* results			Reference
				Engraftment/ Differentiation	**Systemic delivery**	**Functional test**	
iPS cells	**Mouse**	Pax7 integration (IND), EB, PDGF-αR$^+$ Flk-1$^-$ cell sorting	WT cells into mdx mouse, no genetic correction	✔	✗	✔	[128]
		Pax3 LV integration (IND), EB, PDGF-αR$^+$ Flk-1$^-$ cell sorting	μutrn-SB transposon corrected, dys null/μutrn positive cells into dys null/utrn null mouse	✔	✔	✔	[130]
		mesodermal commitment, MyoD-ER LV integration (IND)	WT cells into Sgca-null/immunodeficient mouse, no genetic correction	✔	✔	✔	[9, 132]
		EB and SM/C-2.6 cell sorting	WT cells into mdx mouse, no genetic correction	✔	✗	✗	[137]
		Pax3 transposon integration and EB	NA	✗	✗	✗	[129]
	Human	Pax7 LV integration (IND), EB, purification by cell sorting	HD cells into immunodeficient or immunodeficient/mdx mouse, no genetic correction	✔	✗	✔	[93]
		EB, mesenchymal commitment	HD cells into immunodeficient mouse, no genetic correction	✔	✗	✗	[138]
		treatment with GSK-3 inhibitor (CHIR99021) and bFGF, sorting for AChR$^+$ or CXCR4$^+$/C-MET$^+$	NA	✗	✗	✗	[139]
		EB and treatment with GSK-3 inhibitor (BIO), bFGF and forskolin	HD cells into immunodeficient mouse, no genetic correction	✔	✗	✗	[140]
		mesodermal commitment, MyoD-ER LV integration (IND)	LGMD2D: HD and LGMD2D cells, Sgca-LV corrected cells into Sgca-null/immunodeficient mouse	✔	✔	✗	[9,131, 132]
			DMD: DMD cells and DYS-HAC corrected, *in vitro* only	✗	✗	✗	[9, 131]
		myogenic medium, MyoD AAV	DMD: DMD cells into immunodeficient/mdx mouse, no genetic correction	✔	✗	✗	[133]
		MyoD (IND) PB-transposon integration	HD cells into immunodeficient/Dmd mouse, no genetic correction	✔	✗	✗	[134]
			MM: *DYSF*-PB transposon corrected, *in vitro* only	✗	✗	✗	[134]
			CPT II: no genetic correction, *in vitro* only	✗	✗	✗	[135]
		MyoD LV integration (IND)	HD, DMD, Becker, no genetic correction, *in vitro* only	✗	✗	✗	[136]

Table **2**. Summary of muscle differentiation and genetic correction in iPS cells.
AChR = acetylcholine receptor, AAV = Adeno associated viral vector, bFGF = Basic fibroblast growth factor, Becker = Becker muscular dystrophy, BIO = GSK-3β inhibitor, CPT II = Carnitine palmitoyltransferase II, C-MET = hepatocyte growth factor c MET receptor, CXCR4 = C-X-C chemokine receptor type 4, DYS-HAC = human artificial chromosome containing the entire dystrophin locus, DMD = Duchenne muscular dystrophy, dys = Dystrophin, DYSF = dysferlin, EB = Embryoid bodies, Flk-1 = fetal liver kinase 1, GSK-3 = Glycogen synthase kinase-3 inhibitor, HAC = Human artificial chromosome, HD = Healthy donor, IND = Inducible differentiation, LGMD2D = Limb girdle muscular dystrophy type 2D, LV = Lentiviral vector, μUTRN = micro-utrophin, mdx = X chromosome-linked muscular dystrophy, MM = Myoshi myopathy, MyoD = myogenic differentiation 1, ER= Estradiol receptor, NA = Not assessed (no disease, no genetic correction, in vitro only), N2 = N2 supplemented media, Pax3 = paired box 3, Pax7 = paired box 7, PB = piggyBac, PDGF-αR = platelet-derived growth factor-α receptor, SB = Sleeping Beauty, Sgca = α-sarcoglycan, utrn = Utrophin, WT = Wild Type.

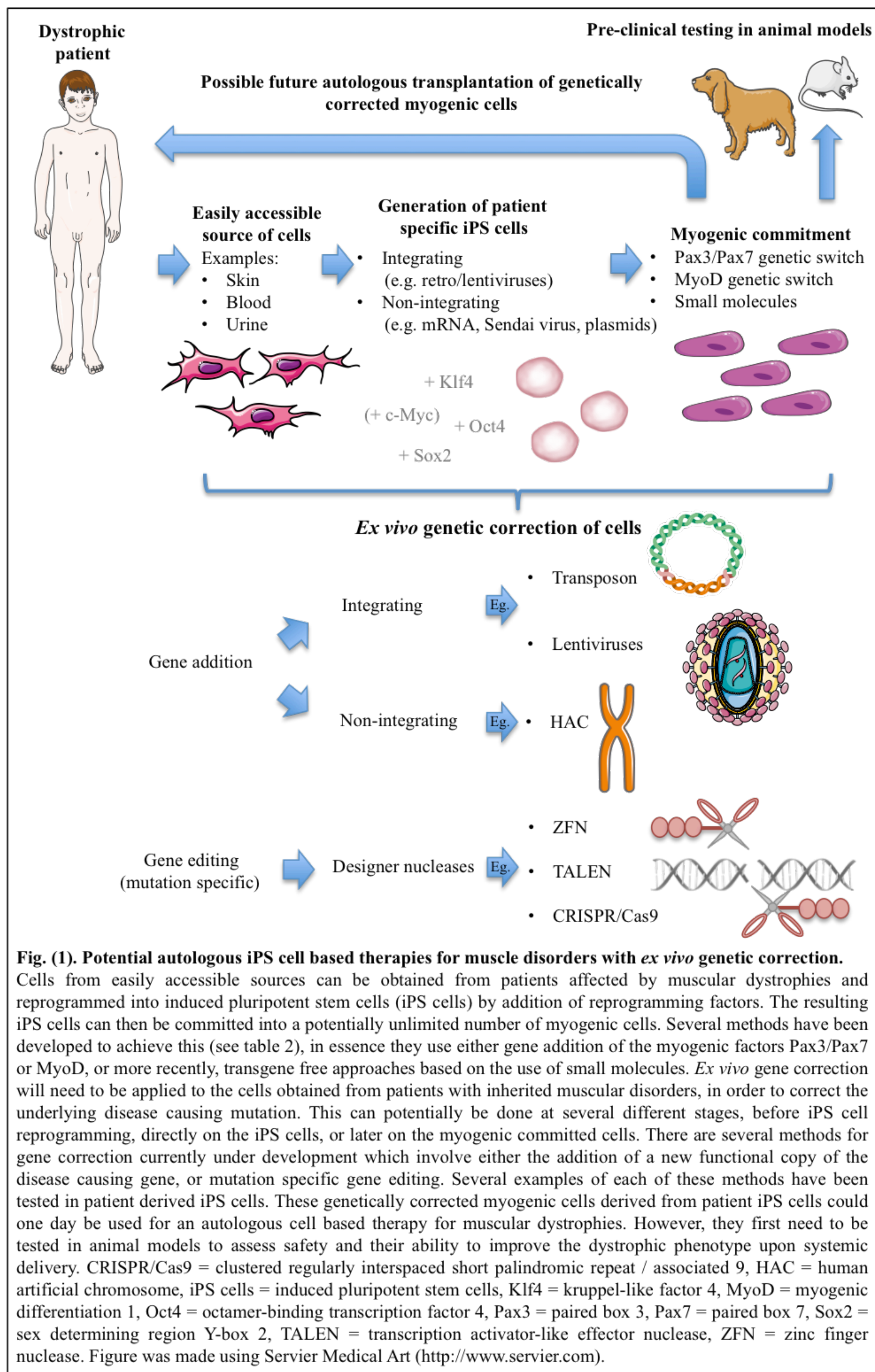

Fig. (1). Potential autologous iPS cell based therapies for muscle disorders with *ex vivo* genetic correction. Cells from easily accessible sources can be obtained from patients affected by muscular dystrophies and reprogrammed into induced pluripotent stem cells (iPS cells) by addition of reprogramming factors. The resulting iPS cells can then be committed into a potentially unlimited number of myogenic cells. Several methods have been developed to achieve this (see table 2), in essence they use either gene addition of the myogenic factors Pax3/Pax7 or MyoD, or more recently, transgene free approaches based on the use of small molecules. *Ex vivo* gene correction will need to be applied to the cells obtained from patients with inherited muscular disorders, in order to correct the underlying disease causing mutation. This can potentially be done at several different stages, before iPS cell reprogramming, directly on the iPS cells, or later on the myogenic committed cells. There are several methods for gene correction currently under development which involve either the addition of a new functional copy of the disease causing gene, or mutation specific gene editing. Several examples of each of these methods have been tested in patient derived iPS cells. These genetically corrected myogenic cells derived from patient iPS cells could one day be used for an autologous cell based therapy for muscular dystrophies. However, they first need to be tested in animal models to assess safety and their ability to improve the dystrophic phenotype upon systemic delivery. CRISPR/Cas9 = clustered regularly interspaced short palindromic repeat / associated 9, HAC = human artificial chromosome, iPS cells = induced pluripotent stem cells, Klf4 = kruppel-like factor 4, MyoD = myogenic differentiation 1, Oct4 = octamer-binding transcription factor 4, Pax3 = paired box 3, Pax7 = paired box 7, Sox2 = sex determining region Y-box 2, TALEN = transcription activator-like effector nuclease, ZFN = zinc finger nuclease. Figure was made using Servier Medical Art (http://www.servier.com).