



Induced pluripotent stem cells and genome editing technology as therapeutic strategies for Duchenne muscular dystrophy

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ABSTRACT

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Duchenne muscular dystrophy (DMD) is a rare genetic, progressive and devastating skeletal and cardiac muscle disorder due to mutation of the dystrophin gene that affects 1 in 3500 young males. Currently, there is no curative management for this pathology. The development of induced pluripotent stem cells (iPSCs) offers a promising cell-based strategy for the treatment of muscular dystrophy. Several techniques have been established to generate functional myogenic progenitor cells derived from iPSCs. In addition, technologies in genetic modification using ZFN, TALENs, or CRISPR/Cas9 demonstrate potent methods to restore dystrophin expression. However, current evidence shows that either iPSCs or gene editing carry a risk of oncogenesis caused by the integration of exogenous DNA into the recipient gene. Thus, the safety issue is a major challenge for translating this method into human clinical applications. This review briefly discussed recent developments and progressions of iPSCs as well as genome engineering technologies relevant to regenerative medicine, especially for the treatment of DMD.

ABSTRAK

Duchenne muscular dystrophy (DMD) adalah kelainan genetic langka yang bersifat progresif dan parah yang menyerang otot rangka dan otot jantung, disebabkan oleh mutasi gen distrofin yang mempengaruhi 1 dari 3500 laki-laki muda. Hingga saat ini, belum ada tata laksana kuratif untuk penyakit DMD. Pengembangan induced-pluripotent stem cells (iPSCs) menawarkan strategi berbasis sel yang menjanjikan untuk pengobatan distrofi otot. Beberapa teknik telah dikembangkan untuk menghasilkan sel-sel progenitor myogenik fungsional yang berasal dari iPSCs. Selain itu, teknologi dalam modifikasi genetic menggunakan ZFN, TALENs, atau CRISPR/Cas9 menunjukkan metode yang potensial dalam mengembalikan ekspresi distrofin. Namun, bukti saat ini menunjukkan bahwa iPSCs atau teknologi pengeditan gen membawa risiko keganasan yang disebabkan oleh integrasi DNA eksogen ke dalam gen inang. Dengan demikian, masalah keamanan adalah tantangan utama dalam penerapan metode ini untuk pengobatan pada manusia. Tulisan ini akan membahas secara singkat perkembangan dan kemajuan teknologi iPSCs terbaru serta teknologi rekayasa genom yang relevan dengan kedokteran regeneratif, terutama untuk pengobatan DMD.

Keywords:

induced pluripotency;
stem cells;
Duchenne muscular
dystrophy;
gene editing;
cell-based therapy;

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, pervasive and the most common inherited muscular disorder that affects young males. The prevalence

of this muscle pathology ranged from 0.9 to 16.8 per 100,000 males, while the prevalence at the time of birth was 1.5 to 28.2 per 100,000 live boys.¹ It is caused by mutation of the X-linked dystrophin gene that is located at locus

Xp21.2. The milder forms of skeletal and cardiomyocyte abnormalities due to dystrophin mutations are named Becker muscular dystrophy (BMD) and X-linked dilated cardiomyopathy (XLDCM), respectively. Dystrophin is a bar-shaped and intracellular protein that is located on the inner side of sarcolemma. Together with transmembrane, cytoskeletal protein and extracellular components, dystrophin forms the dystrophin-glycoprotein complex (DCG). The essential role of DCG is to create a firm linkage between intracellular components and extracellular matrix. The absence of dystrophin leads to sarcolemma breakdown, calcium homeostasis disturbance and progressive muscle fiber destruction.²

Although proximal muscle wasting starts to appear by the age of 3, diagnosis is made mostly when patients are approximately 5 years of age because their locomotion ability is remarkably different from that of other children. Patients show a positive Gowers sign upon standing up and experience difficulty maintaining their normal gait. The other signs are calf muscle hypertrophy and abnormal laboratory findings (elevation of creatine kinase). Naturally, DMD progresses to respiratory muscle insufficiency and cardiac failure. The patients frequently become ambulation-dependent around their 13 years old, and if left untreated, the mean age at death is around the early twenties.³

Although the molecular pathologies of DMD have been well known for decades, unfortunately, no effective medications are currently available. The primary objective of the management is to restore muscle function. In general, there are 3 main approaches for achieving this goal: gene therapy, pharmacotherapy, and cell-based therapy. Gene therapy emphasizes correction of the defective dystrophin gene using exon skipping, gene transfer, RNA interference, and

gene-editing technologies, such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems 9 (Cas9) system. Pharmacology therapy focuses on drug development for treating complications and health conditions related to DMD. Meanwhile, cell-based therapy concerns the replacement of damaged tissues by providing new healthy cell colonies utilizing satellite cells, muscle-derived stem cells, muscle-derived CD133+ cells, embryonic stem cells and induced-pluripotent stem cells.⁴ The anti-inflammatory effect of corticosteroids has been proven to enhance skeletal and cardiac muscle function, slow the occurrence of scoliosis and respiratory abnormalities, and increase the quality of life in patients for more than 2 years.^{5,6} Prednisone and deflazacort are the primary corticosteroids used for DMD therapy. Griggs *et al.*⁷ compared the effectiveness of deflazacort, prednisone, and placebo in improving skeletal muscle strength. They found that deflazacort and prednisone had significantly increased muscle strength compared to placebo. However, several adverse effects related to long-term steroid administration occur, including Cushingoid appearance, skin rash, hirsutism, and increased body weight. The latter was more prominent in the prednisone group.⁷ To overcome the frequent side effects of daily corticosteroid usage, Quattrocchi *et al.*⁸ performed weekly pulse doses of corticosteroids in an animal model with similar efficacy and lower side effects.

Recently, only a few novel therapies have been conditionally approved for DMD patients. The most promising agents are readthrough therapy (ataluren) and exon skipping (eteplirsen). So-called readthrough therapy because this agent is able to restore the expression of dystrophin protein by inhibiting the

termination of protein translation. Oral administration of a total dose of 40 mg/kg bodyweight ataluren daily for 48 weeks improved the 6-minute walk test (6MWT) distance compared to placebo in boys with nonsense mutation DMD. It also has a good safety profile with only mild to moderate adverse events.^{9,10} Eteplirsen, an exon skipping agent, precisely recognized exon 51 and altered the function of splicing enzyme to skip exon 51 of the DMD pre-mRNA, resulting in restoration of mature mRNA and production of shortened yet functional dystrophin protein.¹¹ A randomized controlled study involving boys aged 7 to 13 years old with DMD suggested that eteplirsen was effective in improving dystrophin production as well as increasing the 6MWT in ambulation patients compared to placebo.¹²

This review briefly discussed recent developments and progressions of induced-pluripotent stem cells (iPSCs). Moreover, the genome engineering technologies relevant to regenerative medicine, especially for the treatment of DMD was also discussed.

DISCUSSION

Techniques for Inducing Somatic Cells into Pluripotent Stem Cells

To be characterized as pluripotent lineage, stem cells have to meet a number of criteria, which are able to produce cell types from each of three embryonic germ layers: ectoderm, mesoderm, and endoderm; generate teratoma or chimeric offspring; maintain normal karyotype after serial passaging; and they must preserve their population quantity by self-renewal. Historically, stem cells were first described more than a half century ago from mouse bone marrow cells. Under a specific environment, these cells are able to differentiate into numerous types of mature cells, named pluripotent stem

cells. In 1981, Evans and Kaufman for the first time succeeded in isolating ES cells, which were the inner cell mass (ICM) taken from delayed-implantation blastocysts in mice. Subsequently, these cells developed teratoma when engrafted to the flank region of syngeneic male mice.^{13,14} Later, in 1998, Thomson *et al.*¹⁵ differentiated human blastocyst cells *in vitro* into all three embryonic cell lineages for the first time called embryonic stem cells (ESCs). In addition, following injection into immunodeficient mice, human blastocysts produced teratomas, which verified their pluripotency.¹⁵

There was a major medical science breakthrough in 2006, where scientists succeeded in reprogramming mouse fibroblasts into pluripotent cells using 4 transcription factors: Oct3/4; Sox2; Klf4; and c-Myc, with retrovirus as a vector. These cells are termed induced-pluripotent stem cells (iPSCs).¹⁶ In the next year, two groups of scientists generated human iPSCs from human dermal fibroblasts (HDFs) by using different transcription cocktails. Human iPSCs are indistinguishable from human ESCs in morphology, surface antigens, protein expression, and telomerase activities. This proof of pluripotency was strengthened by transplantation of human iPSCs to immunodeficient mice, resulting in the formation of teratomas consisting of three elementary germ layers: endoderm, mesoderm, and ectoderm.^{17,18}

Narita *et al.*¹⁷ generated iPSCs by exposing retroviruses containing human Oct3/4, Sox2, Klf4, and c-Myc, which were the same reprogramming cocktails used in generating mouse iPSCs, into HDFs under the conditions of human ESC culture.¹⁷ Other variations of the reprogramming cocktail (Oct4, Sox2, Nanog, and Lin28) were adequate to generate pluripotent stem cells from human fibroblasts taken from foetal as well as foreskin of the newborn.¹⁹

Oct3/4 and SOX2 are fundamental

transcription factors for either maintaining pluripotency or inhibiting differentiation. These factors are controlled by Nanog, which is a homeodomain protein essential for preserving ES cell pluripotency. Other crucial molecular effectors, Foxd3 and Setdb1, required for ES cell survival are also controlled by Nanog.²⁰ c-Myc is a proto-oncogene found in human cancer cells. Deletion of c-Myc in mouse embryos resulted in death between 9.5 and 10.5 gestation days with pathological conditions found in the heart, pericardium and neural tube.²¹ In contrast to mouse ES cells, the role of c-Myc in human ES cells provokes apoptosis as well as differentiation. However, by reducing the adhesion of cells to the extracellular matrix, c-Myc does not contribute to self-renewal. Therefore, c-Myc functions are influenced primarily by the cell lineages

and niches of stem cells. Kruppel-like factor 4 (Klf4) is a zinc finger protein containing a sequence of amino acids. During the quiescent phase of the cell, Klf4 levels are highly detectable; in contrast, Klf4 almost disappears during the proliferation period.²² Overexpression of Klf4 in cell culture provokes DNA synthesis inhibition and cell cycle progression.²³ Newborn mice from Klf4 null embryos die in 15 hours with abnormalities found in the skin and colon. This finding suggests the role of Klf4 as a tumor suppressor gene.^{24,25} On the other hand, overexpressed Klf4 is found in both breast cancers and squamous cell carcinomas.^{26,27} It also initiates dysplasia of squamous epithelial cells in basal keratinocytes.²⁸ Consequently, Klf4 can act as both a tumor suppressor gene and oncogene.²⁹

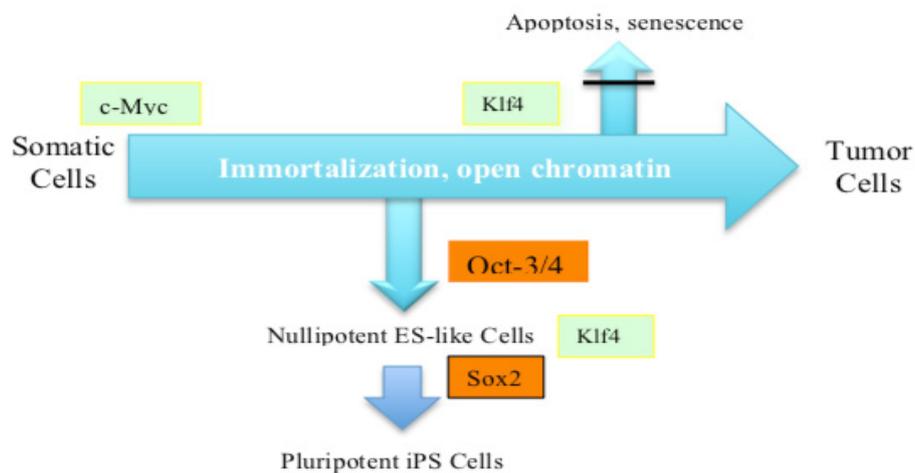


FIGURE 1. Roles of the four transcription factors in the induction of iPSCs. adopted and modified from Yamanaka,²⁸

The first pioneering iPSC trial used retroviral and lentiviral-based methods for transfecting somatic cells. However, utilization of these vectors is related to safety issues because the integration of their viral genes increases the likelihood of activating endogenous oncogenes that can be transformed into malignant conditions. Consequently, recent non-integrating viral vectors have been proposed, for example, adenovirus and Sendai virus. Adenoviral vectors that express c-Myc, Klf4, Oct4, and Sox2 have been successfully used to create human iPSCs from embryonic fibroblasts. Viral DNA was not integrated into iPSCs, as proven by Southern blots and polymerase chain reaction (PCR) analysis. However, the overall efficiency using this technique

was quite low, ranging from less than 0.0001 to 0.001% compared with 0.01 to 0.1% by integrating virus. This is presumably due to viral expression that cannot be maintained long enough to stimulate endogenous pluripotency factors.³⁰ Because the efficiency of the adenoviral vector was extremely low, Sendai virus was introduced for transfecting the target cells. Sendai virus, a family of *Paramyxoviridae*, is a nonpathogenic, single-stranded RNA virus with envelopes that can infect cells by replicating only in the cytoplasm. Their gene will not be inserted into the host genome. Generating iPSCs from human fibroblasts and peripheral blood mononuclear cells through this vector shows an efficiency of 0,1%.³¹

TABLE 1. Overview of current reprogramming methods

Vector	Systems	Integrating Systems	Non-Integrating Systems
Viral Vectors		Retroviruses ^{16,17}	Adenoviruses ³⁰
		Lentiviruses ¹⁸	Sendai virus ³¹
Non-Viral Vectors			Episomal plasmids ³⁴
		PiggyBac transposons ^{32,33}	mRNA ³⁵
			miRNA ³⁶
			Small molecules ³⁷

Apart from viral transfection methods, there are various nonviral approaches, including episomal plasmids, modified messenger RNAs (mRNAs), microRNAs (miRNAs), small molecules and piggyBac transposons. Most of those techniques are non-integrating systems, except for piggyBac transposon technology.³²

Administration of two different plasmids into mouse embryonic fibroblasts, the first contains complementary DNA of Oct3/4, Sox2, and Klf4, while the second plasmid contains complementary DNA of c-Myc created iPSCs without evidence of plasmid integration into host cells. Although this method reduces the risk

of tumorigenicity theoretically, the efficiency is remarkably lower than that of viral integrating methods.³⁴ Convenient and nonmutagenic modified mRNA exhibited an efficiency of 4.4%, which was the highest achievement when 5 reprogramming factors, including Klf4, Sox2, Oct4, c-Myc, and Lin28, were transfected into human fibroblasts combined with valproic acid (VPA)-supplemented media and low oxygen culture conditions.³⁵ miRNA is an endogen, small non-coding RNA that has approximately 22 nucleotides. Because miRNA controls gene expression post-transcription, it can be involved in embryo maturation, stem cell differentiation and apoptosis.³⁶

Moreover, miRNA has the capacity to reprogram a somatic cell with a high level of efficiency. Inducing human foreskin and dermal fibroblasts with miR302/367 resulted in an efficiency up to two orders of magnitude higher than that of standard transcription cocktails. Approximately 10% of iPSC clones were produced by this approach.³⁷

The combination of small molecules may address particular signaling pathways and mechanisms leading to

pluripotent stem cell induction. However, *in vitro* reprogramming efficiency is affected by the type of somatic cells, the condition of culture and the combination of compounds used.³⁸ The PiggyBac transposon method provides high efficiency to reprogram human somatic cells into iPSCs. However, microdeletions have been shown at the excision sites of mouse ESCs that could be an oncogenic transformation.³³

TABLE 2. Summary of reprogramming methods.

Methods*	Advantages	Disadvantages
Retrovirus ^{16,17}	<ul style="list-style-type: none"> • High efficiency • Simple protocols 	<ul style="list-style-type: none"> • Potential to activate an endogenous oncogene • Risk of carcinogenesis
Lentivirus ¹⁸	<ul style="list-style-type: none"> • High efficiency • Simple protocols 	<ul style="list-style-type: none"> • Potential to activate an endogenous oncogene • Risk of carcinogenesis
Adenovirus ³⁰	<ul style="list-style-type: none"> • Non-integrating genome 	viral <ul style="list-style-type: none"> • Extremely low efficiency
Sendai virus ³¹	<ul style="list-style-type: none"> • Non-integrating genome • High efficiency 	viral <ul style="list-style-type: none"> • Complex protocols • High cost
Plasmids ³⁴	<ul style="list-style-type: none"> • Non-integrating DNA 	plasmid <ul style="list-style-type: none"> • Low efficiency
mRNA ³⁵	<ul style="list-style-type: none"> • Higher efficiency than standard viral approaches 	<ul style="list-style-type: none"> • Complex protocols
miRNA ³⁶	<ul style="list-style-type: none"> • High efficiency 	<ul style="list-style-type: none"> • Unstable • Risk of off-target effect
Small molecules ³⁷	<ul style="list-style-type: none"> • High efficiency 	<ul style="list-style-type: none"> • Lack of validated protocols • Limited used in clinical trials
PiggyBac transposon ³³	<ul style="list-style-type: none"> • High efficiency 	<ul style="list-style-type: none"> • Potential causing epigenetic alteration

*Each method has its own benefit and drawback in terms of safety, efficiency, and procedural complexity

Generation of Myogenic Progenitor Cells from Induced Pluripotent Stem Cells

Myogenesis development is initiated during the early embryonic period. Presomitic mesodermal cells are differentiated from pluripotent stem cells by expressing myogenin 1, which works as a key regulator of unsegmental presomitic mesodermal formation.

Segmental somites are formed afterward. Expression of the Pax3 and Pax7 genes in the presomitic mesoderm leads to myogenic progenitor cell generation. Pax3 and pax7 are essential regulatory factors for myogenic progenitor cell formation. Then, upregulation of MyoD and Myf5 in the dorsomedial lip of dermomyotome together with Mrf4 function as a master regulator for differentiation of myogenic progenitor

cells into myoblasts. Eventually, the expression of myogenin triggers the formation of a terminal differentiated

type of cell, which are multinucleated myotubes.³⁹

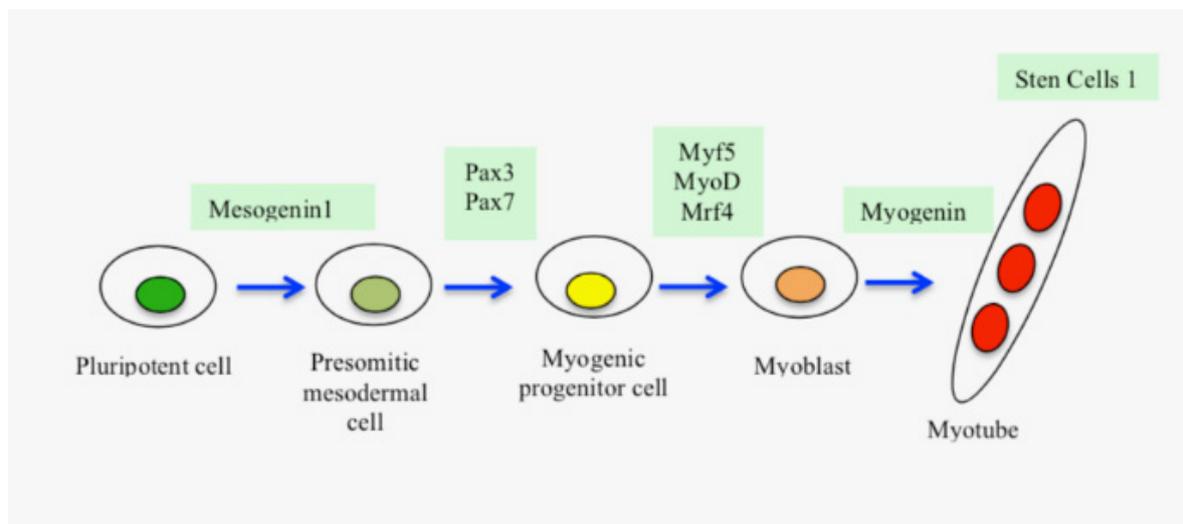


FIGURE 2. Hierarchical master transcription factor cascade for myogenesis. Adopted and modified from Kodaka *et al.*³⁹

Both transgene or transgene-free methods are applicable for generating myogenic progenitor cells from iPSCs. Transgene-free or direct reprogramming uses myogenic reprogramming, for example, Pax3, Pax7 and MyoD. On the other hand, the transgene-free method involves small compounds or cytokines to either impede or stimulate the signaling pathway, particularly for inducing skeletal muscle formation. Despite the ability of a small molecule to generate multinucleated myotubes, it is inefficient, comparatively weak and does not provide a genuine myogenic clone. Therefore, its application in extensive-scale drug screening and cell-based therapy trials is undecided. In contrast, direct reprogramming protocols are quick and efficient, resulting in more than 90% pure myogenic clones.²⁹

Rao *et al.*⁴⁰ transduced iPSCs with lentivirus encoding doxycycline that expresses Pax7 and green fluorescent protein (GFP) in E6 media. More than 40% pure GFP- and Pax7-expressing cells, termed induced myogenic progenitor cells (iMPCs), are yielded by fluorescence-

activated cell sorting by day 20. However, after 3 passages in E6 media, the number of iMPCs was extremely decreased. These iMPCs exhibited copious expression of early myogenic markers, Pax3 (more than 70%) and Myf5 (approximately 80%). In contrast, only a small percentage of later myogenic markers MyoD and myogenin were shown, approximately 4% and 2%, respectively. After culturing for 4 days in differentiation medium, the expression of MyoD and MyoG increased with a subsequent decrease in GFP. iMPCs formed spindle-shaped and obtained elongated nuclei, typical morphology of myocytes. Eventually, following two weeks of differentiation, multinucleated myotubes that contract spontaneously were already available. Overall, up to 200 million iMPCs were obtained by this protocol beginning with only 300 thousand iPSCs.⁴⁰ Both human iPSCs and human ESC-derived myogenic progenitors substantively demonstrated similar expression of surface markers, including CD56, CD29, CD44, M-cadherin, and alpha 7-integrin. Although murine satellite cells and myogenic progenitors

express these surface markers, thus far, only CD56 has been recognized as a human satellite cell marker.⁴¹

One of the transgene-free protocols was established by Choi *et al.*⁴² They employed two combinations of a small compound, CHIR99021 and DAPT. The role of CHIR99021 is to activate the canonical Wnt signaling pathway. Together with the PI3K inhibition pathway, activation of the Wnt signal is adequate for generating myogenic progenitors from iPSCs. Exposure of human iPSCs with CHIR99021 in defined N2 media for 4 days potentially increased the expression of Mesogenin1, a specific marker for the pre-somite mesoderm line, TBX6, and Pax3. Next, muscle progenitor cells that express MyHC, MyoG and MyoD appeared after 40 days of differentiation. Supplementation of the culture between days 4 and 12 with DAPT, an inhibitor of gamma-secretase that blocks Notch signaling, increased the efficiency and effectiveness of myogenic differentiation. In addition, using exactly the same protocol, they could generate human iPSCs derived from DMD patients into myoblasts without evidence of dystrophin gene expression. In contrast, control myoblasts derived from healthy humans displayed dystrophin protein.⁴²

Gene-editing Technology for Correction of Genetic Defect

Approximately 60% of DMD patients have mutations in exons 45-55 that interrupt the translational reading frame of the gene, which aborts the translational process, resulting in dystrophin deficiency. Meanwhile, mutations with preservation of the reading frame create a milder form of skeletal muscle abnormality, named Becker muscular dystrophy (BMD). Hence, deletion of this region by genetic mutations provides a therapeutic approach for DMD patients affected by

exon 45-55 mutations.⁴³

Either a small part or entire gene could be excised from or transferred into a particular chromosome, yielding different gene expression. This approach is referred to as the gene editing or gene correcting technique. In the early 2000s, zinc finger nucleases (ZFNs) were widely applied for genetic modification. A ZFN is a synthetic endonuclease formed by designed zinc finger protein (ZFP) and restriction enzyme FokI. It works by either removing or inserting a specific gene, resulting in gene knockout.⁴⁴ Genetic modification of cells from DMD patients using ZFN technology precisely restored human dystrophin gene expression at the sarcolemma membrane. However, compared to standard nontoxic nuclease (I-SceI), this method had a moderate level of cytotoxicity.⁴⁵

Another engineered nuclease, transcription activator-like effector nucleases (TALENs), is employed for editing genome damage at the desired site. A designed pair of TALENs binds and clamps certain target sites, permitting dimerization of FokI and breaking the double-stranded DNA.⁴⁶ Exposure of TALENs into immortalized myoblasts derived from DMD patients led to the restoration of dystrophin gene expression. In addition, TALENs indicated a low grade of cytotoxicity similar to I-SceI.⁴⁷

Alternative to site-specific nucleases, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems 9 (Cas9) have become an emerging and popular technology that considerably has a facile and efficient method.⁴⁸ (CRISPR/Cas9 was extracted from the bacterial adaptive immune defense to destroy viral and plasmid invasion. The greater efficiency, lower toxicity combined with easy targeting of specific genome sites compared to ZFN and TALENs make CRISPR/Cas9 a promising method for clinical

application.⁴⁹

A number of trials have been conducted by applying CRISPR/Cas9 myo-editing in animal models. Nelson et al. delivered CRISPR/Cas9 using adeno-associated virus to excise the mutated exon 23 from a DMD mouse model. Functional dystrophin protein in skeletal and heart muscle was partially recovered following the deletion of exon 23, as shown by improvement of muscle strength.⁵⁰ Amoasii *et al.*⁵¹ injected adeno-associated virus (AAV) vectors carrying the CRISPR component either intramuscularly or intravenously into DMD dog models. After 6 to 8 weeks, they found significant improvement in muscle, particularly cardiomyocytes.⁵¹ Recently, a similar method was also performed in DMD pigs, resulting in the expression of dystrophin in the diaphragm, heart and skeletal muscle. Moreover, increasing muscle contraction force and twitch amplitude enhanced skeletal muscle function.⁵²

Young *et al.*⁵³ performed *in vitro* deletion of exons 45-55 Cas9 from DMD-derived human iPSCs using a single pair of CRISPR/Cas9. The reframed iPSCs were then differentiated into both functional skeletal myotubes and cardiomyocytes, as demonstrated by improvement of cell membrane integrity and recovery of the dystrophin glycoprotein complex.⁵³ Moretti *et al.*⁵² generated iPSCs derived from DMD patients. Compared to iPSCs from healthy young men, DMD-derived iPSCs expressed notably lower concentrations of skeletal muscle markers and failed to produce functional multinucleated myotubes. Subsequently, utilization of AAV-mediated CRISPR/Cas9 was able to excise exon 51, thus stimulating the development of normal skeletal and cardiac muscle cells.⁵² However, the restoration of functional skeletal and cardiac muscle is directly proportional to the dosage of AAV-mediated CRISPR/Cas9 administered. Comparison of systemic delivery of

high-dose (1×10^{12} vg/mouse) and low-dose (3×10^{11} vg/mouse) AAV-CRISPR/Cas9 in 3-day-old mdx mice showed better formation of dystrophin-positive cardiac muscle as well as contractility of the heart in the high-dose therapy group.⁵⁴ A similar result was also demonstrated in a canine model in which a higher dose of virus particles significantly ameliorated the expression of dystrophin protein compared to a lower dose.⁵¹

Cell-based Therapy Approach for Duchenne Muscular Dystrophy

The significant evolution of human iPSCs opens a new era in life science, including disease modelling, drug discovery and regenerative medicine. Compared to traditional cellular screening, the human iPSC method has enormous advantages, including human origin, reproducibility, infinitely expandability, plasticity to generate almost any cell lines and avoidance of an ethical issue related to human ES cells. Furthermore, personalized medicine that aims to treat medical conditions precisely for each individual has the potential to be developed by using human iPSC technology.⁵⁵

Animals such as mice have offered a meaningful tool for the study of human disease, allowing the recognition of pathological conditions in a certain type of cell for every stage of development. However, due to fundamental species differences between humans and mice, it is impossible to learn the entire spectrum of human disease pathology. Although patient-derived cells are important to learn about human disease, they are difficult to access, for example, brain cells and heart cells. Thus, human iPSCs provide an alternative method because they have the competency to differentiate into almost every cell type. They can also be obtained from easily accessible cells such as skin fibroblasts and blood cells.⁵⁶

Cell-based therapy involves engrafting of myogenic progenitor or skeletal muscle cells competent to produce functional dystrophin-expressing cells into DMD patients, either intramuscularly or systemically. Ideally, the cells should be able to reach not only skeletal muscle but also the heart and diaphragm, thus, systemic

administration is preferred over intramuscular administration. These cells can be obtained from a healthy donor without genetic modification (allograft) or patients' own cells genetically corrected (autologous). Although autograft cells are mutation-free, they increase the risk of rejection from the donor's immune system.^{57,58}

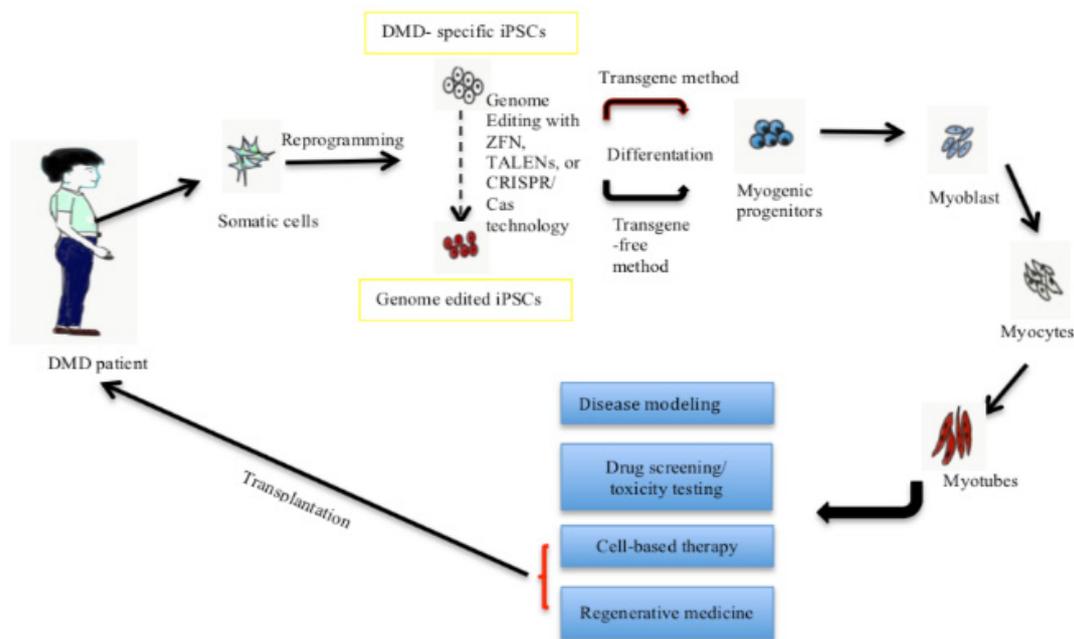


FIGURE 3. Scheme of the iPSC-based therapy potential for Duchenne muscular dystrophy. Adopted and modified from Danisovic *et al.*²⁹

In 2013, scientists successfully transplanted autologous engineered iPSCs to mdx dystrophic mice for the first time. They used tail-tip fibroblasts (TTFs) from dystrophin/utrophin-deficient mice to generate iPSCs using reprogramming cocktails (Oct4, Klf4, and Sox2) by retroviral transduction. The micro-utrophin (UTRN) gene was inserted using the Sleeping Beauty transposon to correct dystrophin lack iPSCs followed by induction of Pax3, which generated myogenic progenitor cells (MPCs). These cells were engrafted to the tibialis anterior (TA) muscle of dystrophic mice, resulting in muscle regeneration and improved contractility

as well as satellite cell compartment seeding.⁵⁹ Kyrychenko *et al.*⁵⁸ generated DMD model iPSCs by removing exons 8 and 9 using the CRISPR/Cas9 system. To correct these DMD-iPSCs, they performed three different methods with CRISPR/Cas9 by deleting exons 3-9, 6-9, or 7-11, resulting in restoration of the dystrophin reading frame, which subsequently became functional iPSC-derived cardiomyocytes.⁵⁸

Another study demonstrated amelioration in mdx mice treated with myogenic progenitor cells from iPSCs of normal mice. It was shown by a decrease in both fibrotic tissue and central nuclei, which are notable characteristics in

muscular dystrophy. Furthermore, they also reported improvement of dystrophin distribution, acetylcholine receptor expression, and Pax7 expression. The latter is a fundamental factor for muscle progenitor biogenesis.⁵⁹

Induced-pluripotent stem cells can be applied for treating skeletal muscle abnormalities as well as cardiomyopathy. However, because of its medical, technical, and safety issues, until now, no human clinical trial using iPSCs has been conducted to treat DMD.⁴ To date, only a few clinical trials in terms of cell-based therapy for human DMD cases have been conducted. Mendell *et al.*⁶⁰ failed to improve skeletal muscle strength in DMD patients after several injections of myoblasts into the biceps brachii muscle donated from the patient's father or brother. Transplantation of autologous muscle-derived CD133 stem cells to DMD patients was a safe and feasible procedure. Muscle-derived CD133 cells display various myogenic markers, including Myf5, Mrf4, and MyHCs. Four of the five patients showed an increasing number of capillaries in each myofiber, and two of them showed a conversion of slow myosin myofibers into the fast type.⁶¹ However, to increase the therapeutic effectiveness of cell-based therapy in DMD, the systemic route for distributing engineered cells to diseased muscle has to be established.

For the first time, in 2015, Cossu *et al.*⁶² performed intra-arterial transplantation of HLA-matched mesangioblasts in 5 DMD patients under an immunosuppressive regimen (tacrolimus). Mesangioblasts were obtained from a muscle biopsy of the patients' brothers. Mesangioblasts are a subsidiary of pericytes from human skeletal muscles. They can be expanded and differentiated into either skeletal or smooth muscles. Interestingly, mesangioblasts have the ability to cross the endothelial barrier when delivered intra-arterially and can also be distributed into muscle

compartments, as demonstrated in animal model experiments. However, measurement of muscle function did not show notable improvement following a series of transplantations. Moreover, unfortunately, one of the patients experienced a severe adverse event, which was an ischemic thalamic stroke.⁶²

Thus, further investigations are required to improve the understanding of the molecular mechanism controlling the characteristics of stem cells in human skeletal muscle tissue to achieve feasible, reproducible and the most important, harmless cell-based therapy in DMD patients.

CONCLUSION

The rapid advancement of induced pluripotent stem cell technology provides the generation of an unlimited number of skeletal muscle cells from other types of cells, such as fibroblasts or white blood cells. This technique is a cell-based therapy approach for the treatment of various incurable diseases, including DMD. Because of mutations in a particular gene, the expression of dystrophin protein is absent in DMD patients, resulting in significant skeletal and cardiac muscle dysfunction. *Ex vivo* modification of the genome using design endonucleases and CRISPR/Cas9 has successfully restored the dystrophin gene from human iPSC-derived skeletal muscle cells. Although animal models exhibit improvement of muscle function following stem cell transplantation, there are several obstacles to applying this method in DMD patients. For example, gene mutation as a result of vector integration could lead to tumor formation. The other issues are related to immune rejection and adverse effects arising from stem transplantation. Therefore, further study should be able to address these issues by adopting this technology into the human clinical territory.

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