Gene therapy for Duchenne Muscular Dystrophy: CRISPR-Cas9 mediated gene editing in DMD patient-derived iPSC

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Abstract

Duchenne Muscular Dystrophy (DMD) is a severe muscle degeneration disease caused by gene mutations in the dystrophin gene. The CRISPR-Cas9 system is an emerging potential gene editing tool that utilizes the Cas9 nuclease and DNA double strand breaks (DSB) to make site-specific gene corrections. Induced human pluripotent stem cells (iPSC) are derived from the human body and reprogrammed to become potential stem cells that can differentiate into various human cell lineages. Using CRISPR-Cas9 in iPSCs to correct certain exon mutations in the dystrophin gene provides great advantages in both efficacy and low immunogenicity. This article reviews four main CRISPR-mediated exon correction methods that correct various mutations occurring across the dystrophin gene. In addition, this review discusses current developments and studies utilizing CRISPR-Cas9 to perform gene editing in iPSC derived from DMD patients and evaluates the feasibility of such an integrated gene therapy. To the end, this review compares the advantages and limitations of this gene therapy to other traditional methods and provides future prospective regarding further implementation.

Keywords: Duchenne muscular dystrophy, CRISPR-Cas9, induced pluripotent stem cells, dystrophin, knock-in, knock-out, exon reframing, exon skipping

1 Introduction

1.1 Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy (DMD) is a progressive muscle degenerative disease caused by a variety of genetic mutations in the dystrophin gene. DMD patients tend to present with short stature, enlarged calf muscles, curvature of

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the spine, and a waddling abnormal gait. Patients also have difficulties in performing simple movements such as walking, sitting down, running, and jumping beginning at a young age. [X19] During adolescence, patients gradually lose ambulance and are subjected to wheelchairs for further support. As the disease progresses and the patient ages, fibrotic and adipose tissue forms and substitutes the functional muscle fibers, making the muscle susceptible to inflammation, contraction injuries, and necrosis. [KE15] Eventually, severe muscle weakness and wasting lead to cardiac and respiratory failure and even death. 1 out of 3500 male live births has muscle degenerative diseases while 1 out of 5000 male live births is affected by DMD. [ea14]

DMD is a X-linked recessive monogenetic disorder that primarily affects males. The dystrophin gene accounting for DMD is located on Xq21. [ea14] Each male has a 100 percent chance of inheriting the mutation from a DMD mother and is certain to exhibit DMD. Each female has a 100 percent chance of being a carrier of the mutant gene from a DMD mother.

The gene that results in DMD, the dystrophin gene, is one of the largest known human genes at about 2.4Mb and consists of 79 exons. [ea14] A wide variety of mutations occur across the dystrophin gene. Mutations split into two parts: Point Mutation which includes nonsense mutation and missense mutation, and Frameshift Mutations which comprise out-of-frame mutations and in-frame mutations. Nonsense mutations and missense mutations are both substitutions of nucleotides with rather different effects. The former results in a pre-mature stop for the synthesis of protein, while the latter generates wrong codon coding for a distinct amino acid. In-frame mutations are the deletions or insertions of codons, or three nucleic acids in a row, which allows the introduction of shorter or longer but still readable mutations in the dystrophin gene, often resulting in milder muscular dystrophies such as Becker Muscular Dystrophy (BMD). Out-of-frame mutations are disruptions of the whole reading frame. It usually creates a premature stop codon that leads to the production of non-functional dystrophin protein. All these mutations disrupt the gene's open reading frame and generate a premature stop codon. Studies have shown, deletion occurs frequently in the exon 45-54 and exon 3-22, and the most prevalently duplicated areas are at exon 3-11 and 21-37. [ea14] DMD breakpoints for exon deletions mainly occur in introns 43-55, and for exon duplications in introns 2 and 7. [ea14] Approximately 60 percent of mutations on the dystrophin gene occur in exon 45-55 as depicted in Figure 1, thus making this region an ideal spot for gene editing.

Dystrophin is a 427kDa protein providing strength, flexibility, and stability for muscle fibers as depicted in Figure 2. This large intracellular protein links the dystrophin-glycoprotein complex (DGC) residing on the sarcolemma of the myofiber and the actin filament to provide integrity and stability to the muscle cell. Dystrophin protein is constructed with three parts. The N terminus binds itself with muscle fiber actin filaments, whereas the C terminus connects with DGC. The central rod domain, as shown in Figure 3, functions as a string that bridges the two ends. [eaC16] The 24 repeats in the rod domain can in some cases be shortened to produce functional but less flexible dystrophin. With the

Figure 1: The 79 exons of the dystrophin gene. Mutational hot spots exons are colored in red.

absence of dystrophin, macrophages invade muscles while adipose replaces muscle fibers, allowing inflammation to occur. Additionally, the loss of dystrophin pushes excess calcium into the cell membrane, leading to excess water in the mitochondria, and thus bringing dystrophy, impaired cell signals, mitochondria dysfunction, and myocyte necrosis to the muscle. [ea14]

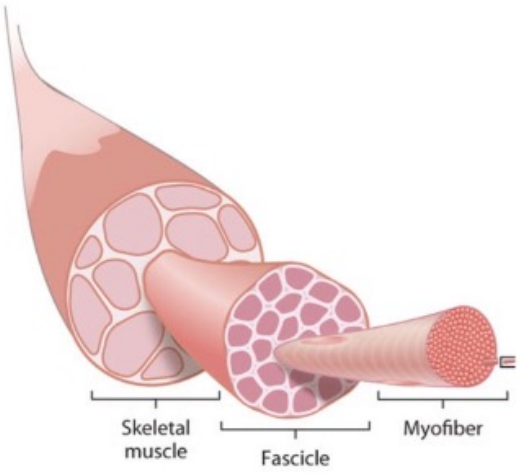

Figure 2: The muscle structure. The muscle is constituted of bundles of myofibers. [eaC16]

To briefly describe the whole process, various genetic mutations in the dystrophin gene are the basis of DMD. A single or multiple exon mutation will disrupt the open reading frame of the dystrophin gene, and as a result, affects functional dystrophin production. The alteration or loss of dystrophin further affects the integrity and stabilization of the muscle cell and enhances its susceptibility to contraction damage. In all cases, dystrophin mutations render muscle degeneration and weakness.

Current treatments for DMD are based on three main aspects: the rescue of dystrophin protein expression, the correction of the dystrophin gene, and the managing of symptoms to improve the quality of life. [Y19]

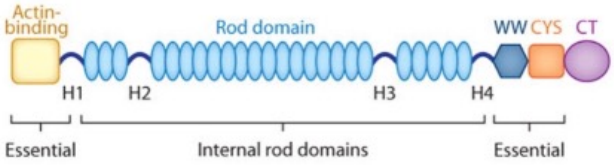

Figure 3: The dystrophin protein. The N terminus(left) binds itself with muscle fibers' actin filaments. The C terminus(right) is part of the DCG which resides on the sarcolemma of myofibers. The central rod domain(middle) functions as a string that bridges the two ends. [eaC16]

To produce functional dystrophin, approaches have used adeno-associated viral vectors (AAV) to deliver genes encoding a correct dystrophin protein. However, the use of AAV to deliver the 12kb dystrophin coding sequence is challenging due to the 4.7kb packaging limitation by the virion. Therefore, scientists have generated a mini dystrophin that encodes a shortened form of the protein with unimportant parts removed. [ZJ17]

Additional approaches have also been aimed to protect muscle. For example, approaches have been developed in driving muscle growth, speeding muscle repair, protecting muscle from damage, fighting inflammation in the muscle, blocking muscle fibroids, maximizing blood flow to the muscle, and defending the dystrophin-deficient heart.

Another approach is to use different site-specific genome correcting methods such as exon skipping, exon reframing, knock-in, and knock-out and gene editing tools such as CRISPR, TALENs, and zinc-finger nucleases to correct the dystrophin gene. Studies have already developed exon-skipping drugs utilizing the capacity of antisense oligonucleotides (AONs) which mask exonic splicing enhancers in pre-mRNA to restore biologically active dystrophin protein. [A18] The approach that employs both gene-editing tools and exon correction methods is increasingly accepted and used by scientific researchers. This review will mainly discuss and evaluate current studies and development on the use of the CRISPR-Cas9 system to carry out those exon correction methods in iPSCs derived from DMD patients.

1.2 CRISPR-Cas9 system

DNA double strand breaks (DSBs) are the basis for generating mutations in a specific genome sequence, thus the foundation for genome editing. The DNA repair mechanism Non-Homologous End Joining (NHEJ) is an imprecise correction that roughly links the DNA breaks. NHEJ accounts for exon deletion (gene knock-out) and reframing. Another DNA repair mechanism is Homology Directed Repair (HDR). When the desired template complementary to the target mutation site is presented, HDR replaces the DSB, or the mutation, with the desired template, or called the exogenous DNA sequence, through homologous

recombination. HDR takes place mainly in proliferating cells, unable to perform in many differentiated myocytes. HDR accounts mainly for exon insertion (gene knock-in) and exon reframing. By introducing DSBs to a specific site, genome editing can occur accurately and efficiently. [FM16]

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 system is a bacterial anti-viral immune system repurposed into a tool for genome editing. [FM16] The use of CRISPR-Cas9 has unprecedented potential for scientists to manipulate the genome of any animal on the earth.

The CRISPR-Cas9 system consists of three main parts as depicted in Figure 4: CRISPR-Associated Nuclease 9 (CAS9), CRISPR RNA (crRNA), and trans-activating RNA (tracrRNA). Among them, CAS9 is an RNA-guided endonuclease (RGN) that can introduce double strand breaks into a specific site. crRNA includes twenty nucleotides that are homologous to the target site DNA. tracrRNA, fused with the crRNA, provides a scaffold for combining the Cas 9 nucleases. The crRNA and tracrRNA are usually hybridized into one single guide RNA (sgRNA) shown in Figure 4, where the first twenty nucleotides are antisense to the target genome sequence and the other end is bound to the Cas9 nuclease. [FM16] The discovery of this single guide RNA(sgRNA) by Doudna and Charpentier was awarded the Nobel chemistry prize in 2020, marking the unprecedented power of CRISPR to make corrections in possibly any eukaryotic cells. When the gRNA's 5' end has identified a Protospacer Adjacent Motif (PAM) that is sequence NGG or NAG (N can be any nucleotide), it can recognize its complementary target genome sequences and introduce DSB with the Cas9 nuclease. Thus, the PAM sequence is critical to safeguard the accuracy of the Cas9 nuclease, allowing it to introduce DNA DSBs. [FM16]

Three main delivery methods are used to realize CRISPR gene editing. The first is a plasmid-based -CRISPR-Cas9 system which incorporates the Cas9 mRNA and sgRNA all together in one vector. The transcription of mRNAs all from one vector avoids multiple transfections and leads to greater stability. The second method delivers the mixture of Cas9 mRNA and sgRNA into targeted sites. The mRNAs are then translated into proteins in the body and packaged into the CRISPR-Cas9 system. This mixture method promises lower cytotoxicity, fewer off-target effects, and less time. The final strategy would be the Cas9 protein and sgRNA mixture delivery which is quick without the need to select promoters and optimize codons and also safe due to less toxicity and immune response. [K17] Viral delivery of CRISPR components is an unneglectable force to induce CRISPR correction. Viral vectors such as lentivirus and AAV are widely applied to deliver plasmid-based CRISPR-Cas9 system. Physical or non-viral delivery such as electroporation and microinjection are also important aspects because of their safety and cost-effectiveness. [KL17]

This review will discuss four main CRISPR-Cas9 gene editing strategies – exon deletion, exon reframing, exon skipping, and exon insertion – all aimed to correct these mutations illustrated above.

Exon deletion occurs through NHEJ. Usually, two sgRNAs recognize specific sites within the exon of interest and Cas9 will make multiple cuts in the mutated exon or exons, ensuring total knockout. Approximately 65-72 percent of DMD

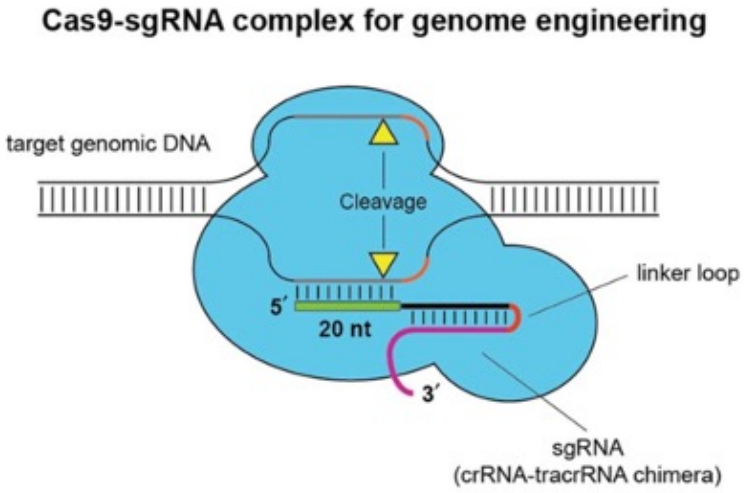

Figure 4: The CRISPR-Cas9 system. CAS-9 nuclease (blue) introduces DSB breaks in the target genome. crRNA(green) includes twenty nucleotides that are homologous to the target site DNA. tracrRNA provides a scaffold for combining the Cas 9 nucleases. The crRNA and tracrRNA are hybridized into one single guide RNA (sgRNA). Protospacer Adjacent Motif (orange) serves as a marker that helps cas9 recognize target genome sequence. [eaC16]

patients have a deletion on one or more exons in the hot spots region of exons 45-55, resulting in a premature stop codon of the dystrophin gene.

Exon skipping occurs mainly through disruption with exons splicing donor or acceptor sites, which produces a shortened but functional dystrophin protein. The production of such protein accounts for Becker Muscular Dystrophy (BMD). Antisense-oligonucleotide (AON), exon-skipping drugs, are based on this method which masks exonic splicing enhancers in pre-mRNA. This drug has been already approved by the FDA in the treatment of exon 51 in DMD patients. [T18b]

Exon reframing is another possible method that relies on both deletion and insertion to correct the dystrophin gene reading frame. Successful exon reframes have been performed on exon 18,38,40,73,79 on the dystrophin gene. [eaC16] The open reading frame (ORF) is restored through exon reframing.

Lastly, exon insertion utilizes HDR to insert a desired mutation from the donor template, thus restoring the full-length protein. Exon 44 has been replaced to its original site through HDR, effectively completing the mRNA reading frame. Exon insertion is even proven to be the most effective correction method according to one recent research. [ea15]

1.3 Induced pluripotent stem cell

Induced Pluripotent Stem Cells(iPSCs) are derived from human somatic cells such as fibroblasts and then reprogrammed with certain gene expressions into a pluripotent stage. As such, they are capable of differentiating into any type of cells and tissue lineages in the body. The rise of iPSCs application has generated a great deal of novel insights into disease modeling, drug screening, gene corrections, and organ regeneration. Moreover, the combination of iPSC and contemporary gene-editing tools have created tremendous areas for personalized treatments of diseases, especially for muscular dystrophies.

The generation of iPSCs generally contains three stages as shown in Figure 5. [W17b] The first stage is the preparation of iPSCs where somatic cells such as fibroblasts, peripheral blood cells, cardiac, and neuronal cells are reprogrammed with transcriptional factors such as OCT4, SOX2, and KLF4 into iPSCs. Then, the iPSCs undergo the second stage: vast expansion where iPSC cells divide and aggregate into a cell colony. The third and final stage for iPSCs is differentiation and application where they can be induced into various cell lineages such as T-lymphocytes and myoblasts. These cells will then be utilized for various means. Some will function as gene editing platforms; some will be used for research including disease modeling and drug screening; others will be further differentiated into tissues or even organs. [W17b]

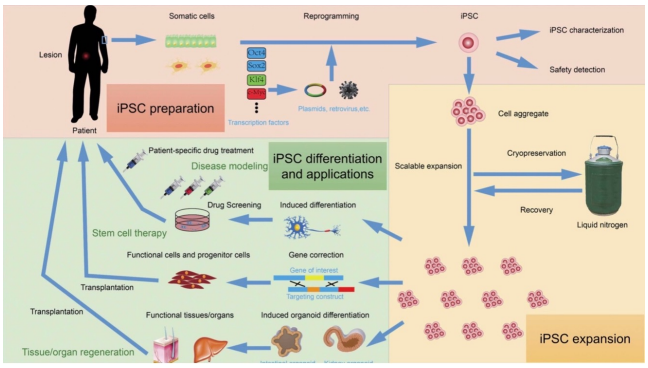

Figure 5: The three stages of the generation of iPSCs. iPSC preparation stage: somatic cells are reprogrammed with transcriptional factors into a pluripotent stage. iPSCs expansion stage: iPSC cells divide and aggregate into a cell colony. iPSCs differentiation and application stage: iPSCs differentiate into various cell lineages. iPSCs will then be utilized for various means. [W17b]

To realize the complete generation of iPSCs, certain delivery systems are often required. During stage one, transcriptional factors are delivered mainly by viral vectors such as AAV (adeno-associated viruses), lentiviruses, and retroviruses which carry specific genes and deliver them into target sites. Alternative delivery approaches rely on physical methods such as microinjection and electroporation which, compared to viral deliveries, avoid certain infections from

viruses and are cost-effective. During the differentiation and application stage, differentiated cells need to be transplanted into the body which is currently a major hardship for the implementation of iPSCs. Intravenous cell delivery may lead to cell entrapments in organs, thus leading to unwanted immune response and low cell quantity at the function site. [R19] Therefore, the intra-arterial route has several benefits. In one recent study [R19], gene-edited cells are differentiated into myogenic cells by inducing MyoD expression. Then, the differentiated cells are transplanted into a null mouse model for using intra-arterial injection through femoral arteries. However, there are limited reports on the use of intra-artery cell delivery in humans, and translation from animal models may prove difficult. The transplantation or delivery of edited cells into humans is still a major challenge for iPSCs requiring more study and research.

iPSCs are a desirable platform for disease modeling and drug screening. Compared to animal models which are widely used, iPSCs derived from human somatic cells have the same genetic background and cell behavior of the human population, something which animal models are lacking. Therefore, study results on iPSCs can potentially be directly applied to human physiology and pathology. In addition, iPSCs have a quick expansion rate and generally requires lower cost to produce compared to animal model research. [A19]

iPSCs can also be readily used for gene editing through such tools as CRISPR-Cas9. Various gene editing for muscular dystrophies can be effectively realized by iPSCs, for example, exon deletion of mutant and out-of-frame DMD exons on DMD patient-derived iPSCs and exon skipping using antisense oligonucleotides (AON) on DMD-iPSC cardiomyocytes. [T18e] Therefore, iPSC had showcased its tremendous potential for the development of personalized gene therapy.

There are two main current challenges for the use of iPSCs: the ability to generate large quantities of pure and functional cells and the development of cell delivery routines that render the least immunogenicity while enabling the greatest on-target effect. Despite these major setbacks, iPSCs still hold enormous capacity for gene therapy developments in the clinic and disease study, leading to future research and discoveries.

2 iPSC and CRISPR-cas9 integrated gene therapy for DMD

2.1 4 CRISPR mediated exon correction strategies and their applications on the dystrophin gene

2.1.1 Exon deletion

Exon deletion is the removal of one or several exons. Generally, two gRNAs target two separated exons or introns to introduce cleavages on them. The following exons within the two cutting sites are deleted, thus generating a DSB which is then corrected by NHEJ. The use of the CRISPR-Cas9 system to perform exon deletion is especially efficient and safe. Through exon deletion, the dystrophin

gene's open reading frame is corrected. Among all correction methods, exon deletion is probably the most efficient and widely applied approach, serving a large range of mutations and patients. Moreover, CRISPR-mediated exon deletion is approximately available to 83 percent of DMD patients. [ea16a] Many facts of exon deletion attribute to its wide popularity. First of all, exon deletion generally involves NHEJ DNA repair mechanism which is comparatively easy to occur and is also possible to perform in all cell types. Additionally, compared to HDR, NHEJ does not require an extra delivery of donor template. Lastly, deletion strategy is generally applicable to a variety of mutations and serves a larger pool of patients.

The following incorporated examples show how effective CRISPR mediated exon deletion is when applied to mutations in the dystrophin gene.

Exon 2 is one of the most frequently duplicated areas on the dystrophin gene, leading to frameshift mutations. CRISPR-Cas9 has been exploited to target the duplicated exon 2 in patient-derived myoblasts which are undifferentiated cells capable to differentiate into muscle cells. Only a single gRNA is utilized for this system and lentiviral packaging is incorporated to maximize delivery capacity and efficiency. Compared to traditional deletion strategies which generate partial functional protein, exon duplication deletion produces a full-length and complete wild-type dystrophin protein. [ea17]

Exon 23 contains a nonsense mutation which induces a premature stop. To realize exon 23 deletion, a study has used two Cas-9/gRNA complexes targeting intron 23 and intron 24 to cleave out exon 23. The two gRNAs and Cas-9 are delivered into target sites by AAV serotype 8 vector(AAV8) [ea16a]. Another study has also applied paired gRNAs targeting exon 23 on disease alleles of primary muscle stem cells in a mdx mice, a relevant dystrophic mouse model. The results successfully demonstrated CRISPR-Cas9's editing ability in both differentiated cells and satellite cells and its efficacy in restoring dystrophin expression. [ea16b]

Exon 44 deletion leads to the splicing of exon 43 and exon 45 as shown in Figure 6, thus generating a premature stop codon that alters the open reading frame. To correct this mutation, CRISPR-Cas9 is employed to delete the splice acceptor and donor site of exon 43 or exon 46, thus successfully splicing exon 42 and exon 45 together or exon 43 and exon 46 together. In both scenarios, the premature codon is erased through the connection of nearby exon regions. These two methods have been demonstrated in a research publication which utilized electroporation to deliver the CRISPR editing components to patient-derived iPSCs lacking exon 44. The edited cells were then differentiated into cardiomyocytes where expression of dystrophin protein was successfully detected. [ea19]

CRISPR-Cas9 system is also capable of generating large genomic deletions. A recent study successfully introduced a 336kb large genomic deletion on the mutational hotspot exon 45-55. Skeletal myoblasts from DMD patients treated with this approach demonstrated the excision of exon 45-55 in the mRNA and expression of dystrophin protein. The method to exclude multiple exons such as exon 45-55 was applied to over 60 percent of DMD patients. [CA15]

Another study has generated a humanized DMD mouse with exon45 deletion

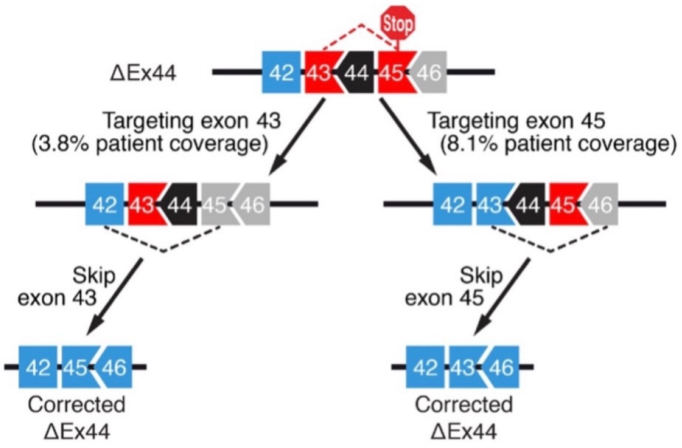

Figure 6: Exon 43 deletion and exon 45 deletion. Splicing of exon 43 and exon 45 generates a premature stop codon. On the Left, CRISPR deletes the splice acceptor and donor site of exon 43, thus linking exon 42 and 45. On the right, CRISPR deletes the splice acceptor and donor site of exon 45, successfully connecting exon 43 and exon 46. [ea19]

to apply exon45-55 multi exon deletion. Via electroporation, two gRNAs with Cas9 nucleases are delivered to intron 44 and intron 55 to cut down exon 45-55. The deletion of this whole exon region bypasses the mutated exon 45, as a result, restores the dystrophin genes reading frame. Dystrophin expression has been detected in hDMD del45 mdx and mdxD2 muscles, successfully validating the ability of CRISPR-Cas9 to target the human genome in vivo. [W17a]

In addition to exon 45-55, multi exon deletion approaches can also be applied to exons 3-9, another mutational hot spots on the dystrophin gene. CRISPR-Cas9 can again be used in human iPSCs to carry out the correction. Three methods are developed to delete exon 3-9: targeting of intron 2 and 9 thus bringing the deletion of exon 3-7, targeting of intron 5 and 7 leading to the deletion of exon 6-9, and targeting intron 6 and 11 subsequently deleting exon 7-11. In all, the approach to delete exon 3-7 demonstrates the greatest efficacy in both restoring the whole function of the dystrophin protein and maintaining the stability of the protein in iPSC-derived cardiomyocytes. [T18a]

To summarize, CRISPR-mediated exon deletion therapy utilizes the nuclease, Cas9, to target the correction site. The nuclease then cuts down the objective exon to introduce deletion.

2.1.2 Exon skipping

Exon skipping is the process where mutant genes are skipped over either by disrupting the splicing procedure, which allows introns to be removed and exons to be spliced together or by suppressing the expression of the gene. Ultimately, exon skipping restores the open reading frame and produces functional but shortened forms of dystrophin protein which can convert the severe symptoms of DMD into the milder type of BMD.

Antisense-oligonucleotide is commonly applied for exon skipping therapy. Antisense-oligonucleotide (AO) is a short synthetic nucleic acid sequence hybridized to target pre-mRNA sequence. [T18a] AO masks splice donor or acceptor sites in the dystrophin gene's pre-mRNA, suppressing the expression of particular genes, thus producing a truncated but functional dystrophin protein.

In addition, CRISPR-Cas9 is employed for exon skipping. When sgRNA targets the exon junction site, the Cas9 nuclease can make cuts at the junction point. The DSB break generated by CRISPR-Cas9 together with the exon splice donor or acceptor site near the junction point will then be repaired through NHEJ. The repair mechanism deletes the exon splice donor or acceptor site, leading to splicing of the next exon, thus skipping the mutated exon.

Approximately 70 percent of DMD patients with deletion mutation and 47 percent of DMD patients with nonsense mutation can be treated by single exon skipping therapy. [T18a]

Deletion of exon 44 renders the wrong splicing of exon 43 and exon 45, thus generating a premature stop codon. By using AOs, a study [H18] has presented exon 45 skipping on human iPSCs of DMD patients with exon 44 deletion. A piggyBac(PB) transfection vector was also designed to express a myogenic master regulator MYOD1 in hiPSCs which induces differentiation. This study effectively shows the efficacy of AOs and their feasibility to integrate with iPSCs.

Deletion of exon 50 in the dystrophin places exon 51 out of frame, thus leading to the loss of whole dystrophin protein. AON exon skipping drug can mask an exonic splice enhancer (ESE) on exon 51, resulting in the skipping of exon 51 as seen in the diagram in Figure 7. The ORF can be restored and a functional protein can be produced. One of the AON drugs targeting exon 51 skipping is Eteplirsen which is applicable to approximately 14 percent of all DMD patients and has been approved by the US FDA for clinical use. [T18d]

Multi exon skipping serves for a wider range of mutations especially for the mutational hot spots at exons 45-55 and exons 3-9. Approximately 80-90 percent of DMD patients can be treated with multi exon skipping. [T18a] Moreover, multi exon skipping for exon 45-55 can be applied to 46.9 percent of DMD patients. [T18d] Yet, not every AOs in the AO cocktail harbors benefits to patients, for it requires toxicological tests for every one of the AOs. Therefore, additional studies should be carried out in order to promote the AO cocktail approach for multi exon skipping.

The canine X-linked muscular dystrophy model (CXMD), or simply the dog DMD model, includes a point mutation on exon 6 hence resulting in exon 7 deletion. Exon 7 deletion causes the splicing of exon 6 and exon 8 which gen-

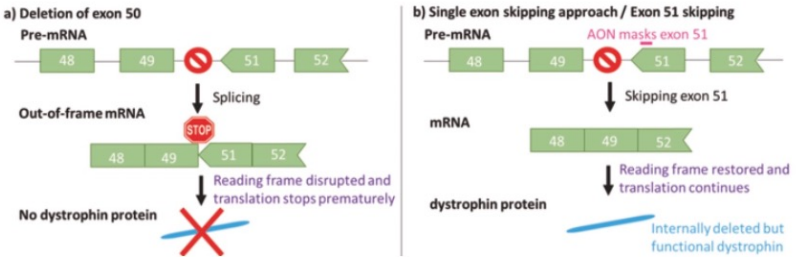

Figure 7: Exon 51 skipping. Deletion of exon 50 in the dystrophin places exon 51 out of frame, thus generating a premature stop codon and leading to the loss of whole dystrophin protein. AON exon skipping drug masks an exonic splice enhancer (ESE) on exon 51, thus skipping exon 51. The ORF is restored and functional dystrophin protein is produced. [T18d]

erates a premature stop codon. AOs are capable of effectively binding to exon 6 and exon 8, resulting in them being spliced out and thus restoring the ORF. This mixture of AOs, or multi-exon skipping of 6-8, successfully recovers the synthesis of functional dystrophin protein. It is also necessary to point out that because of the likeness between humans and dogs, the AO drugs serving dogs can be applied to DMD patient cells. [T18a]

However, AO exon skipping for animal models cannot be directly introduced to human DMD patients owing to the genome difference between species. In order to assess AO exon skipping approach on patient-derived primary cells in vitro before implementing this approach into human cells in vivo, a study [T18c] has redesigned AO based on the ones for the canine model. The regenerated AO performs exon skipping on DMD patient cells with exon 7 deletion. The corrected cells are MyoD transduced fibroblasts that can convert to myotubes with the successful expression of dystrophin mRNA and protein.

Though AO drugs are promising tools for exon skipping, there are several setbacks to their implementation. AO drugs are applicable to limited types of mutations and can serve rather small percentage of patients. For example, exon 51 skipping is only feasible for 10 percent of DMD patients. [ea17] AO drugs also require repeated injection which sometimes could last life long, making this approach costly and inconvenient, not to mention the toxicity and tissue uptake of frequent injection of AO drugs. In contrast, CRISPR exon skipping leads to a permanent correction with only one administration. Lastly, to remove each exon on the gene, a distinct AO is designed, while CRISPR-mediated exon skipping only requires two necessary nucleases to delete any mutations. Therefore, using CRISPR-Cas9 to implement exon skipping may be more successful.

2.1.3 Exon reframing

Exon reframing, also called NHEJ reframing, is the restoration of the open reading frame through NHEJ DNA repair mechanism. The main goal of exon

reframing is to generate INDELs (insertions and deletions) in one particular exon. INDELs can either be the deletion or insertion of a whole exon or the deletion or insertion of a single nucleotide. In all, exon reframing modifies the reading frame to help produce a functional dystrophin protein. It is a readily applicable strategy to reserve the maximal dystrophin gene sequence. [eaC16]

A recent study [MYCFLH20] has generated three DMD mouse models with the corresponding deletion on exon 43, 45, and exon 52, successfully representing three frequently deleted regions on the dystrophin gene. Exon 52 deletion puts the following exons out of frame. In exon 52 deleted mouse model, a 3n+1 INDEL (inserting or deleting one additional nucleotide) is successfully introduced into exon 53 through NHEJ DNA repair mechanism, as depicted in Figure 8. As a result, the reframing on exon 53 retrieves the open reading frame and constructs a functional dystrophin protein. In addition to the mouse model, sgRNA generates 26 percent of 3n+1 genomic INDELS in human iPSCs too.

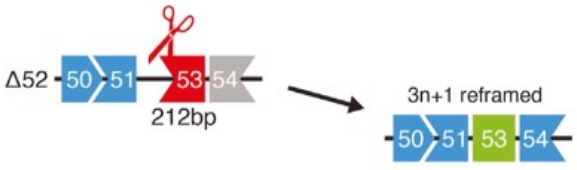

Figure 8: Exon 53 reframing. Exon 52 deletion renders exon 53 out of frame, creating a premature stop codon in exon 53. A 3n+1 nucleotide insertion or deletion successfully reframes exon 53, thus restoring the open reading frame. [MYCFLH20]

In the mouse model with exon 43 deletion, which generates a premature stop codon near exon 44, a 3n-1 deletion on the proximity of the splice donor site of exon 44 can be employed to restore the open reading frame. Aimed at exon 45 deletion, which also leads to out-of-frame mutations, a 3n-1 INDEL (inserting or deleting one additional nucleotide) can be directly used to reframe exon 44, as shown in Figure 9. Also, the introduction of 3n-1 INDEL to reframe exon 44 of human DMD iPSCs allows the overall exon 45 corrections.

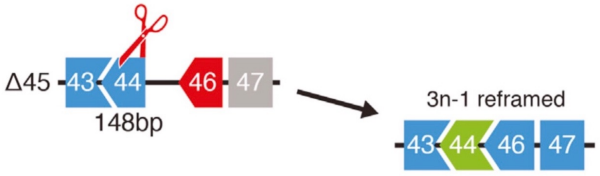

Figure 9: Exon 44 reframing. Exon 45 deletion leads to out-of-frame mutations. A 3n-1 nucleotide deletion on exon 44 reframes exon 44, thus restoring the ORF. [MYCFLH20]

Moving on to a true scenario, a study has generated patient-derived iPSCs from a DMD patient with exon 44 deletion. Exon reframing has been tested on exon 43 and exon 45. The introduction of a 3n+1 INDEL (inserting one additional nucleotide) on exon 43 or a 3n-2 INDEL(deleting two nucleotides) on exon 45 can both restore the reading frame. As a result, the study, after sequence analysis of the clones, found 13 of 34 clones incorporated 3n+1 or 3n-2 reframing. Furthermore, exon skipping and reframing for exon 44 deletion are capable of treating 12 percent of DMD patients, a testimony to exon reframing's great ability to correct mutations. [ea19]

Another study has applied CjCas9, the smallest Cas9 ortholog, mRNA, sgRNA, and additional components all into one AAV9 vector. The AAV9 is then delivered through intramuscular injection onto the TA muscles of DMD mice. Exon reframing with CjCas9 is performed at the region upstream of the premature stop codon in exon 23. The introduction of INDELs successfully restores the reading frame and also verifies the efficiency of all in one vector delivery and the potential to correct mutations with a single sgRNA through NHEJ DNA repair mechanism. [ea18]

2.1.4 Exon insertion

Exon insertion (knock-in) is the introduction or replacement of exons which restores the complete reading frame of the dystrophin gene. Exon insertion is supported by HDR DNA repair mechanism, requiring a donor template with its end homologous to the ends of the DSB. The corresponding donor template is then exchanged onto the DSB breaks through homologous recombination, effectively bringing back the full-length reading frame. Despite the result of having a complete reading frame restored through HDR, HDR has lower accuracy than NEHJ. Moreover, it is also limited to mitotic cells and the template length is confined due to the limited packaging ability of delivery systems. Nevertheless, exon insertion approach has been used in various researches and showed great potential. [eaC16]

In an effort to evaluate exon skipping, exon reframing, and exon insertion strategies over exon 44 deleted iPSCs from a DMD patient, a study developed an exon insertion method that replaces exon 44 in front of exon 45, as shown in Figure 10. They designed a donor template with exon 44 and exon 45 hybridized together, both sharing the same splicing acceptor site. The donor template and CRISPR-Cas9 system vector are both electroporated into the cells. Results show that approximately 90 percent of the examined clones exhibited donor template replacement on the target site. Through the results, exon insertion strategy was proven to be a much more efficient and precise strategy over all other correction methods, capable of restoring the full-length dystrophin protein. [ea15]

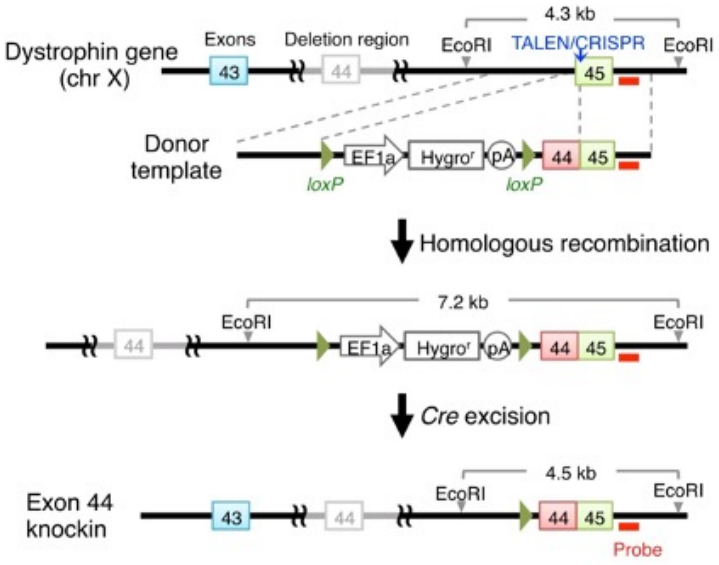

Figure 10: Exon 44 insertion. CRISPR cuts are adjacent to exon 45. A donor template with exon 44 and exon 45 hybridized together, sharing the same splicing acceptor site, is designed. Through homologous recombination, the donor template is exchanged onto the DSB break generated by the CRISPR cut near exon 45. After cre excision, exon 44 is successfully knocked in, thus restoring the full-length dystrophin protein production. [ea15]

3 Conclusions and discussions

CRISPR-Cas9 and iPSC integrated therapy utilizes CRISPR-Cas9 system to induce genomic corrections on DMD patients' iPSCs. As shown in the schematic in Figure 11, human somatic cells are first derived from DMD patients with mutations across the dystrophin gene. Then they are regenerated into iPSCs containing the dystrophin gene's mutation. Next, CRISPR-Cas9 system is introduced to perform various CRISPR corrections according to distinct mutation properties. As a result, genome-edited-iPSCs are generated for differentiation, which are either transplanted into the body or preserved in vitro. The edited iPSCs, differentiated in vitro, can develop into mature cell types such as myoblast and cardiomyocytes. They are then delivered into the body to restore dystrophin expression in the myofibers.

Several inherent features of the dystrophin gene, CRISPR-Cas9 system, and iPSCs make such an integrated approach possible, delivering a number of advantages for DMD patients.

First of all, the dystrophin gene is a viable platform for genome correction. DMD is one of the largest genes and may present thousands of mutations. To

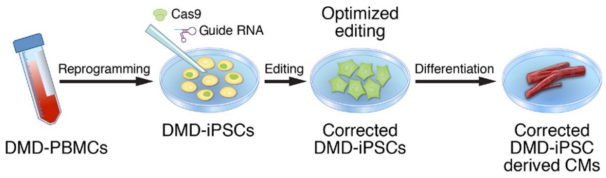

Figure 11: The whole process of iPSC and CRISPR-Cas9 integrated therapy for DMD patients. [ea19]

date, approximately four thousand mutations have been identified. Mutations on the dystrophin gene usually cause a premature stop codon that halts the synthesize of protein. They also lead to frameshift mutations that disrupt the overall reading frame. Simple correction methods such as exon deletion are capable of restoring the open reading frame and expressing proteins. Moreover, owing to the unique features of dystrophin protein that is they can remain functional even when missing parts of its domain, removal of internal but essential exons of the dystrophin gene can restore the reading frame. It produces functional but truncated forms of dystrophin protein that can lessen the severity of DMD. Whereas for other genetic diseases, the production of shortened forms of protein may not lead to an amelioration of symptoms, but only a near-normal level restoration of protein can. Additionally, DMD is a typical example of monogenetic hereditary disease, thus only one mutated allele needs to be corrected [CA15]. As such, applying gene editing may be the best treatment for DMD. Lastly, DMD is a muscle degeneration disease, whereas skeletal muscle cells are syncytium in which several nuclei share a single cytoplasm. Gene editing in a small portion of DMD patients' cells guarantees distributions of dystrophin protein throughout the muscle fibers.

Secondly, the CRISPR-Cas9 system is a supreme gene editing tool showing great potential. CRISPR correction has improved accuracy because of sgRNA's high-on-target recognition. It relies on nucleotide base pairing to the target genome site and demands an additional requirement of the PAM sequence before recognition. The system is also simple including only two parts: the Cas9 nuclease and the sgRNA, making it easy to produce and transport. Furthermore, compared to exon skipping methods, AAV encoding mini dystrophin protein, and other protection therapies, CRISPR-Cas9 system can materialize life-long correction with only one administration and bind to millions of genome sites. [AR17] In sum, the CRISPR-Cas9 system is effective, efficient, simple, and holds tremendous potential for genome editing, capable of functioning in any type of eukaryotic cells.

Lastly, iPSCs are ideal for CRISPR correction. To begin with, iPSCs are easily accessible from any patient cells or tissues, resulting in potentially unlimited resource availability for production. iPSCs have great pluripotency, competent to differentiate into many cell lineages. Secondly, corrected genes on iPSCs can enter into differentiated mature cells, allowing continual expression of corrected

protein and enabling tissue-wide expression of functional protein. [ea16c] This feature is extremely useful for muscular dystrophies because of the body-wide requirement of functional muscle. Furthermore, the mutations investigated under iPSCs7 represent the human genome, hence the learnings gained from iPSC studies can be more readily applied to human biology and diseases. Genomic correction on individual cells also allows the development of personalized treatment. Fourthly, genome correction using CRISPR on iPSCs results in a lower immune response. One of the major setbacks of CRISPR is its cytotoxicity and immunogenicity over the human body resulting from the CRISPR components and AAV vector for its delivery. The straightforward CRISPR correction in vivo may induce immune responses, yet the correction made on iPSC, which is in vitro, causes a lower immune response. Lastly, iPSCs have great advantages compared to other stem cell therapies such as Embryonic Stem Cell (ESC). [NL19] iPSCs are derived from human somatic cells, while ESCs are from embryonic cells. As such, the use of iPSCs provokes fewer ethical concerns. The incorporation of iPSCs into CRISPR correction to treat DMD patients is evidently beneficial.

It is equally important to highlight that the realization and the advantages brought by the integrated gene therapy count on the dystrophin genes' capability for CRISPR correction, CRISPR's competence to make corrections on iPSCs, and iPSC's suitability to rescue protein production particularly for DMD. The three parts, CRISPR-Cas9, the dystrophin gene, and iPSC, function in unity. Breaking up any link among the three will only fail the entire integrated therapy.

4 Current challenges

It is necessary to mention the current challenges of such an approach. CRISPR-Cas9 system renders immune response to the body due to the CRISPR components and viral vector for its delivery. Off-target effects of the CRISPR-Cas9 system are also unavoidable. AAV vector may express in more than one year, making the target site susceptible to off-target mutations. [T18a] Additionally, the accuracy of CRISPR editing needs perfection. The mostly used DSB repair mechanism NHEJ is still an imprecise repair system, whereas the much more refined mechanism HDR is limited down to a certain period of the cell cycle. Concerning iPSCs, there are limited transplantation methods for cells to enter the body. Intramuscular cell delivery activates immune responses. Intra artery cell delivery may prove to be feasible, yet limited researches have reported on the success of the Intra artery method to deliver cells into humans. Meanwhile, the ability to generate a large quantity of pure and functional cells is still a remaining question. Also, the variation of the genetic background of individual iPSC cell lines may render small phenotypic differences between cell lines rather than disease-relevant phenotypic differences. With respect to ethical issues, the rising ability of CRISPR gene editing in iPSC leads to genome editing even in fertilized eggs. Editing may be performed without control if no regulations are enforced to supervise this area. Future developments and improvements should

take aim at these concerns.

5 Future prospective

Despite all these hurdles and setbacks, CRISPR editing on DMD patients' iPSC cells has great potential for genome correction and disease modeling. Not just for the treatment of DMD, the combination of human iPSC cells with the CRISPR-Cas9 systems has showcased its potential in various other inherited diseases, such as immunological, neurodegenerative, cardiac, and metabolic diseases. The combination of CRISPR editing and iPSCs also shed light on researches regarding principles of human cellular biology, such as tumor suppression and cell immortality [R17]. Being effective, efficient, and safe, this integrated method will be tremendously rewarding for future biomedical researches and treatments.

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