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Review Article

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CRISPR/Cas9 and its Delivery Methods for Duchenne Muscular Dystrophy (DMD) Gene Therapies

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Abstract

Duchenne Muscular Dystrophy (DMD) is a X linked genetic disorder that causes difficulty in walking, followed by progressive skeletal muscle degeneration and some cardiac muscle related issues, that threatens the life expectancy of patients. This condition is due a mutation in a gene that produces cytoskeletal protein termed as dystrophin. Targeting this gene to correct or bypass the mutation would benefit in effective therapies for DMD. CRISPR/Cas9 (Clustered Regularly Interspaced Palindromic Repeats) technology has created an evolution in precise gene modification techniques. With the help of a guide RNA, Cas9 (a DNA endonuclease) can create a double strand breaks to carry out the targeted gene modifications. By simply modifying the guide RNA sequences, Cas9 can be used for flexible programming of new target sites. The prime factor that determines the therapeutic efficiency of gene editing is the delivery vector. Lot of attempts has been made to create an efficient therapy for DMD with CRISPR/Cas9, but still, the major hurdles rely on delivery techniques. Therefore, optimization of the delivery methods will support the complete regenerative therapy for DMD in future. This review mainly concentrates on the various aspects of CRISPR/Cas9 technologies and its delivery methods used in developing therapies for DMD and its optimization possibilities.

Keywords: CRISPR/Cas9; Delivery methods; Duchenne Muscular Dystrophy (DMD)

Introduction

Duchenne Muscular Dystrophy (DMD) is characterized by the loss of functional dystrophin protein and its progressive muscle weakness. The milder version of this disease is Becker muscular dystrophy (BMD) which leaves the patients with some reasonable percentage of functional dystrophin protein. Since it is an X linked genetic disorder, male children of carrier mothers inherit this disease. Dystrophin, as the third largest gene in size (2.1Mb), consist of 79 exons, which encodes a protein of 3685 amino acids. There are several types of mutations accounted so far in dystrophin gene, which includes duplications and gross deletions. These mutations may result in reading frameshifting (70%) and occasionally point mutations (30%) that result in loss or abnormal function of dystrophin protein domains. Dystrophin is a cytoskeletal protein of cell membrane, that links intracellular γ-actin to dystrophin associated protein complexes (DAPC) in the cell membrane and hence connects with the extracellular matrix by laminin [1]. There four functional domains in dystrophin, a caboxy- terminal domain (homologues to utrophin), a cysteine-rich domain, a central rod domain and an actin binding amino terminal domain (ABD) [2]. Targeting all the muscle cells to regain its active dystrophin, is the ultimate aim of gene therapies [3,4]. Several methods like, premature stop codon suppression, exon skipping, stem cell therapies have been attempted in animal models like mdx mice and these approaches stepping ahead to clinical trials leaves promising hope on treatment possibilities of DMD patients [5-7].

Literature Review

Genotype correlating phenotype

The reading frame rule is not followed by approximately 10% of genetic mutations, that is, BMD appears, when the mutations are out of reading frame and the patients with mutations inside the reading frame causes DMD [8]. The clinical phenotype does not appear, when the mutations are out of frame, but in cases where the reading frame is disrupted, it results in abnormal or truncated domain of dystrophin protein and hence the disease severity. However, three notable exceptions are there,

1. In some cases, the non-sense or frame shift mutations in exon 8 can result in BMD, which is due to a mutations that creates the alternative translational initiation site in exon 6 or 8 (for example a stop mutation in exon 1 or deletion mutation of exon2 may cause translation initiation at exon 6). Apparently, it depends on patients and their mutation types that decides, if the translational site is altered or not [9-11].

2 Nonsense mutations in in-frame may result with BMD [10]. In this case the mutations may disrupt the exon recognition sequences, and the exon is skipped rarely which would bypass the mutation and the reading frame is maintained.

3 A mutation flanking exon 44 presented with moderately milder version of DMD, where, exon 44 is skipped spontaneously at very low levels, and the dystrophin levels are higher than DMD patients with other types of frame shift mutations [12,13].

In some cases of cardiomyopathy, the patients showed only the cardiac phenotype with the void of skeletal muscle problems [14,15]. This is due to the presence of functional dystrophin in skeletal muscles but not in cardiac muscles. Also most of the carriers of mutated dystrophin gene do not experience the symptoms, but some female

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Discovery of CRISPR/Cas9

Ishino et al. in 1987 first reported the presence of nearly palindromic pattern of unusual repeating sequences that are separated by non-repeating sequences in *E. coli* [16]. Mojica et al. in 1993 described them as 'curious sequences' when they found similar kind of palindromes in *Haloferax* and *Haloarcula archae* [17]. The functional importance of these sequences remained mystery, but the identification of them in 20 more microbial species and then in 90% archaea and in 40% bacteria confirms some functional importance [18]. Then acronym CRISPR (Clustered Regularly Interspaced Palindromic Repeats) was preferred to call out these palindromes. Then a set of genes adjacent to the CRISPR locus were determined, and soon it was proposed to be termed CRISPR-associated system 9, or Cas9 [19]. Attempts to understand the functional relationship of the CRISPR spacers and Cas9 were made [20]. A significant progress came after the discovery of these CRISPR spacer sequences in foreign chromosomal DNA [21], particularly in few bacteriophages [19,21,22]. The bacteria that contain these sequences of phage DNA at CRISPR locus was found to be naturally resistant to the infection by that phage, and so it was hypothesized that, CRISPR could be the reason of this adaptive immunity [23]. Experimental evidences for this mechanism supported this hypothesis strongly [24]. Even though it was hypothesized to follow the RNA interference mechanism in the initial stages, it was soon proved to be the genomic memory of invading phages. Cas9 proteins uses these CRISPR sequences to search for the invading pathogens and preclude them by creating double strand breaks (DSB) [25].

CRISP/Cas9 as gene editing tool

The adaptive immune capacity of CRISPR/Cas9 from the bacterial species such as *S. pyogenes*, *S. thermophilus*, and *N. meningitides* are now being used to target the human genes and edit them as desired [26]. For gene editing purposes the Cas9 nuclease is co expressed along with a single guide RNA (sgRNA), these sgRNA forms complex with Cas9 at its 3′ end, making them as RNA guided DNA endonuclease. The 5′ end of sgRNA forms complementary base paring with the protospacer adjacent motif (PAM) at the target site for gene modification and create a double strand break (DSB) [27]. By simply changing the PAM sequences of the sgRNA, Cas9 can be targeted to new genomic sites and make the desired gene modifications. The target sequences should be immediately followed by PAM is the major restriction in using this as tool. The host genome protects itself from self-cleavage, if the PAM sequence is absent at the CRISPR locus. PAM sequences for Cas9 of several bacterial species have been studied to make them target specific for human system. For example the PAM sequence defined for the Cas9 from *S. pyogens* (spCas9) is 5′-NGG-3′. CRISPR/Cas9 system can be used to target multiple genomic loci simply by co expressing single Cas9 protein along with multiple sgRNAs making them unique in gene editing techniques [28].

Induced pluripotent stem cells (iPSCs) as diseased models

The use of iPSC derived from patients, to test the therapeutic potentials of CRISPR/Cas9 is creating huge attention. The urine/blood samples or any other easily accessible tissues are collected from DMD patients, because of its pluripotent nature, the iPSCs derived from these samples are used to differentiate them into relevant cell types, which can be used in studying the CRISPR/Cas9 gene editing (Figure 1) [29]. For example, the cardiomyocytes derived from iPSCs can exactly mimic the physiology and developmental progression of human heart that is inadequately represented in animal models. The severity of cardiomyopathy is less in mice compared to that in DMD patients. Also, the regulation of myosin function, ion channel activity and cardiac vasculature are regulated differently in mice and human [30]. Animal models like dogs and mice exhibits increased tolerance and resistance to the cardio toxic effect of drugs. These differences may lead to imprecise representation of drug activity in human [31]. In addition, without the need of obtaining the cardiac biopsies from patients, the iPSCs would replicate the patient mutation in cardiomyocytes. Also obtaining the cardiac muscle cells directly from patients would irreversibly damage heart [32,33]. Over 7000 types of DMD mutations can be modeled with iPSCs [11]. The skeletal myotubes differentiated from iPSCs offered similar advantages as like iPSC derived cardiomyocytes.

Repair of Double Strand Breaks (DSB)

The sgRNA directs Cas9 to a specific genomic target by

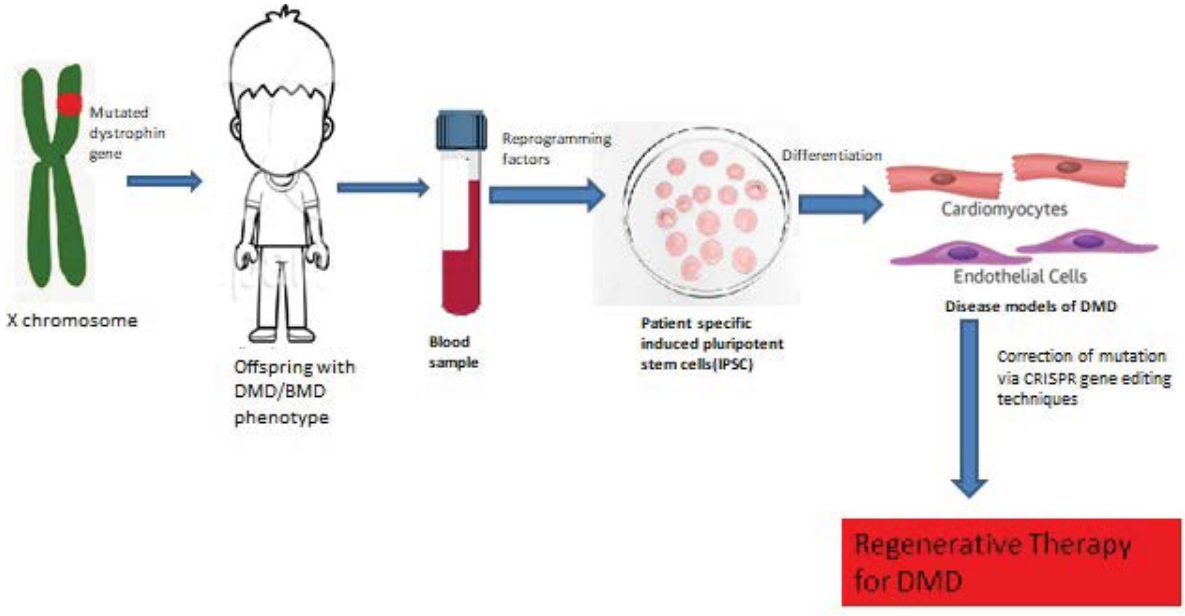

Figure 1: Creating the diseased models for *in vitro* DMD studies using induced pluripotent stem cells (iPSCs).

complementary base paring with PAM sequences, where the Cas9 creates a double strand breaks (DSB) [34]. These breaks are repaired by endogenous repair system, in order to complete the intact double strands back, which may happen in either of following two ways,

1. Homology directed repair (HDR), where the repair is done with the help of exogenous template DNA or sister chromatids, which contains homologous sequences flanking the broken region. This kind of repair results in sealing of the break in error free manner.
2. Non homologous end joining (NHEJ), where the two ends of broken ends are repaired by directly joining them. This leaves the sealing with imprecise indel (insertion/deletion) mutations.

Both of these repair systems are active in majority of eukaryotic cells, but HDR is restricted to happen only if a template is available, which is naturally presented in endogenous repair system as sister chromatin during the late S2 and G2 phase of cell division [35,36]. In cases, if the HDR is to be carried out in gene editing by CRISPR/Cas9, the artificial template DNA should be added. The HDR efficiency is determined by several factors including, the amount of template presented during repair, length of the homology arm, cell cycle stages and the regulation of endogenous repair systems. To obtain the proper sealing of DSB, HDR optimization for cell lines is indeed, particularly in case of immortalized cells [37].

Factors affecting efficiency of the CRISPR/Cas9 gene editing

CRISPR/Cas9 (Clustered regularly Interspaced Palindromic Repeats) technology has created an evolution in precise gene modification techniques. By simply modifying the guide RNA sequences, Cas9 can be used for flexible programming of new target sites. The factors that need to addressed for the higher efficiency of gene editing includes, selecting a target DNA site for modification, designing the sgRNA for particular PAM sequences, determining the off target effects, cutting efficiency of Cas9, mode of delivering the CRISPR/Cas9 components and DSB repair incidences via NHEJ/HDR. Lot of attempts has been made to create an efficient therapy for DMD with CRISPR/Cas9, but still, the major hurdles rely on delivery techniques. Therefore, optimization of the delivery methods will support the complete regenerative therapy for DMD in future.

CRISPR/Cas9 delivery systems

As CRISPR is speeding up the way to human therapeutics, the delivery means poses major challenge. CRISPR/Cas9 components that are delivered for gene editing purposes could be any of the combinations below. (1) a guide RNA along with a mRNA for Cas9 translation (2) A plasmid DNA that codes for both mRNA and Cas9 protein and (3) guide RNA and Cas9 protein as ribonucleoprotein complex (RNP). Because of the fragile tertiary structure and higher molecular sizes, the RNPs face poor cell membrane permeability, which demands an appropriate shield to escort them into the nucleus [38]. The type of CRISPR/Cas9 components to be delivered will precisely depend on the delivery methods. The altogether concentration of Cas9 protein present in the system reflects the tightly monitored gene editing efficiencies. In case of delivering the Cas9 DNA in plasmid, the concentration of functional Cas9 present inside the system is much difficult to be assessed. Vehicles used to deliver the CRISPR/Cas9 components can be classified into three categories (Table 1). (1) Viral delivery methods, (2) Physical delivery methods, and (3) Non- Viral delivery methods. Viral delivery methods are the most attempted delivery technique for CRISPR/Cas9 gene editing, the most commonly used viral species that is precisely engineered are, Adeno- associated virus (AAV), Adenovirus (AdV) as full sized forms and Lenti virus (LV). Physical delivery methods suit well for much of the *in vitro* studies carried so

Mode of delivery	Delivery vehicle	Hotspot targets	Advantages	Downsides	References
Viral delivery Methods	Adeno -associated virus (AAV)	Dmd exon 20, 23, 51 and 53	Persistent presence of exogenous DNA, Simple and most suited for invitro and *in vivo* studies with minimal immunogenicity.	Size restriction which demands the use of dual vectors.	[43,45,46,48,50,51]
	Adeno virus (Adv)	Dmd exon 21, 23, 44, 51, 52 and 53	Can infect both dividing and non-dividing cells without integrating with genome, larger insertions are better tolerated than AAV	Elicit strong humoral immune responses, difficult scaled production.	[58-60]
	Lenti virus (LV)	Dmd exon 2, 27, 43, 51, 47 and 58	Persistent gene transfer.	Prone to gene rearrangements and high off target effects.	[58,61,62,84]
Physical delivery methods	Microinjection	Dmd exon 23 and 51	Not limited by molecular weight of CRISPR/Cas9, delivers the CRISPR/Cas9s directly to target site within cell, controlled delivery of known quantity of CRISPR/Cas9s, efficient in creating animal models.	Possibility of *in vivo* setting is very less, time consuming.	[34]
	Nucleofection	Dmd exon 45, 55, 51, 6, 7 and 11	Directly delivers the CRISPR/Cas9s into the nucleus.	*In vivo* delivery is least possible.	[75-77,94]
	Electroporation	Dmd exon 45, 55, 21, 23 and utrophin A/B promoter	Less dependent on cell types, well known technique.	Cell damages caused by high voltage and application time, most limited for *in vitro* applications	[60,61,67,71]
Non -viral delivery methods	Lipotransfection	Dmd exon 23, 50 and 54	Well suited for nucleic acid and RNP delivery invitro and *in vivo*	CRISPR/Cas9s directed to lysosomal pathways, insufficient to shield plasmid	[59,93]
	Gold nanoparticles	Dmd exon 23	Inert carriers, readily used for invitro and *in vivo* experiments, bind large variety of proteins	Lowered HDR frequencies due to high levels of cytotoxicity.	[56]
	Polyethenimine (PEI)	Dmd exon 2 (duplicated exon removal)	High charge density and p H buffering ability, limitless options for customization, favors endosomal escape.	Branched PEI are highly cytotoxic.	[84]

Table 1: CRISPR/Cas9 delivery methods in DMD studies.

far, which includes, electroporation, nucleofection and micro injection. Non-viral delivery methods are not well established as like the other two methods, but optimization of this method is a budding area of research, this includes Polyethenimine, lipid and gold nanoparticles.

Viral delivery methods

Adeno associated virus (AAV): Adeno associated virus (AAV), a single stranded DNA virus is extensively used as a carrier vehicle for gene therapies. It is not related with any type of diseases in humans and the availability of many serotypes offers the transfection of cells with various specificities [39]. CD8 T cell immune response is shown towards the viral capsids, but still the advantage of AAV serotypes with wide tropisms makes it a best carrier, in spite of the immune response issues it raised [40]. The AAV delivered plasmid present persistently into the cells either as integrated form into the host genome or remains as exogenous DNA [41]. The final goal of modifications decides, if this persistent presence is either advantageous or disadvantageous.

HEK293 T cells are created by CRISR/Cas9 using AAV. After when the AAVs are created with limitations to infect specific cell types, they will carry on the infection in the way similar to that of native viral particles. This makes the transferred contents of CRISPR/Cas9 as persistent one inside the cells.

AAVs can be used as delivery method for CRISPR/Cas9 in one of the following ways:

(1) Single vector system, where the SpCas9 and sgRNA are packed into a single plasmid DNA and delivered to cells by single AAV particle. Together SpCas9 and sgRNA will make 4.2 kb of size, whereas the 20nm size of AAV can hold upto roughly 4.5-5 kb of external genomic contents to be packaged [42]. The plasmid construct demands the space to include other elements like fluorescent tags (in order to ensure the delivery of CRISPR/Cas9 components), template DNA (if HDR is a preferred repair mechanism) and multiple sgRNAs (if Cas9 has more than one target site). Therefore, size remain a major hurdle in using a single vector as delivery method [43].

(2) Dual vector system, where sgRNA and SpCas9 are constructed in two separate AAV and co infect the target cells [44]. The major issue of size restriction has been addressed by the use of this dual vector approach, but still this added more difficulties in constructing plasmid than with the single vector system. Higher cutting efficiencies have been obtained by using the dual vector of AAV, where the CRISPR/Cas9 was targeted to carry out single exon deletion(exon23) and achieved more than 10% of dystrophin restoration in skeletal and cardiac muscles [45]. Another attempt on dual vector approach to carry out multiple exon deletion also yielded dystrophin restoration in cardiac (34%) and skeletal muscle (10-50%), where the DSB repair was achieved by HDR [46]. Rather than usual Cas9 version of SpCas9 (obtained from *Streptococcus pyrogenes),* a new Cas9 variant was identified from *S. aureus* (designated as SaCas9). With the similar potential cutting efficiency, SaCas9 is 30% lesser in size than SpCas9. This SaCas9 smaller size leaves some extra room of 1 kb in plasmid to accommodate markers and multiple different tags in single vector [47]. SaCas9 in AAV particles have been used to target DMD mutations in mice [45,48]. Similar shorter variants of Cas9 have also been identified from *Streptococcus thermophiles* and *Neisseria meningitidis* [37,49]. Smaller Cas9 variants identified so far has longer PAM sequences, which limits the sequence availability in the target region. Higher target specificity was observed in another Cas9 variant CjCas9(*Campylobacter jeguni*), with which a mice model with the deletion of exon 23 in *dmd* gene was created [50].

Exon 51 duplication mutation of *dmd* gene in a dog model was corrected, where dystrophin levels of 92% was observed in cardiac muscles. This is the first reported study to use dog model in testing DMD therapeutic potential of CRISPR/Cas9 [51]. The immune response triggered by Cas9 exhibits several hurdle for its progress in therapies [52]. This may be due to the common exposure of the bacteria like *S. pyogenes* and *S. aureus* from where the Cas9 enzymes were derived [53]. Efforts addressing to avoid these immune responses are in earlier stage [54]. This includes engineering of Cas9 nucleases with the void of immunogenic properties and using regulatory T-cells to lower these responses [53,55]. However further safety issues should be studied in order to proceed these regulatory controls to the next level [56].

Adeno viral vectors (AdV): Using AdV in CRISPR/Cas9 gene editing is the way similar to that by AAV. Infecting both the dividing and non-dividing cells without integration into the genome is one of the major advantages in using AdV for CRISPR/Cas9 studies. This advantage limits the off-target effects to some extent. The most widely used serotype is type 5 AdV. The backbone of this serotype is derived from one among the well-studied AdV. As like AAV, AdV can also be effectively used in *in vitro* and *in vivo* studies. Advs were used to deliver the CRISPR/Cas9 components targeting *dmd* exon 51 and 53, where SpCas9 with two gRNA in plasmid construct was used and the editing efficiency was equally similar to that with single gRNA [57,58]. Transduction efficiency levels were higher when the delivery was carried with template DNA for HDR [59]. Multiple exon deletions were also carried out in mdx mice, targeting *dmd* introns 20 and 23. Around 50% of dystrophin restoration and sarcolemmel localization with membrane integrity was observed [60]. The important aspect in previous studies revealed, immunotoxicity of liver and humoral immune responses associated with AdV, particularly cellular immune response was detected while using Spcas9. Immunogenicity studies for AdV are indeed to be carried out before considering it for therapies [57].

Lenti viral vectors (LV): The provirus of HIV generally acts as backbone of LV. Similar to Adv and AAV, LV can be feasibly applied for *in vitro* and *in vivo* studies. To make the LV particularly useful in gene modification techniques like CRISPR/Cas9, the cellular tropism of LV is altered by pseudotyping with other viral proteins (for example G protein of vesicular stomatitis virus). The reconstitution of native viral particles inside the transfected cells are prevented by splitting up of LV plasmids till three generations. The transfection mechanism is almost similar to that of AAV and Adv, the major difference relies with the size, where LV and AdV are approximately 90nm in diameter, compared to AAV, which is just 20nm in size. Therefore, LV can accommodate larger insertions. LV is particularly useful for CRISPR/Cas9, in case where additional packaging is desired to include one or more sgRNA or Cas9 constructs of varying sizes in order to carry out multiplex genome editing [57]. In attempt of duplicated exon(ex 18-30) removal from *dmd* gene, the patient derived primary fibroblast were transfected with CRISPR/Cas9 components by LV and restored native full length dystrophin. Multiple exon deletions have also been carried using LV, gRNAs designed favored the expression of almost native tertiary structure of dystrophin [61,62].

Immune responses for AdV and LV poses a major hurdle in using it as a delivery method for CRISPR/Cas9 [63]. Also creating the integration deficient HIV provirus is prime factor to be taken care. Even though LV integrates less likely with the genome, eliminating the integration chances completely is least possible [64]. In case of

integrations also, there is no assurance that it would happen only at the target sites. Incidences of off targets effects will be immeasurable, if the integration happens at the site of crucial cellular proteins [65].

Physical delivery methods

Electroporation: It is one of the most opted conventional transfection techniques for *in vitro* studies. Application of high voltage electric current pulses into the buffers containing cellular suspension enables the transient opening of nanometer sized pores in the cell membrane. This transient gateway in cell membrane allows the transfer of components with hydrodynamic diameters into the cell.

Irrespective of cell types, this technique can be applied for transfection of CRISPR/Cas9 components into the cells effectively. The large amount of voltage used remains a barrier for the application of this technique in *in vivo* studies. Mammalian cells are highly sensitive to the voltage ranges applied for electroporation compared to that of bacterial cells. Specially in case of using the immortalized cells lines, the issue of high voltages remains major barrier. Many attempts are still being carried to use electroporation in CRISPR/Cas9 component delivery, because of its easy transfection procedures [57]. Electroporation of DMD patient derived iPSCs to target the DMD exon 44 mutation by both NHEJ and HDR methods were performed [66]. With the aid of drug selection system, high percentage of HDR events has been accomplished. The off-target effects were also minimized by using a unique k-mer approach which allows the visualization of targetable regions in the entire genome.

In an attempt to address a deletion mutation in *dmd* gene, skipping of exon 51 was carried out by multiplexing capability of CRISPR/Cas9. The combination of SpCas9 and two sgRNA flanking exon51 was electroporated into DMD patient myoblasts. The target exon skipping was achieved cells only when SpCas9 with both sgRNAs were presented into the cells [67]. By deleting few common exons in *dmd* gene covering some mutational hot spots, could help in treating all the phenotypes related to that deleted exons. Multiplexing ability of CRISPR/Cas9 have been exploited to delete around 336bp (exon 45-55), which would in turn represent the DMD patients with the deletions in exon 45-55 regions. At the same time the decrease in size of 336bp reduces the efficiency of the therapy [68]. In order to achieve the gene modifications to turn a gene to its wild type, developing a complete personalized patient specific therapies are needed.

The abnormal reading frames in *dmd* gene were corrected by creating small indels that are created during NHEJ repair of DSB. SpCas9 with sgRNA targeting exon 51 was electroporated into DMD patient myoblasts with exon 48-50 deletion, where the NHEJ repair mechanism carried out to seal DSB created small indels in exon 51 and the dystrophin protein expression was observed [67]. Upregulating the utrophin protein expression gave another angle of treating DMD with CRISPR/Cas9. As a protein homologous to dystrophin, utrophin is expressed in muscles of myotendinous and neuromuscular junctions [69]. This homology can be exploited by CRISPR/Cas9 to treat DMD by simply upregulating utrophin. In this case the nuclease activity of Cas9 is not desired, therefore a new version of Cas9 called dCas9 (catalytically dead Cas9) is used along with some transactivators like VP16, which can be designated as dCas9-VP16. A gRNA have been designed to take this dcas9-VP16 to the target site, where VP16 favors the transcriptional activators and enhance the selected gene expression [70]. In case of utrophin expression the utrophin promoters (UTRNA and B) were targeted by gRNA and along with dSpcas9-VP16, they were electroporated into DMD patient cells with deletion mutations in dmd exon 42-52. The increased utrophin expression was observed, which was reflected the dystroglycan expression [61].

Excision of around 23 kb region from X chromosome by CRISPR/Cas9 was able to cover up exon 23 mutation in *mdx* mice model, which restored dystrophin expression in skeletal muscles and normalized calcium dynamics [29]. CRISPR/Cas9 plays significant role in creating the diseased models. A mouse model reflecting dystrophic phenotype of human (hDMD mice) was created using CRISPR/Cas9 by deleting exon 45 in *dmd* gene [71]. This model was utilized to study multiple exon deletion (exon 45-55) strategy of CRISPR/Cas9 and obtained the proper localization of dystrophin *in vivo*. But due to voltages applied in electroporation and extensive culture, resulted in lower frequencies of dystrophin fibers [67]. The corrosive solutions used in pretreatment of zygotes to favor the uptake of CRISPR/Cas9 components by electroporation resulted in the compromise of embryo viability [72]. Also, the recent electroporation protocols require the use of specialized electroporater devices. The EEZs (Easy Electroporation of Zygotes) have optimized the electroporation of CRISPR/Cas9 components that improves the embryo viability with widely used electroporaters, showed high efficiency in creating transgenic mice models [73]. Additionally, electroporation of pre-assembled Cas9/sgRNA proved to enhance transgenic capabilities [74]. These EEZs protocols can be adopted in future attempts to create DMD mice models and its further improvised gene editing.

Microinjection: Microinjection confers promising delivery of CRISPR/Cas9 components into the cells *in vitro*. The CRISPR/Cas9 components may be either plasmid DNA coding sgRNA and Cas9 protein or mRNA coding sgRNA and Cas9 protein or Cas9 protein with sgRNA as ribonucleoprotein. Either of the above CRISPR/Cas9 components can delivered into the cells using 0.5-5.0 lm diameter needle under the microscope. Using this microinjection technique, the barriers coupled with extracellular, cell membrane and cytosol are easily crossed by CRISPR/Cas9 components. The molecular weight of the components delivered is not the limiting factor in this case. Also, the known quantity is delivered to the desired cell of study, which lowers the incidence of offtarget effects [57]. In a study targeting nonsense mutation in exon 23 of *dmd* gene, the mouse zygote were microinjected with sgRNA and Cas9 along with HDR template [34]. This method of correcting a disease in its germ line gives an opportunity to set back all the cells in body to its wild type, including myogenic progenitors [34]. The modified germline cells resulted in 2-100% of wild mosaicism and normal structure and functions of muscles were restored. Microinjecting technique is widely used in creating desired type of animal models. Typically, microinjection is best suited type of delivery method in *in vitro* studies, because microscopic setting to inject the CRISPR/Cas9 components for *in vivo* studies is technically impractical.

Nucleofection: Nucleofection can be termed as a specialized electroporation technique, which can directly deliver the CRISPR/Cas9 components into the nuclei of target mammalian cells. This technique requires specific electric parameters and specialized solutions to carry out the delivery components into the nucleus. The delivered components resulted in enhanced gene expression, because it doesn't demand a cell to be in state of division or breaking of nuclear envelope to deliver the CRISPR/Cas9 components. The iPSCs derived skeletal muscles from DMD patients with frame shift mutations at hot spot (exon 45-55) region, were targeted to carry out multiple exon deletion (exon 45-55) by nucleofection of CRISPR/Cas9 components. This resulted in deletion of 725 kb, which is the highest recorded deletion

carried out using CRISPR/Cas9 technique in DMD studies [75]. These corrected iPSCs were transplanted to tibialis anterior of mdx mice and dystrophin positive fibers were observed. The 3D engineered heart muscles, which are created from iPSC derived cardiomyocytes showed to have better contractile force, when they were nucleofected with CRISPR/Cas9 components to target mutations in exon 50,51& 54 of *dmd* gene [76]. In order to target the mutations in amino terminal actin- binding domain (ABD-1), exons regions (3-9, 6-9, 7-11) of *dmd* gene were targeted by CRISPR/Cas9 components using nucleofection technique. By assessing the calcium cycling of cardiomyocytes derived from iPSCs, the least functionality of dystrophin was observed for exon7-11 deletions, whereas exon 3-9 deletions showed higher functionality of dystrophin [77]. The development of this technique to be used in true *in vivo* settings can strengthen the further clinical studies leading to its therapeutic usage.

Hydrodynamic delivery: Hydrodynamic delivery works by rushing up higher volume of solution (10% of body weight) along with the CRISPR/Cas9 components into the animal blood stream, particular tail vein, in cases of mice model. This increase in pressure makes the temporary permeability of parenchymal and endothelial cells possible, which allows the CRISPR/Cas9 components to penetrate into the cell membrane. The CRISPR/Cas9 components can be either nucleic acids or proteins. Since this means of delivery depends on transient increase in pressure, it works well with the closed system and hence suitable for *in vivo* studies alone. This mode of delivery with CRISPR/Cas9 components has been extensively studied in liver, but it can also be highly appealing to cells of heart, lungs, kidneys and muscles [57].

Even though this mode of delivery have not yet been tried to deliver the CRISPR/Cas9 components for treating DMD, attempts have been made to deliver the full-length mouse dystrophin gene to skeletal muscles throughout the hind limbs of the mdx mouse model and separate groups attempted to deliver the CRISPR/Cas9 components against chronic HBV infection [78,79]. The physiological issues created by sudden increase in blood pressure could result in liver expansion and cardiac dysfunctions [80]. This could pose a threat to patients' life. Therefore, before stepping into clinical settings, these complications are indeed to be addressed in detail.

Non-viral delivery vehicles

Lipotransfection: Nuclei acid deliveries have been extensively reported with lipid nanoparticles. The higher anionic nature and hydrophilicity of nucleic acids obviously needs some net cationic carriers to pass through cell membrane and escort it from enzymatic degradations. Lack of viral particles and its immune response issues throws high hope with lipid nanoparticles for delivery of CRISPR/Cas9 components. Similar to viral particles, lipid nanoparticles also find equal applications with *in vitro* and *in vivo* applications. When lipid nanoparticles are used, the CRISPR/Cas9 components may be either nucleic acid or ribonucleoprotein complexes [81]. Due to the higher anionic properties of ribonucleoproteins, this delivery method is well suited with sgRNA-Cas9 as RNPs [82]. However, the extra and intracellular barriers play a major hurdle in using it as effective transfection method. When the lipid nanoparticles along with CRISPR/Cas9 components have passed through the cell membrane, it should effectively escape the endosomal degradation pathway in order to successfully carryout the therapy. Even then it should translocate through the nuclear membrane to reach the target site of action. Commercially available cationic lipids like lipofectamines is well studied lipid nanoparticle for nucleic acid delivery [57]. This cationic lipid formulation allows the nucleic acids to easily pass the anionic cell membrane and favors endosomal escape in cytosol. Lipofectamine transfection efficiency was very low in myoblasts under the standard lipofectamine 2000 transfection. But still with some optimized protocols, insertion deletions were performed in *dmd* exon 50 and 54 of DMD patient derived iPSCs [83]. In another study CRISPR/Cas9 components containing two sgRNAs and Cas9 along with template DNA for HDR correction to target *dmd* exon23 was transfected to mouse muscle derived fibroblasts using lipofectamine 3000 and the dystrophin positive fibers were observed in corrected satellite cells [59]. Lack of viral components and its related immune responses developed much hope on lipid nanoparticles as delivery method. Considering the liposome surfaces to target specific cell types, improved packaging of CRISPR/Cas9 components and endosomal escaping capacities, will help in taking lipotrasfection as an efficient delivery method.

PEI (polyethenimine): Polyethenimine nanoparticle used in gene editing techniques, works by electrostatic interactions with the CRISPR/Cas9 components. FuGENE-6 is the widely used commercial reagent, which is a combination of some proprietary compounds with lipids. The vast used polymeric vector for nucleic acid delivery is Poly (L-lysine) and Polyethenimine (PEI). High charge density of branched PEI favors the efficient packing of plasmids and the endosomal escape is facilitated by its buffering ability. To carry out duplicated exon (exon 2) removal in *dmd* gene, PEI was used to transfect the CRISPR/Cas9 components to immortalized muscle cells derived from DMD patients with duplication mutation. Use of single sgRNA was shown to remove a duplicated exon, with dystrophin rescue [84]. The cytotoxicity of PEI is the important concern to be minimized in order to optimize this technique for efficient gene therapies [57].

Gold nanoparticles (AuNPs): Apart from the applications in biomedical sciences as inert carrier and imaging agents, gold nanoparticles are scoring its space in gene editing techniques like CRISPR/Cas9. By non-specific electrostatic forces gold nanoparticles can bind to a few variety of proteins including Cas9 RNPs[56]. AuNPs find application in CRISPR/Cas9 gene editing, due to its capacity to be internalized by different cell types [85]. CRISPR-gold containing Cas9 RNPs with template DNA for HDR, can be delivered *in vivo* by local administration. Along with endosomal disruptive polymers, CRISPR-gold can favors the escape of endosomal degradation pathways, due to polymers cationic nature [86]. These polymers facilitate the disruption of endosomes and favors the release of CRISPR/Cas9 components into the cytosol [85].

AuNPs of 15nm size were conjugated with thiolated DNA oligonucleotides along with CRISPR/Cas9 components, template DNA strand and endosomal disruptive polymer [56]. This conjugate was then used to target a DMD mutation in mice and restored dystrophin expression by 5.4% of corrected cells in a single injection. Reduced levels of fibrosis and 50% restoration of muscle functions was observed. Due to inert nature of AuNPs, the immune responses observed was very low, but still cytokine production have been recorded [87]. The cytotoxic effects caused by CRISPR/Cas9- gold nanoparticles seem to reduce the HDR efficiency on higher concentrations.

Discussion

This review delivers the comprehensive source about the delivery methods used for CRISPR/Cas9 in developing therapy for DMD. Optimizations of the delivery techniques are in demand to translate CRISPR/Cas9 for clinical settings. In case of DMD it is mandatory to prove that the gene editing is restoring the proper muscular functions

in skeletal and cardiac muscles with void of off target effects and leave the patients with very minimal cytotoxic or immune responses. Studies using animal models have certain limitations such as difference in regulation of cytotoxic levels, drug tolerance, ion channel activities, myosin functions which failed to represent the exact effects of drugs in human, therefore prior to the *in vivo* studies the preclinical studies that use iPSCs and its derived myotubes leads to the accurate understanding of the DMD patients mutations and drug effects. Also use of iPSCs is highly preferred in case if, biopsies are not available for patients with specific mutations.

Application of AAV in *in vitro, ex vivo,* and *in vivo* work, makes them highly flexible delivery vehicles. Also, AAV based Cas9 delivery has the potential to cause significant off-target genomic damage, due to the prolonged expression of Cas9 [60]. Several methods like inducible promoters, anti CRISPR proteins and chemical control of protein stability have been used to have certain control over Cas9 activity, but they cannot remove Cas9 and also few of those demands the use of additional non-human protein moieties [88-90]. Recent development of self-deleting AAV CRSIPR/Cas9 system which uses CRISPR gRNA that splice Cas9 coding sequence *in vivo,* gives a promising solution for permanent Cas9 expression in genome editing by CRISPR [33]. This self- deleting AAV CRISPR/Cas9 can be opted for the future *in vitro* and *in vivo* studies of DMD, where prolonged expression of bacterial components won't be a threat anymore. The small packaging size of AAV demands the use of multiple viruses to deliver Cas9 RNP and donor DNA *in vivo*, this reduces the incidences of HDR. In spite of some inspiring pre-clinical trials based on AAV, viral titer levels, immune responses against Cas9 and viral capsid proteins are indeed to be addressed in detail. Body wide correction of DMD in animal models have been achieved with dual vector approach, but still for maximum efficiency and translatability, using single vector system would be much effective [48]. Delivering Cas9 and sg RNA as RNPs would minimize the off-target cleavage occurring due to prolonged expression of Cas9 endonuclease, this can be achieved by improving the capacity of the delivering vehicles, which is a remarkable limiting factor with viral vector delivery systems despite of its hopeful *in vivo* delivery.

Improving the nanoparticles efficiencies to target the specific cell types, escaping the endosomal pathways and entering the nucleus could make these nanoparticles as potential delivering method for CRISPR. So far, the repair of DSBs via NHEJ are focused much in DMD gene editing because of its efficiencies in correcting frameshift mutations by single base editing without the need of a template DNA, ultimately making them the most adopted strategy. Though NHEJ repair is much reliable compared to repair via HDR, the chances of converting the various other mutant DMD gene to its wild type are least possible [46]. Since CRISPR/Cas9 carried with HDR have the potential to correct mutated genes back to their wild type sequence, the vast majority of genetic diseases can be targeted with this method [56]. CRISPR/Cas9 carried out based on HDR strategy can be successfully implemented either by the use of alternative CRISPR associated nucleases (such as Cpf1 or Cas9-nickase) or by inhibiting genes involved in NHEJ which may increase the efficiency of precise gene editing, if the HDR events were occurring in mitotically active myogenic precursors. Cytotoxicity and their nonspecific serum protein interactions were the major barriers for lipofectamines despite of its excellent delivery capacity [91-93].

Nanoparticles with DSPE-PEG (distearoylphosphatidylethano lamine-polyethylene glycol) created by some surface modifications mitigated this problem and improved its quality as delivery vehicle which may possibly implemented in developing a therapy for DMD [94]. The multiple exon deletion strategies and gene deletions covering the mutational hot spots are said to treat a wide range of DMD mutations but still, these strategies of gene editing could result in truncated dystrophin protein, which is again a milder version of DMD or BMD. So, it would not be supportive in developing complete cure therapy for DMD, but the prolonged life expectancy of patients. Therefore, development of well-established gene editing strategies of CRISPR/ Cas9 with optimized delivery should be accomplished in order to achieve a personalized gene therapy to get the complete cure of individuals with DMD, which is through a much laborious procedure.

Conclusion

Great power always couples greater responsibilities; recent creation of HIV resistant twins using the CRISPR technique leaves the entire world with fusion of fear and excitements. There is no doubt that CRISPR technique leaves peak of hope on treating genetic disorders, but patience will render its fruit. The off-target effects and ethical issues should be well studied on long run before stepping into clinical therapies.

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