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CRISPR/Cas9 as a Therapeutic Approach to Duchenne Muscular Dystrophy

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Abstract
Transhumanism, designer babies, gene therapy, and super-soldiers are founded upon the same concept—genetic engineering. Clustered Regularly-Interspersed Short Palindromic Repeats (CRISPR) is a natural bacterial immune response method that takes advantage of gene manipulation to prevent an infection from mobile genetic elements. Since Mojica et al. (2005) first suggested the relationship between the CRISPR/Cas system and prokaryotic immunity, significant advancements have been made in understanding the mechanism and subsequent applications of CRISPR. CRISPR, has three main subtypes based on unique proteins and interference pathways and serves as an accurate and effective method for gene editing. Its mechanism consists of spacer acquisition, crRNA production, and interference. This highly dynamic form of genetic modification generates significant CRISPR sequence differences in species that are almost identical when comparing the rest of their genome. CRISPR/Cas9 demonstrates the simultaneous alteration of multiple gene loci in individual cells with a high degree of specificity and precision. Duchenne muscular dystrophy (DMD) is a genetic neuromuscular disorder characterized by progressive muscle loss and eventual death in the late teens to early twenties. DMD affects calcium homeostasis, vasculature, genetic regulation, muscle movement, glycosylation, tissue remodeling, and inflammatory response mechanisms. Current treatments include antifibrotic pharmaceuticals, calcium maintenance, myostatin inhibitors, upregulation of uthrophin, nonsense suppression drugs, vector-mediated gene therapy, and cell transplantation. This review describes the mechanism of CRISPR/Cas9 and its application as a therapeutic approach to treating Duchenne muscular dystrophy.

Keywords: CRISPR, genomic engineering, Duchenne Muscular Dystrophy, Cas protein, interference mechanism, PAM recognition

Introduction

DNA sequencing assays are highly efficient and economical and dramatically increase our understanding of genetic diseases. Once we identify and comprehend the cause of the disease, the quandary is to figure how to reverse the mutation and cure it. The ability to modify genes in mice serves as a crucial function to model human diseases (Yang et al., 2014). Conventional approaches to study and develop disease models utilize homologous recombination, such as retroviral insertion (Kuehn et al., 1987), in embryonic stem cells, to knockout the gene of interest (Thomas and Capecchi, 1987). Genetic variations among embryonic stem cells (Ledermann, 2000) coupled with relatively long time requirements (Markel et al., 1997) cause significant limitations to this approach (Carbery et al, 2010). Other methods used in gene editing include zinc-finger nucleases (ZFNs) (Kim et al., 1996; Geurts et al., 2009) and transcription activator–like effector nucleases (TALENs) (Nanjidsuren et al., 2016; Tesson et al., 2011).

Plasmids, bacteriophages, and transposons promote adaptation and survival by inducing the development of toxic compound degradation, antibiotic resistance, and other evolutionary advantages (Frost el at., 2005). Among mechanisms used to resist harmful infections and monitor the entry of genetic material, bacterial species utilize clustered regularly interspersed short palindromic repeats (CRISPR) (Labrie et al., 2010). CRISPR, a defense mechanism ensuring resistance against viral invasion, is exhibited by an estimated 40 percent of eubacterial and 90 percent archaeal species (Hatoum-Aslan et al., 2011). Kunin et al. (2007) used PILAR-CR, an algorithm that identifies CRISPR repeats, and found 561 arrays in 44 percent of the genomes tested. A population of ~1031 viruses coupled with ~1025/s rates of infection promoted the evolution of defense pathways that effectively identify foreign genetic elements and mount a response to degrade harmful foreign nucleic acids (Hendrix, 2003; Richter et al., 2012). These repeats are ubiquitous in archea and evident in some bacteria (Lillestøl et al., 2006).

While sequencing the Escherichia Coli iap gene in 1987, a researcher identified an "unusual structure" at the 3' end flanking the gene, noting five repetitive sequences of 29 nucleotides each having unique 32 bp spacer segments (Ishino et al., 1987). In 2005, Francisco Mojica was first to suggest a relationship between the CRISPR-Cas system and bacterial immunity. This observation later turned out to be extremely significant, yielding a dramatic benefit to genetic research. CRISPR, an adaptive bacterial immunological response mechanism, consists of alternating sequences, one repetitive and the other a segment of a viral genome or a plasmid sequence. CRISPR utilizes CRISPR associated (Cas) proteins and small non-coding RNAs for its function. Abutting each CRISPR loci, CRISPR-associated (CAS) genes encoding for various enzymatic proteins couple with CRISPR to form a multitude of different CRISPR/CAS pathways (Horvath and Barrangou, 2010). Incorporation of phage DNA into the spacer portion of a CRISPR array in Streptococcus thermophilus yielded resistance towards viral infection of the corresponding phage (Barrangou et al., 2007). CRISPR can be involved in several processes such as; replicon partitioning in

halo bacteria (Mojica et al., 1995), DNA rearrangements within a replichore (Deboy et al., 2006), and thermal adaptation in E. Coli (Riehle et al., 2001). There is a direct correlation between an increased sensitivity to viral infection and a mutation to the Cas genes or the spacer sequences of the corresponding virus (Barrangou et al., 2007; Brouns et al., 2008; Oost et al., 2014). CRISPR/Cas systems can be used to manipulate genes with substantial precision and accuracy, effectively giving researchers the ability to develop causal linkages between known mutations and observed phenotypes (Hsu et al., 2014). This review article will assess the application of the CRISPR/Cas system as a therapeutic approach to Duchenne muscular dystrophy.

Methods

An analysis of scholarly articles with a focus on papers published in peer-reviewed journals with high impact factors were performed through access to databases of the Touro College Online Library, Medline, Proquest, NCBI Pubmed, and Google Scholar. In-print articles were obtained from the Touro College library in the Avenue J campus. An analysis of both review and experimental research articles were conducted to delineate the mechanism and outline recent applications of CRISPR in a clinical setting. In each database, the search word "CRISPR" prompted recent publications on that topic. Articles that were labeled as "similar" to papers published recently were also used. Proteins associated with CRISPR discussed in this paper were analyzed using the uniprot database. Original research papers describing aspects pertaining to the discovery, mechanism, and applications of CRISPR were found on the webpage of Dr. Lluís Montoliu's Lab at Centro Nacional de Biotecnología.

Results

Spacer Acquisition

The genetic interference pathway of the CRISPR/Cas system is initiated with spacer acquisition upon entry of foreign genetic material (Swarts, 2012; Richter et al., 2012; Marraffini, 2010a). This step is highly dynamic and involves the recognition of foreign DNA by the host as well as its first integration into the spacer portion of a CRISPR array. Identification of the foreign genetic element is essential to the CRISPR mechanism. Proto-spacer adjacent motifs (PAMs) or spacer precursors are important components of the CRISPR systems. Each CRISPR-CAS variant can correspond to a specific spacer precursor or proto-spacer that will be evident on the foreign DNA particle. The interference target is determined by a specific short motif sequence that corresponds to each CRISPR variant. Using the classification of CRISPR variants determined by Kunin et al. (2007), a sequence of either two or three nucleotides abutting each proto-spacer was found to be conserved in six main groups (Mojica et al., 2009). This finding suggests a correlation between PAMs and each CRISPR-CAS system.

Various Cas genes are found adjacent to all CRISPR arrays with the exception of Thermoplasma acidophilum (Marraffini and Sontheimer, 2010a). More than 45 distinct CRISPR associated protein families have been identified using Hidden Markov models (Haft, 2005). Each subtype of CRISPR is classified based on associated Cas genes as well as its distinct repeat characteristics (Gesner, 2011). Three main subtypes are classified based on the presence of a unique Cas protein: type 1 has Cas3, type 2 has Cas9, and type 3 has Cas10 (Gleditzsch et al.; 2016, Makarova et al., 2011a; Richter et al., 2012). A fourth subtype, with its mechanism and function still uncharacterized, is called CRISPR type U (Koonin and Makarova, 2013).

Cas1 and Cas2, a metal-dependent nuclease (Wiedenheft et al., 2012) and a pH-dependent nuclease (Ka et al., 2014), respectively, are necessary in initiating spacer acquisition by incorporating non-self DNA into the leading end of the CRISPR array (Mojica et al., 2009). Strains of E. Coli lacking the endogenous cas genes prevented spacer acquisition from occurring without affecting further steps in the pathway, outlining their importance in the first step. Both Cas1 and Cas2 are evident among almost all CRISPR systems (Makarova et al., 2011b), possess a crucial role in spacer acquisition (Yosef et al., 2012), and contain highly conserved motifs. CRISPR type U is the only known form of CRISPR that does not possess a CRISPR array or Cas1 (Koonin and Makarova, 2013). These findings outline the importance of Cas1 and Cas2 in spacer acquisition (Oost et al., 2014). Cas1 and Cas2 form a complex determined by a 2.3Å resolution crystal structure (Nuñez et al., 2014).

Both DNA recognition and spacer acquisition will be prevented if the Cas1-Cas2 complex formation is disrupted by a mutation (Nuñez et al., 2014). In addition to its role in the CRISPR/Cas system, Cas1 is believed to be involved DNA repair (Babu et al., 2010). The recognition of a variant-specific adjacent short sequence on the foreign DNA particle prompts the incorporation of a spacer precursor. This completes the first step in the CRISPR defense mechanism (Mojica et al., 2009). Replication of the inserted repeat begins with the repeat most proximal to the leader portion of the array (Yosef et al., 2012). The CRISPR response is amplified through the increase in spacer sequences corresponding to a specific foreign DNA element (Swarts et al., 2012).

crRNA Expression

The successful incorporation of a foreign DNA segment into the spacer region of the CRISPR array and the production of a multiunit precursor (Koonin, 2006) permits the subsequent processing of the precursor CRISPR RNA (pre-crRNA) (Oost et al., 2009; Wiedenheft et al., 2012). The crRNAs specific to each CRISPR array are integral to the CRISPR pathway. Analysis

of the CRISPR/Cas system in Escherichia Coli K12 determined that both the repeats and the spacers within the CRISPR are transcribed into a long precursor RNA (Marraffini and Sontheimer, 2010a). The crRNA transcript transcribed from the CRISPR array requires cleavage prior to activation.

Cas proteins catalyze the conversion of precursor RNAs (pre-crRNA) into small crRNAs (Gleditzsch et al., 2016; Marraffini and Sontheimer, 2010b). Eight Cas genes were identified in this strain: cas123 and casABCDE (Brouns et al., 2008). After knocking out each individual Cas gene using in-frame single-gene deletions (Baba and Mori, 2008), the resulting transcript determined the position of each gene on the CRISPR array. Further, the RNA cleavage assays did not require ATP or divalent metal ions to progress. CasE, an endoribonuclease resembling an RNA-binding protein, fused together with the maltose binding protein (MalE) in Escherichia coli K12, did not require any other Cas proteins to cleave the pre-crRNA. Northern blot analysis determined His20 residue to be essential for catalysis of the pre-crRNA. A casE knockout prevents the processing of pre-crRNA in Escherichia coli K12 outlining its importance in the pathway (Brouns et al., 2008). Cascade (CRISPR-associated complex for antiviral defense), a ribonucleoprotein, is a 405 kDa undecamer made of five different Cas proteins. Cascade is coupled with a 61 nucleotide crRNA structure spanning the length of the protein complex that has a 5'-hydroxyl and 2',3'-cyclic phosphate termini forming a seahorse configuration prior to target DNA binding (Jackson et al., 2014, Jore et al., 2011).

Cas3, essential to all type I CRISPR systems, functions both as an ATP-dependent type A superfamily 2 helicase and a ss-DNA nuclease (Brouns et al., 2008; Gesner, 2011; Huo et al., 2014; Sinkunas et al., 2011). Cas3, together with the mature crRNA as a guide and Cascade as a targeting complex, catalyze the degradation of double stranded DNA elements (Huo et al., 2014). Brouns et al. exposed Escherichia coli to virulent Lambda phage in various scenarios to determine the role of Cascade and Cas3 in resisting phage infection. Two Escherichia coli strains each contain a CRISPR variant that targets four important lambda genes: the coding strain produced crRNAs complimentary to both the coding and non-coding strand of the four genes, while the template strain produced crRNAs complementary to the proto-spacer regions. With both Cascade and Cas3 present, results showed a hundred-fold and ten million-fold decrease in sensitivity to phage infection with the coding strain and template strain, respectively. Based on the aforementioned experiment, the presence of Cascade and Cas3 is crucial to phage resistance in the CRISPR defense mechanism (Brouns et al., 2008).

High resolution X-ray structure analyses of Cse3, a component of Cascade, both before and after cleavage of pre-crRNA, suggests a molecular basis for the mechanism of crRNA recognition by the Cascade. Three structures of Cse3 bound to different RNA products all displayed a stem loop complex, suggesting the involvement of Cse3 in RNA recognition (Gesner et al., 2011). Processing of pre-crRNA into mature crRNA allows effective interference by the CRISPR-Cas system.

Interference

The interference stage differs mechanistically among the three main subdivisions of CRISPR: type I, II, and III. Each subtype accomplishes the same goal of foreign DNA degradation, differing only in the route of interference. The goal of CRISPR interference is to degrade the foreign genetic elements that correspond to the acquired spacer sequences in the CRISPR array.

CRISPR type I, evident in both bacteria and archaea, exploits Cas3 for target degradation. Cascade is the multi-subunit crRNP (CRISPR ribonucleoprotein) complex that is unique to CRISPR type I. Different routes, depending on the subtype of CRISPR type I, can induce a conformational change in the crRNP complex which may cause the recruitment of Cas3 for degradation (Oost et al., 2014). The interference in CRISPR type I is initiated when the mature crRNA binds to a variant of Cas6, an endoribonuclease. The variant will depend on the subtype of CRISPR type I (Richter et al., 2012). The crRNA forms a stem loop within each repeat and bind to the corresponding Cas6 protein. Cas5d, the Cas6 variant of type I-C/Dvulg, processes the pre-crRNA. Further, Cas5d binds to the mature crRNA (Nam et al., 2012) and recruits the Cascade which induces a conformational change in the complex (Oost et al., 2014). In type I-E, CasA or Cse1, functions to discriminate between self and foreign DNA through the recognition of a proto-spacer adjacent motif (PAMs) (Sashital et al., 2012; Westra et al., 2013) as well as induce interactions between DNA and Cascade (Jore et al., 2011). The initial interaction with the foreign DNA employed by a short loop on Cse1 recognizes a sequence of seven nucleotides near the 5' end in addition to the PAM (Richter et al., 2012).

The CRISPR Type II system uses trans-activating crRNAs (tracrRNA) in crRNA processing. Strains lacking tracrRNA did not yield mature crRNA, demonstrating the importance of tracrRNA in crRNA processing. The tracrRNA base pairs with 24 nucleotides (Deltcheva et al., 2011) of the crRNA and recruits RNase III for cleavage. The fusion of tracrRNA and crRNA to become a single-guide RNA (sgRNA) can be easily programmed and is used in the modification of multiple DNA sequences simultaneously (Bolukbasi et al., 2016; Cho et al., 2013; Mali et al., 2013). The process of cleaving tracrRNA and crRNA also

requires a Cas protein called Csn1 determined by in-vivo in-frame deletions of the gene. The subsequent appearance of mature crRNA and cleaved tracrRNA after the induced expression of Csn1 further supports the hypothesis that Csn1 is vital to the processing of crRNA and tracrRNA (Deltcheva et al., 2011). However, Csn1 only serves to stabilize the interaction between pre-crRNA and tracrRNA without a direct contribution to the catalysis, further emphasizing catalytic role of RNase III (Fonfara et al., 2013). CRISPR-associated endonuclease Cas9 or Csn1 is Mg2+-dependent and contains two endonuclease domains. One is a RuvC-like nuclease domain and the other is a HNH nuclease domain cleaving the target DNA non-complimentary and complimentary to the crRNA, respectively (Jinek et al., 2012; Anders et al., 2014; Nishimasu et al., 2014). Csn1 undergoes a conformational change upon binding to the tracrRNA and mature crRNA, effectively activating its nuclease activity (Jinek et al., 2014). Although both type I and type II use PAM recognition to bind to DNA, there are two technical differences. Firstly, the PAM motif is adjacent to the 5' end of the crRNA in type I and the 3' end in type II. Secondly, the PAM motif of the target DNA in type I is the strand that directly interacts with the crRNA whereas it is on the displaced strand in the mechanism of type II. After the ribonucleoprotein complex is in its active form, the subsequent activation of the nuclease domains mediates site-specific double stranded breaks of the foreign DNA via Cas9 (Oost et al., 2014). Sternberg et al. classified the interaction between the RNA-Cas9 and the target DNA to be through a three-dimensional collision outlining the specificity of the CRISPR mechanism (2014).

The crRNP complexes in CRISPR type III are structurally alike and have similar roles to type I. Type III-A and type III-B are associated with csm and cmr complexes, respectively. The type III-A system displays the ability to degrade double-stranded DNA and single-stranded RNA (Niewoehner and Jinek, 2016), whereas the type III-B system targets RNA (Hale et al., 2009). Type III-A, present in staphylococcus epidermidis RP62a, contains nine csm genes (Hatoum-Aslan et al., 2014) and does not rely on PAM recognition for target degradation (Marraffini and Sontheimer, 2010b). Instead, the csm complex uses the csm3 and cas10 subunits to target and degrade single-stranded RNA and double-stranded DNA, respectively. Csm6, a single-stranded RNA-specific endoribonuclease noted for its higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domain, is integral to the function of the csm complex. Ribonuclease activity was determined to be a common feature among orthologs of csm6, outlining its importance in the type III pathway. Although the mechanism of csm6 interference is currently inconclusive, a notable theory posits that when the csm complex is unsuccessful in resisting foreign invasion, csm6 somehow activates and targets its own nucleic acids inducing apoptosis. Such regulatory methods serve as an important defense in preventing further infection. Structural analysis of csm6 in Thermus thermophilus describes multiple binding domains which could indicate that there are ligand-dependent levels of catalytic activity (Niewoehner and Jinek, 2016). Type III-B in Thermus thermophilus uses the cmr complex, an 11 subunit protein complex comprised of six distinct proteins aptly labeled cmr1-6, for RNA degradation. The cmr complex is a Mg2+-dependent endoribonuclease that targets the RNA strand at multiple sites complementary to the crRNA. Degradation begins at the 3' end of the target RNA strand and cleaves toward the 5' end with each cleavage separated by six nucleotides. The distance between two cmr4 subunits is consistent with the six nucleotide intervals present in the cleavage mechanism which suggests characterizing cmr4 as a ribonuclease within the cmr complex (Staals et al., 2013). A significant distinction in the molecular mechanism of CRISPR type III is that it relies on Cas10/Csm or Cas10/Cmr complex for target interference whereas type I and type II require a recognition sequence (Samai et al., 2015).

It is important to note the ability of the CRISPR/Cas system to differentiate its own genetic material from foreign nucleic acids. Without the basic capacity to distinguish self versus non-self, all CRISPR mechanisms would act on their own DNA and subsequently induce autoimmunity and cell death. The basic mechanism behind preventing self-degradation relies on a genetic distinction between target and self. Since the sequences incorporated in the spacers are meant to assist in the recognition of foreign genetic elements, they are the same and should cause self-interference. However, analysis of the CRISPR array in Staphylococcus epidermidis yielded dissimilarities between sequences of target DNA and the CRISPR DNA that are not in the spacer region. The higher degree of base complementarity between CRISPR DNA and crRNA in regions flanking the spacer sequences prompts evasion of interference. Adding fifteen base pairs matching both sides of the spacer's flanking sequences onto a target strand showed that interference was unsuccessful in the plasmid that had the 5' flanking sequence. The exact "protective region" was determined to be eight base pairs flanking the spacer sequence. It is the variable complementarity within sequences abutting the spacer region that prevents autoimmunity in all CRISPR systems (Marraffini and Sontheimer, 2010b).

CRISPR/Cas9 Genome Engineering

Jinek et al. (2012) was the first to propose the application of CRISPR/Cas9 for genomic manipulation. CRISPR/Cas9 is a form a CRISPR type II and is well understood (Ran et al., 2013). The purpose of CRISPR/Cas9 is to induce double-stranded breaks at specific locations within a chromosome. Cong et al.

(2013) investigated the application of CRISPR/Cas9 in genomic engineering by reconstructing the CRISPR type II locus of Streptococcus pyogenes SF370 in mammalian cells. A spacer sequence that resembles a specific portion of the EMX1 gene adjacent to the proper PAM sequence was designed and then transfected into 293FT cells along with RNase III, Cas9, tracrRNA, and pre-crRNA. Notably, cleavage activity was prevented when a base mismatch between the protospacer and the guide RNA sequence was within eleven nucleotides of the PAM sequence, demonstrating the specificity and accuracy of CRISPR/Cas9. Results yielded effective cleavage even without the addition of RNase III. This finding outlines three essential components required for this method of genetic engineering; Cas9, mature crRNA, and trans-activating crRNA.

Cas9 is an endonuclease that requires a Mg cofactor in order to bind to target DNA (Jinek et al., 2012) and is inhibited by EDTA (Jiang et al., 2016). The target sequence needs to abut the 5' end of a protospacer adjacent motif. Each ortholog of Cas9 requires a specific PAM sequence. The crRNA contains the twenty nucleotide sequence used to target the gene of interest that leads to the PAM. Furthermore, the crRNA requires tracrRNA for its activation into discrete units. When used in genome editing, the crRNA and tracrRNA are fused together to become a complex commonly called single-guide RNA (sgRNA) or guide RNA (gRNA) (Ran et al., 2013).

Mali et al. (2013) described how the CRISPR/Cas9 system can be used to both stimulate homologous recombination and modify a locus. They developed human embryonic kidney HEK 293T cells that contain a green fluorescent protein (GFP) sequence with an interruption that prevented functionally fluorescing GFPs. Two gRNAs were designed to target the region that disrupted florescence. After transfection of a donor to repair the sequence, Cas9, and a gRNA, flow-activated cell sorting (FACS) began detecting fluorescing cells at ~20 hours, elucidating the efficiency of CRISPR/Cas9 in inducing homologous recombination with a repair donor. To demonstrate how CRISPR/Cas9 can modify genomic loci, Mali et al. (2013) introduced two gRNAs that would each target nineteen base pairs with one base pair in-between them and then a double stranded donor sequence to take its place at the AAVS1 locus. PCR and Sanger sequencing assays confirmed the integration of a foreign sequence in a genome. These findings demonstrate important applications of CRISPR in genomic engineering.

Another important application of CRISPR/Cas9 is the single-step induction of mutations in multiple genes. Developing disease models through targeted deletions and engineering in multiple chromosomes gives extensive insight into the formation of various illnesses. Maresch et al. (2016) exploited electroporation to introduce CRISPR/Cas9 vectors in pancreatic cells and demonstrated optimal results when targeting a "few hundred cells" in each organ. To investigate pancreatic tumorigenesis, sgRNAs for thirteen tumor-suppressor genes and two neutral genes were transfected into a mixture of C57BL/6J and 129S mice strains. Magnetic resonance imaging determined the average time for tumor development to be 10.7 weeks and a 54% tumor incidence at 24 weeks. Next generation sequencing analysis confirmed a significant number of mutations in the target sites of tumor tissue samples. Furthermore, it did not find any significant mutation rates in the target sites of the tissue surrounding the tumors which is explained by the electroporation protocols that target only a few hundred cells in relatively small area (Maresch et al., 2016). All the aforementioned capabilities of CRISPR/Cas9 amalgamate to produce a highly efficient and effective method of genomic engineering with a short time period required to develop disease models. Prospective applications of CRISPR/Cas9 can involve the introduction of targeted mutations that can prevent the acquisition of diseases such as Human Immunodeficiency Virus (Lombardo et al, 2007). The future applications of CRISPR engineering are limitless and serve as an extremely viable option for use as a therapy for Duchenne Muscular Dystrophy.

Duchenne Muscular Dystrophy

In 1836, Gaetano Conte was the first to describe a case of Duchenne muscular dystrophy (DMD) (Nigro, 2010), an X chromosome linked recessive disorder that is classified as a severe progressive muscle wasting disorder. This neuromuscular disease is caused by a mutation in the DMD gene that codes for dystrophin (Hoffman et al., 1987), a 427 kDa rod-shaped cytoskeletal multi-domain protein made of 3,685 amino acids (Koenig et al., 1988) that is expressed in all human muscle cell types. Dystrophin interacts with dystrobrevin alpha (Sadoulet-Puccio et al., 1997), alpha-1-syntrophin (Ahn et al., 1996), and beta-1-syntrophin (Ahn and Kunzel, 1995). The primary function of dystrophin is to connect a cytoskeleton component, actin, with the extracellular matrix (Norwood et al., 2000). DMD occurs when mutations affecting the open reading frame cause premature termination of dystrophin during translation, resulting in a protein with complete loss of function (Yiu, 2015). The end result of a deficiency in dystrophin is muscle fiber degeneration and is believed to be secondary to factors such as sarcolemma impairment, structural damage to the cytoskeleton, and an aberrant calcium homeostasis. Patients with DMD require respiratory, cardiac, orthopaedic, and nutritional management throughout their lives (Yiu, 2015). Life expectancy for DMD patients is usually 25 years with the cause of death commonly being cardiomyopathy or lung issues (Long et al., 2014).

Pathophysiology

DMD, being a neuromuscular disorder, detrimentally affects the body's mechanical abilities, calcium homeostasis, vasculature, genetic regulation, glycosylation, tissue remodeling, and inflammatory response mechanisms. The absence of dystrophin or other proteins within the dystrophin associated complex could significantly decrease normal contractions that increase tension as the muscle lengthens, or eccentric contractions, thereby damaging the membrane of the muscle fibers. A muscle biopsy will show muscle fiber degeneration or necrosis. Eventually, the continual attempts to regenerate the muscle fibers leads to a burnout and begin substituting muscle for connective and adipose tissues (Deconinck and Dan, 2007).

DMD induces a leak within the calcium channels of the cell. The lack of dystrophin affects the structure of the membrane causing compensatory mechanisms that maintain calcium levels to eventually become ineffective. When there is a prolonged influx of extracellular calcium, proteases are activated, causing further degradation of the membrane, which leads to a further increase in intracellular calcium. This glut of calcium can presumably lead to cellular death.

Neuronal-type NO synthase (nNOS) is normally localized to the membrane of white muscle fibers and produces nitric oxide, which is a short-lived highly reactive signaling molecule with important biological functions (Nelson and Cox, 2013). The absence or deficiency of dystrophin causes a reduction in nNOS activity as well as its delocalization from the sarcolemma (Brenman et al, 1995; Crosbie et al., 2002). Dabiré et al. (2012) demonstrated a link between vascular endothelial dysfunction and the expression of endothelial and neuronal nitric oxide synthases in DMD patients.

The conversion of mechanical stimuli into electrical or chemical signals is known as mechanotransduction and is involved in genetic expression and other important physiological processes (Katsumi et al., 2004). Goldspink (1998) demonstrates the effects of muscle activity on mechanotransduction. The progressive loss of muscle tissue occurs secondary to the lack of autocrine insulin-like growth factor-1 production, which is used to repair muscle tissue.

CRISPR/Cas9 Therapy

The human dystrophin gene contains seventy-nine exons and seventy-eight introns (Kole and Krieg, 2015) with at least seven promoters. Alternative splicing yields different variants of dystrophin depending on the stage of development and type of tissue (Im et al., 1996). A spontaneous mdx gene mutation in a colony of C57BL/10ScSnJ mice resulted in increased serum levels of specific proteins as well as histological similarities compared to that of human muscular dystrophy (Bulfield et al., 1984). This spontaneous point mutation yielded a stop codon affecting exon 23 in the dystrophin gene (Sicinski et al., 1989). Biochemical similarities coupled with cross-breeding analysis of mutant and normal mice set the precedent to use the mdx mouse as a model for muscular dystrophy (Bulfield et al., 1984). Although not entirely equivalent to the human disease, researchers utilize the mdx mouse as the predominant model to investigate pathogenic mechanisms of DMD (Partridge, 2013).

The primary goal is to cure DMD by correcting any harmful mutations. Treatments either alleviate the symptoms or aim to cure the disease itself. Pharmacological approaches improve muscle function with corticosteroids (Mendell et al., 1989), maintain calcium homeostasis (Zhao et al., 2012), inhibit the IKK/NF-κB signaling pathway (Acharyya et al., 2007), reduce inflammation and induce the upregulation of uthrophin as a surrogate to dystrophin (Gordon et al., 2013). Glucocorticoids, such as prednisone or deflazacort, are beneficial to muscle function and are the only accepted drug therapy of DMD (Matthews et al., 2016). Van Deutekom et al. (2001) describes a form of gene therapy that attempts to correct the reading frame through the induction of skipping an additional exon. An exon 45 deletion exhibited in DMD patients causes a stop codon in exon 46. The open reading frame is restored when exon 46 is deleted. The resulting protein is still dysfunctional yet displays a milder form DMD, outlining a method of treating the disease. Other attempted strategies of treatment include antifibrotic pharmaceuticals, myostatin inhibitors, nonsense suppression drugs, vector-mediated gene therapy, and cell transplantation (Shimizu-Motohashi, 2016).

The aforementioned therapeutic approaches to treat DMD focus on either reestablishing the expression of or compensating for a deficiency in dystrophin. The efforts to treat DMD are, in many instances, relatively transient and compensatory without any curative effects. CRISPR/Cas9 is revolutionary for its attempt to treat the underlying cause of the disease— mutation or mutations in the dystrophin gene. Long et al. (2014) demonstrated the application of CRISPR/Cas9 to repair the genetic defect in an animal model of DMD. The zygotes of the mdx mouse, containing a nonsense mutation in exon 23 of the dystrophin gene (Sicinski et al., 1989), were injected with a 20 nucleotide single-guide RNA containing a PAM sequence, Cas9, and 90 base pair single stranded template. This template strand incorporates four silent mutations as well as a TseI restriction site for data analysis. After a double-stranded break was induced by Cas9, the strands were repaired by either homology directed repair (HDR) or nonhomologous end-joining (NHEJ). The optimized condition involved injecting the Cas9, sgRNA, and template into the zygote and then performing re-implantation into a female

mouse. The offspring of that mouse determined the results of the experiment. Analysis of eleven repaired mdx progeny revealed adult development of all mice without signs of abnormal phenotypes. Control groups were used to test for off-target effects and yielded data consistent with previous genome-wide studies outlining the specificity of Cas9. Using histological analysis of different muscles, the results of this experiment demonstrated the capability of CRISPR/Cas9 to repair the primary mutation that causes DMD, thereby preventing the symptoms associated with the disease. The determined threshold for sufficient repair was 17% which effectively displayed a dystrophin level comparable to the wild-type mouse. This finding suggests a mechanism of selective advantage for the repaired skeletal myocytes. Soles and heart tissue immunostaining of a three-week old mdx repaired mouse of 40% displayed myofibers without dystrophin while the nine-week old mdx repaired mouse of 41% did not reveal any myofibers without dystrophin. The dystrophin expression levels between the three and nine week old mice were insignificant, suggesting a compensatory mechanism of rescue by the repaired nuclei in a myofiber. Immunostaining revealed myofibers containing dystrophin secondary to a fusion between a repaired cell and dystrophic muscle, providing further evidence of a rescue mechanism. Additionally, the serum creatine kinase levels were inversely related to the percentage of genomic repair, which is consistent with previous data in this paper. A higher level of serum creatine kinase signifies muscle breakdown. The repair of only a percentage of cells can induce a total rescue, suggesting an unknown mechanism that induces muscle regeneration in mice treated with CRISPR/Cas9. The results of this experiment yield a breakthrough in our approach to cure previously incurable diseases by effectively correcting the underlying cause of the disease.

Discussion & Conclusion

Analysis of the CRISPR/Cas system, both in terms of its mechanism and bioengineering applications, yields a plethora of data on various diseases. The short time requirements (Markel et al., 1997) coupled with the simultaneous manipulation of multiple genes (Bolukbasi et al., 2016) characterizes CRISPR as an advanced and highly efficient approach to modeling and thus understanding maladies. Applying a form of CRISPR type II to induce double stranded breaks provides researchers the ability to target a portion of DNA with high specificity and then stimulate homologous recombination to modify a specific locus (Mali et al., 2013). Duchenne muscular dystrophy is a debilitating neuromuscular disease commonly affecting males and generally leads to death before the age of 25 (Long et al., 2014). DMD is a prime example to be used in the application of CRISPR/Cas9 for its well defined mutations that cause the disease. The data presented in experiments using CRISPR/Cas9 to modify a gene seem to be consistent and display accuracy and efficiency (Cong et al., 2013; Mali et al., 2013; Maresch et al., 2016). Based on current data, CRISPR/Cas9 genomic engineering is a promising and hopeful route to effectively reverse disease-causing genetic mutations such as Duchenne muscular dystrophy.

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