CRISPR: A Revolutionary Tool for Modeling and Treating Cancer and Duchenne Muscular Dystrophy

By

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A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.)

School of Pharmacy

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April, 2022

Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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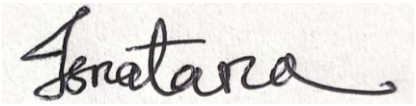

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Approval

The thesis/project titled "CRISPR: A Revolutionary Tool For Modeling And Treating Cancer and Duchenne Muscular Dystrophy" submitted by Israt Ara Jannat Eva (18146047) of Spring, 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on April, 2022.

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Ethics Statement

This study did not involve any human or animal trial. Proper credit is given to the original information collection sources.

Abstract

The novel CRISPR/Cas system is an effective and widely used gene-editing tool that has created the opportunity to study gene function and understand the pathogenic mechanisms underlying various genetic disorders. It has uncovered the pathophysiology of several previously unsolved medical conditions and is currently an essential genome-editing tool being employed to develop new drugs and innovative gene therapies for potentially life-threatening diseases such as, cancer and Duchenne muscular dystrophy (DMD). The specificity, accuracy, high probability of successful outcomes, cost and time effectiveness of CRISPR/Cas systems have made it a reliable means to treat cancer and DMD over other previously used genetic modification tools. This review will discuss how CRISPR/Cas can be utilized to design in vivo and in vitro disease models, target cancer cells, and aid in the identification of novel drug targets to treat cancer and DMD patients.

Keywords: CRISPR/Cas9 mechanism; In vivo and in vitro modeling; CRISPR/Cas9 delivery; Mutation targeting in cancer and DMD; Chromosomal rearrangement; Mouse modeling; Off-target limitation; DMD mutation in human iPSCs; Myoediting of DMD; Therapeutic application of CRISPR.

Dedication

Dedicated to my parents, my brother and respected faculties.

Acknowledgement

First and foremost, I am grateful to my Almighty to give me the strength, wisdom, hope and patience to complete this project. I am grateful that Almighty gave me this great opportunity to involve myself in this novel path of being pharmacist and blessed me to come this far. Without HIS blessings I would have never come this far of my graduation life.

Secondly, I would give my sincere gratitude to my project supervisor, Kazi Fatema Rahman, Lecturer of School of Pharmacy, Brac University. Her sincere encouragement, guidance in every little things and direct suggestions made my working process smooth and boost my confidence about my work. Without her motivational word and appreciation I would have never able complete my project work properly. Then, I am grateful to my School of pharmacy and respected faculties to give me the opportunity to complete this project and pursue my undergraduate degree from Brac University without hardship. And finally, I am thankful to my family and friends for their constant support, love, motivation and understanding my work throughout the whole time to complete this project.

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List of Acronyms

CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
gRNA	Guide RNA
PAM	Protospacer adjacent motif
TracrRNA	Trans-activating CRISPR RNA
NHEJ	Non-homologous end joining
HDR	Homology-directed repair
DMD	Duchene muscle dystrophy
ORF	Open reading frame
AML	Acute myeloid leukemia
GeCKO	Genome-scale CRISPR/Cas9 knockout screening
TSGs	Tumor suppressor gene
HEK293	Human embryonic kidney 293 cells
IDH2	Isocitrate dehydrogenase 2
LAN	Lance array nano-injection
AAV	Adeno-associated virus
PD-1	Programmed cell death-1
EGFR	Epidermal growth factor receptor
MASTL kinase	Microtubule Associated Serine/Threonine Kinase
Human iPSC	Human induced pluripotent stem cells
AON	Antisense oligonucleotide
HCC	Hepatocellular carcinoma
ICC	Intrahepatic cholangiocarcinoma

Chapter 1

Introduction

Clustered regularly interspaced short palindromic repeats known as CRISPR is a revolutionary technology in the field of gene therapy that opened a new era to the scientific field. CRISPR is a powerful genetic engineering tool that can cut, remove or add genetic material to correct, enhance or deactivate a defective gene, inhibiting or inducing the production of desired and undesired proteins in the body. CRISPR was initially identified as an important adaptive immune defense mechanism in bacteria which conferred protection from bacteriophages. The CRISPR system adapted from bacterial defense mechanism and preen it to use biotechnologically for recovering disease mutated genes of animal and human disease (Mojica & Rodriguez-Valera, 2016). CRISPR/Cas system is a programmable genome editing tool that works as a molecular scissor. DNA or RNA- binding proteins is mainly engineered to design CRISPR RNA (crRNA) and (Guide RNA) gRNA, those bind at the specific target site where protospacer adjacent motif (PAM) is positioned to recognize these CRISPR element. crRNAs or gRNAs containing spacers that complement the target sequence, Cas nuclease and gRNA make CRISPR process to edit the targeted gene more precisely and effectively. Consequently, simplicity and efficiency of CRISPR/Cas because of its RNA-based nature, made it superior to the previously used technologies such as Zinc Finger Nuclease (ZFN) and Transcription Activator–Like Effector Nuclease (TALEN). The large number of studies dedicated to CRISPR and CRISPR-associated (Cas) sequences published in recent years demonstrates the field's rapid evolution and the widespread application of CRISPR-based technologies across microbiology, biology, biotechnology and genetics (Pourcel et al., 2005).

A wide range of Cas-mediated molecular machineries are now available that enable genome editing, transcriptional control, and epigenetic modifications. Cas proteins' characteristics and benefits such as portability, programmability, target selectivity, cost-effectiveness and efficiency, have enabled geneticists all over the world to easily modify the genetic material and transcriptome of all hereditarily categorized species (Barrangou & Horvath, 2017). Subsequently, Cas can even ensure to modify iPSC, embryo cell line and mature cell of both mouse and human. Unlike other diseases, CRISPR/Cas9 remarkably proved to be an efficient tool for precisely targeting cancer

cell mutation, suppressing tumor development, and pre-clinical and clinical studies to treat malignancies of various origins (Floc'h et al., 2018). On the other hand, genetically inherited Duchene muscle dystrophy (DMD), a muscle-degenerating disorder can potentially be treated by harnessing CRISPR/Cas system to modify the causative mutations responsible for degenerative/non-functional dystrophin, which can help to restore muscle strength and reduce patient mortality.

This review will provide a comprehensive overview of how novel disease models are being generated using the CRISPR-Cas9 system specifically in cancer and DMD. In addition, the review will also discuss the mechanisms by which genetic defects in these disease conditions can be altered using CRISPR as a therapeutic and curative tool.

1.1. Background of CRISPR

The Journey of CRISPR started in 1987 when Japanese scientist Yoshizumi Ishino and team, discovered a unique set of repetitive DNA sequence interleaved with spacer sequences when analyzing the alkaline phosphatase converting gene of Escherichia coli. They accidently cloned a part of palindromic DNA repeats in an intergenic region upstream of the iap (isozyme conversion of alkaline phosphatase) gene (Ishino et al., 2018). Later, in 1989, Francisco Mojica revealed that *Haloferax mediterranei* R-4 archea have the same repetitive DNA sequence in their genome. Subsequently, in early 2000s, documentation of the existence of CRISPR arrays was being more explored. In 1993, following the mystery of arrays and spacer sequence, J.D.Van Embden discovered distinct strains of *Mycobacterium tuberculosis* which exhibited various spacer sequences between the DNA repeats (Mojica & Rodriguez-Valera, 2016). Mojica collaborated with Ruud Jansen to name these repetitive sequence particularly CRISPR and hypothesized it as an adaptive immunity system of bacteria and archea in 2005. Later, in 2007, outcomes of different studies established CRISPR/Cas as the adaptive immune system of bacteria and archaea and Cas9 remained a mystery in the inactivation of invading phage. John van der Oost, in 2008, showed that tiny RNAs can guide Cas9 to the target DNA which are spacer sequence of *E-scherichia coli* transcript (Brouns et al., 2008). However, the Streptococcus thermophilus model was the first CRISPR–Cas system in which the synthesis of CRISPR RNA (crRNAs), as well as three primary kinds of CRISPR–Cas systems, were discovered (Carte et al., 2014).Timeline of CRISPR development has been illustrated in figure 1.

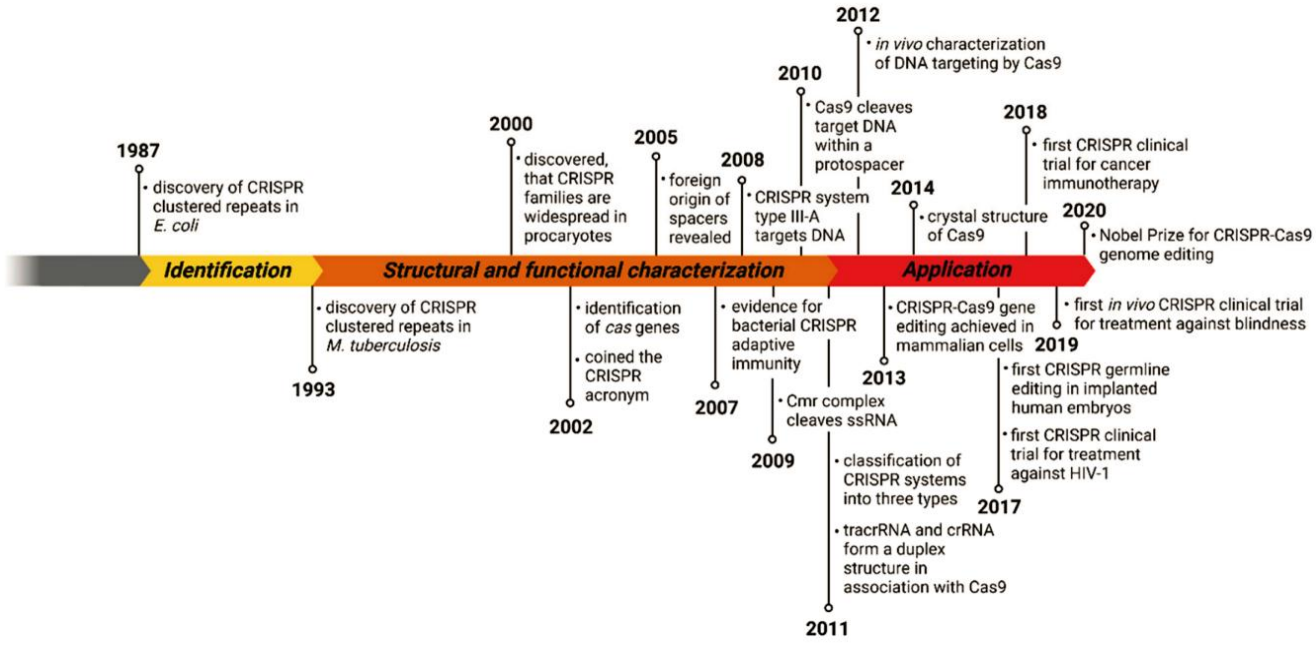

Figure 1: History of CRISPR development (Nidhi et al., 2021).

Emmanuelle Charpentier and Jennifer Doudna presumed about other fundamental parts of CRISPR in 2011. They showed a crRNA and tracrRNA (Trans-activating CRISPR RNA) formed complex that leads Cas9 nuclease to the *Streptococcus pyogenes* target. They proved that CRISPR-Cas9 system was able break isolated DNA properly if guide RNA is changed accordingly. Additionally, they collaborated with Martin Jinek in 2012, and proved that CRISPR/Cas could be used to cleave targeted double-strand DNA breakage of DNA in vitro where PAM also included (Jinek et al., 2012). In the following year 2013, Zhang and his team adopted CRISPR-Cas 9 for genome editing in eukaryotic cell successfully. They also worked to demonstrate this technique to precisely cut genome sequence of human and mouse cell (Cong et al., 2013). Later, E. Charpentier and J. Doudna received the Nobel Prize in 2020 for their incredible successful discovery of the programmable genetic scission on any predetermined site and develop CRISPR/Cas as fundamental genetic engineering tool. So over the time, the race of commercialization made CRISPR/Cas one of the effective gene modification tool.

Chapter 2

How CRISPR/Cas works

Major elements of CRISPR systems are Cas nuclease, gRNA, sgRNA, TracrRNA, crRNA and PAM sequence. Generally, mechanism of action of CRISPR/Cas-9 genome editing is divided into three steps: recognition, cleavage, and repair. In the first step, choosing Cas nuclease is the most important CRISPR process. Here, two type of Cas nuclease is used in editing: Cas9 and Cas12, among those Cas9 is widely used. Cas9 is the crucial element that cleaves the delivery vector at precisely selected sites, bind to the targeted DNA to cut it off, also activates the gene of interest, guided by a guide-RNA (Arora et al., 2018). Cas9 is guided by gRNA which is designed into two types that they are: single guide RNA (sgRNA) and dual guide RNA. These gRNA's binding site depends on the place of PAM sequence of specific DNA cleavage site. Subsequently, combination of Cas9 and gRNA called ribonucleoprotein (RNP), is delivered to the specific site of the genome through different delivery system such as electroporation, lipofection and many more. After the genomic modification, repair system take place to rejoin the DSB (double-strand breakage) of DNA sequences. There are two key system to repair DSB, non-homologous end joining (NHEJ) or homology-directed repair (HDR).

There are two class of CRISPR/Cas depending on the effector nuclease that controls target cleaving: class I (Type I, III, and IV) including multi-protein complex, or class II (type II, V, and VI) including single protein. Type I, II, and V systems identify and breaks DNA; type III modifies both DNA and RNA and type VI can function on DNA or RNA which is unclear (Arora et al., 2018).

The figure 2 below shows a flowchart of the general mechanism process of CRISPR system.

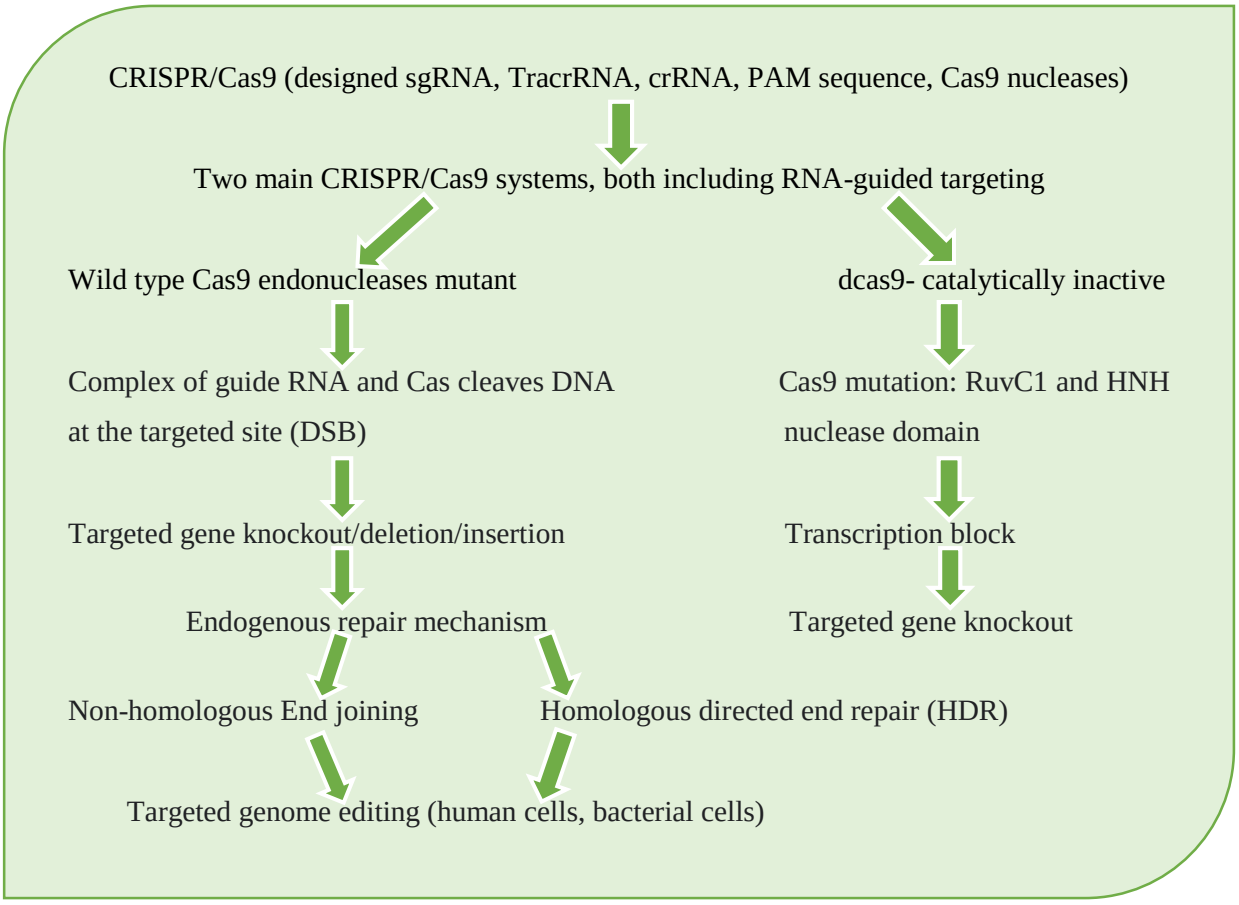

Figure 2: Steps involved in the mechanism of CRISPR/Cas.

Cas consists of multiple domain are mainly acts as genetic scissor. HNH cleaves complementary and RuvC cleaves non-complementary DNA strands in crRNAs and both are main nuclease domains in Cas9. These domains decides which type of breakage process will occur in the DNA sequence whether single or double-stranded breakage. Usually, the sgRNA is designed with a complementary guide sequence domain at the 5′ end of DNA (Wong et al., 2015). sgRNA can detect certain regions and control the Cas9 protein's activity substantially simplifying the genome editing process (Brierley et al., 2002). A diagram of CRISPR system cutting and modifying the DNA is shown in figure 3.

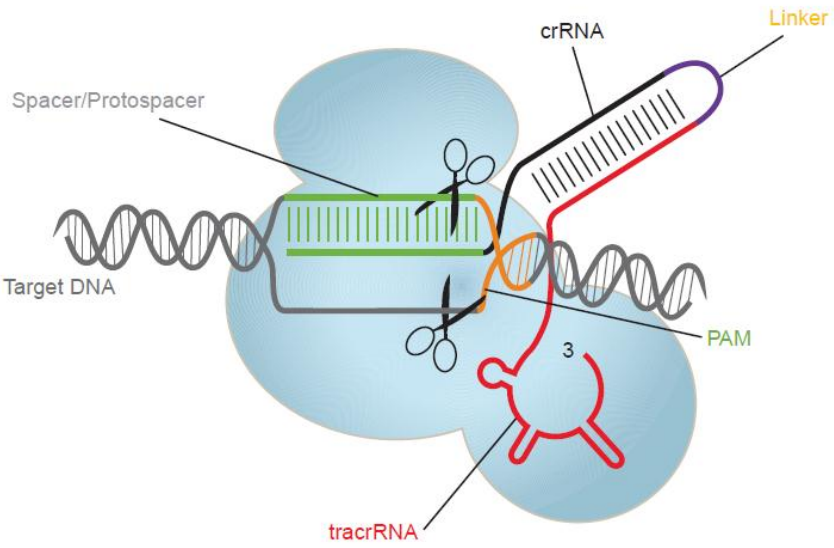

Figure 3: Type II Cas9 working principle of CRISPR. Blue shaped Cas9 enzyme cuts the DNA. The gRNA (fusion of tracrRNA and crRNA) forms a hairpin that binds to Cas9. In the presence of PAM, Cas9 cuts both complementary and non-complementary strands (Kozubek, 2016).

The crRNA of sgRNA specifies the genomic target for Cas9, while tracrRNA serves as a hairpin RNA between the crRNA and Cas9. 16–22 nucleotides made up crRNA, deriving from 3′ end of the repeat sequence and 20 nucleotides complementary to the targeted DNA strand of protospacer. Additionally, TracrRNA is responsible for activating RNase III to help pre-crRNA maturation (Ishino et al., 2018). To trigger cleavage, mature crRNA joins forces with tracrRNA and Cas9 (Z. Liu et al., 2020). On the other hand, the 5′-NGG-3′ sequence (short nucleotide sequence) found in Cas9's protospacer adjacent motif region, which is placed at the 3′ end of the target sequence. Next, Cas9 explores PAM sequence and actively creates a double-strand breakage at the PAM site because of the trigger of prepared specific sgRNA (Barrangou & Horvath, 2017). Consequently, cleavage of plasmid DNA resulted in blunt ends three base pairs upstream of the PAM region (Jinek et al., 2012). The PAM pre-requisite improves the guidance system's specificity and prevents the complex from cutting CRISPR loci. In type II system Cas9 can initially recognize potential protospacer and PAM sequence together in the targeted DNA. So, Cas9 showing this dual duty, justifies it as a rational method to guarantee that the protospacers chosen are directly adjacent to the appropriate PAM sequence, acquisition for future interference, and cleavage when required (Riordan et al., 2015).

The specificity of CRISPR/Cas9 enables the possibility repair of many genes and correction occurs simultaneously. Following the DSBs, two possible pathways called NHEJ and HDR work to rejoin

the engineered genome. Non-homologous end joining is the most active pathway of DNA repair mechanism. NHEJ causes minor indels in the target DNA, which cause amino acid deletions, insertions, or frame shift mutations, resulting in premature stop codons inside the targeted gene's open reading frame (ORF). Ligase I/Ligase III-dependent end-joining is more error-prone which can results in one or many varieties of mutations. On the other hand, homology-directed repair, which is extremely rare in eukaryotic organisms and shows limited error because of the presence of tailored template DNA having desired sequence with homologous arms to heal DNA damage unlike NHEJ. However, HDR is confined to the S and G2 phase of the cell cycle. HDR may allow to insert or delete old or new DNA sections of the genome, leading to a variety of unique alterations such as gene insertion, removal, alteration, reorganization, and repression (Sharan et al., 2009; Vasquez et al., 2001). Because of high efficiency of Cas9 cleavage and low efficiency HDR, NHEJ is used to repair a large percentage of Cas9-induced DSBs.

Chapter 3

Disease modeling using CRISPR/Cas system

CRISPR has emerged as a powerful tool for modifying the genome and developing prospective in vitro and in vivo disease modeling because of its precision, simplicity, and durability. In recent times, the application of CRISPR has extended beyond genome editing due to widely used for gene regulation, live-cell monitoring of chromosomal loci, epigenetic modification, high-throughput RNA screening, editing etc (Gilbert et al., 2013). Diseases such as cancer, DMD, sickle cell anemia, blindness, cystic fibrosis even in recent days COVID 19, CRISPR has already started to rule and paving the way to model and treat them as quickly as possible.

3.1. Cancer modeling

Cancer is a multiple-hit disease characterized by uncontrolled cell function, cell division and growth. In tumorigenesis, genetic mutation and epigenetic alterations plays crucial role. The uncontrollable cell growth is not only confined to the specific organ, also can spreads to the whole body termed as metastasis. This metastasis leading to organ failure, poor survival and increased mortality rate creating big concerning issue in therapeutic sector. Development in the field of CRISPR such as flexible methodology, use of molecular tools, avoidance of viral insertion, short processing times and potential for cancer screening have propelled this technology to become more effective than conventional treatment strategy of cancer. Following the urge to cure cancer, different types of genetically engineered experimental models have been developed using human, mouse, rat, zebrafish, and cells of rhesus monkey etc. through CRISPR (Hsu et al., 2014; Torres-Ruiz & Rodriguez-Perales, 2015).

Figure 4 schematically represents a brief idea of how CRISPR works to model cancer.

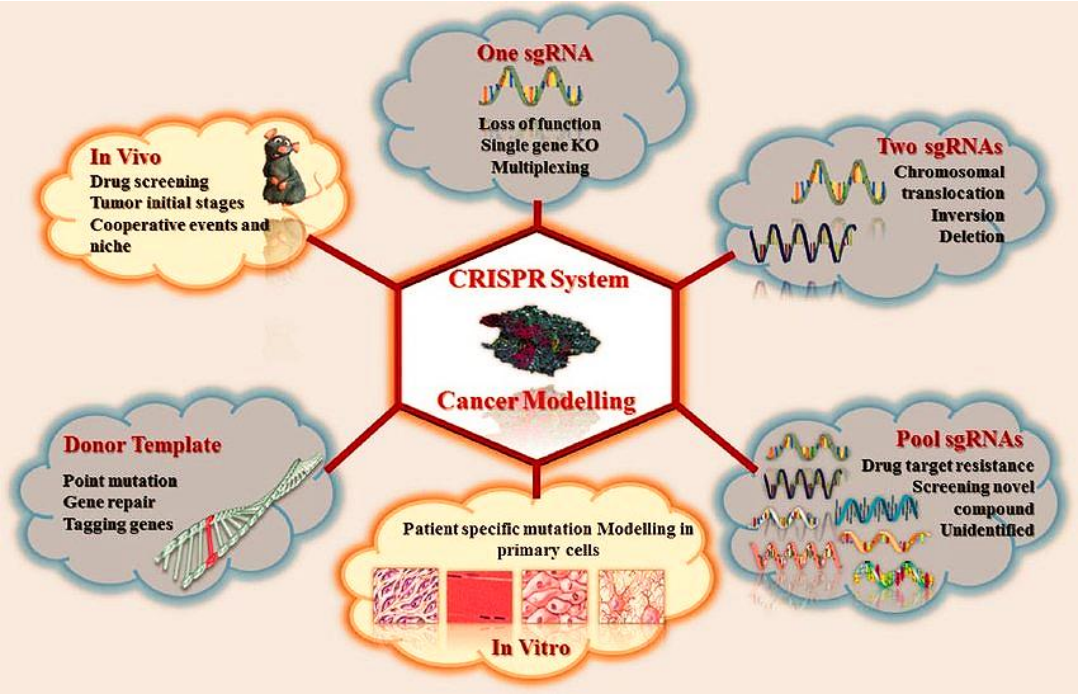

Figure 4: Schematic representation of cancer modeling by CRISPR/Cas. Precise genetic corrections in in vitro and in vivo, sgRNA specificity to target specific genomic loci, screening & modifying has widely made CRISPR key system to modeling of cancer (Ratan et al., 2018).

3.1.1. In Vitro Modeling of Oncogenic Alterations and Chromosomal Rearrangement

To simulate the pathophysiology of cancer, correctly recreate the complicated genetic scenario associated with carcinogenesis in certain types of cells or animals is essential for gene editing (Das Thakur et al., 2014). Significant chromosomal rearrangements such as point mutations, translocations, duplications, deletions, or inversions are reasons for which oncogenes activation or tumor suppressor genes (TSGs) deactivation can occur and repair mechanism can be altered. Not just because of its ease of use, but also because of its great accuracy and efficiency, CRISPR-Cas9 has emerged as the tool of choice for modeling these circumstances. Various variants of the CRISPR system have allowed exact recreation of chromosomal reconfigurations in in vitro. For example, CRISPR-mediated chromosomal translocation t(11;22) which is hallmark of cancerous Ewing sarcoma of human primary mesenchymal stem cells in HEK293 cell line has been investigated by Torres-Ruiz *et al*.

Figure 5 shows the chromosomal translocation in Ewing sarcoma through CRISPR/Cas9.

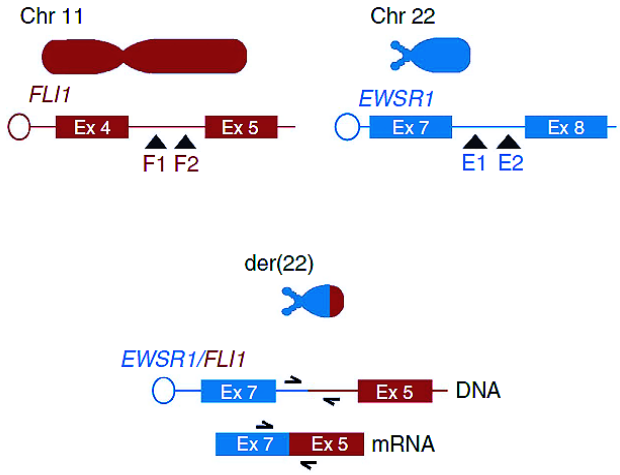

Figure 5: CRISPR/Cas9-mediated chromosomal translocation between the EWSR1 and FLI1 loci, and sgRNA. sgRNAs (arrowheads) and Cas9 induce DSBs that mapping to introns in FLI1 (red) and EWSR1 (blue) (Torres et al., 2014).

The result showed that the erroneous EWSR1-FLI1 transcription factor interfered with the activation of specified downstream target genes, mimicking human cancer consequences. Recreating the chromosomal translocation in acute myeloid leukemia (AML) characterized in HEK293 and CD34+ human hematopoietic progenitor cells, demonstrated the universality of this technique. To prove the CRISPR system's ability to recreate chromosomal rearrangements is strongly influenced by the location of the breakpoints (Torres et al., 2014).

Approach of CRISPR technology in vitro modeling regarding modification and targeting of some disease have been summarized in Table 1.

Table 1: List of in vitro cancer modeling by CRISPR-Cas9 technology (Torres-Ruiz & Rodriguez-Perales, 2015).

Approach	Modification	Disease	Target cell	Gene	Delivery	Reference
In vitro	Loss-of-function and directed mutation	Colorectal tumor	Organoids intestinal epithelium (human)	*APC*, *SMAD4*, *TP53*, *KRAS*	Electroporation	(Drost et al., 2015)
In vitro	Gain-of-function	Melanoma	melanoma cell line (human)	A375 SAM library	LV	(Konermann et al., 2015)
In vitro	Loss-of-function	Melanoma	A375 melanoma cell line (human)	GeCKO library	LV	(Ran et al., 2013)
In vitro	Loss-of-function	Lung cells (mouse)	Lung adenocarcinoma	*Nkx2.1*, *Pten*, *Apc*	LV	(Sanchez-Rivera et al., 2014)
In vitro	Chromosomal rearrangement	Lung adenocarcinoma	HEK293 (human)	*EML4-ALK*, *KIF5B-RET*	Plasmid transfection	(P. S. Choi & Meyerson, 2014)

In parallel to the previous example, Choi and Meyerson used CRISPR-Cas9 technology to create a chromosomal rearrangement and two inversions that are important in lung cancer development. They also produced two types of inversions where both rearrangements were successfully replicated in the HEK293 cell line, with a paracentric inversion efficiency of 8.9% and a pericentric inversion efficiency of 1.62 percent proving the influence of CRISPR in rearrangements (Sanchez-Rivera et al., 2014).

Ghezraoui and colleagues investigated the method of genomic rearrangements engaged in the deposition of changes in tumor cells using the TALEN, ZFN, and CRISPR systems in combination. They investigated the bonding processes specifically the function of NHEJ pathways occurs in human genomic translocations. So, this study revealed that in the modified HCT116 cell line

(colorectal carcinoma), wild-type Cas9 induces correct DSB merging and paired nickase-Cas9 generates insertion/ablations in the t(2;5)/NPM-ALK translocation derived chromosomes' junction sites of anaplastic large cell lymphoma (Chiou et al., 2015).

3.1.2. Targeted mutation modeling

Targeting and identifying the mutated genome, their position, and their state is one of the key function of CRISPR. In acute myeloid leukemia (AML) model development, researchers inserted loss-of-function genetic mutations that are repeatedly suppressed in myeloid malignancies introduced into hematopoietic stem and progenitor cells (HSPCs). Tet2, Runx1, Dnmt3, Nf1, Smc3, p53, and other genes that express epigenetic regulators, transcription factors, and cytokine signaling intermediates were used to imitate the genetic combinations found in people with AML, resulting development of myeloid clonal growth and progression to AML. Furthermore, CRISPR elements were created in mouse HSPCs using a two-vector lentiviral approach, allowing researchers to modify up to five genes ex vivo in a single cell clone (Torres-Ruiz & Rodriguez-Perales, 2015). Another successful model of AML using *IDH2* R140Q mutation is made by RNA-guided CRISPR/Cas9 system. In this model, a DNA template is introduced by homologous recombination at the endogenous gene locus can delete or reproduce AML-associated mutations in human leukemic cells. Here, CRISPR/Cas9 provides specific DNA DSB in *IDH2* gene of K562 ML cell and the specific DNA template inserts into the *IDH2* gene locus with the *IDH2* R140Q point mutation by HR. After effective genome editing, K562 cells also shows a good proliferation and colony formation in in vitro human myeloid leukemia. Also, CRISPR/Cas9 associated with lentivirus can remove or edit the *IDH2* R140Q gene mutation from primary AML patient successfully (Brabetz et al., 2017).

Furthermore, in colon cancer cell line, combination of CRISPR/Cas9 and single-stranded oligodeoxynucleotides (ssODNs) repairs, restores protein function, and decrease tumor growth of a *PKCβ* gene mutation. Here, in a xenograft model of colon cancer, CRISPR/Cas9-mediated loss-of-function PKCβ mutant fixing inhibited anchorage-independent growth and decreased tumor development (Antal et al., 2015). Thus, CRISPR/Cas9 is being used in different mutation targeting or identifying, result in targeting specific site and even shows the capacity to edit several targets at once that opened a wide path to scientists to attempt making disease model.

3.1.3. CRISPR-Cas9 genetic screenings

CRISPR screening is a process by which small number of crucial gene or genetic sequence can found from large number of genetic sequences that express specific cell type's function or phenotype. The CRISPR/Cas9-based screen has a higher targeting capability, lower off-target effect, targeting both coding and non-coding regions throughout the genome. Knockout screen, knockdown screen and activation screen are the main categories of CRISPR screens, shares almost common working mechanism (He *et al.*, 2021).

The development of sgRNA libraries identifying thousands of genes has taken use of this advantage for the systematic analysis of genes implicated in malignancy. Zhang and colleagues published the in vitro findings of two investigations in 2014, using a library of loss-of-function and a genome-wide gain-of-function sgRNA libraries to identify genes linked to melanoma resistance to the BRAF protein kinase inhibitor vemurafenib (Konermann et al., 2015). Author employed the GeCKO (Genome-scale CRISPR/Cas9 knockout screening) library of CRISPR-Cas9 that could target over 18,000 human genomes, search genes whose ablation is linked to resistance of vemurafenib, and discovered validated genes with novel possibilities to treat the resistance problem (Torres et al., 2014). Robert et al. used a pooled CRISPR knockout screen in transplantable tumors in immunotherapy-treated mice. The researchers discovered that knocking down PTPN2 improved IFN-mediated antigen presentation and growth inhibition, boosting immunotherapy efficacy (Manguso et al., 2017).

3.1.4. In vivo oncogenic alteration modeling: Embryos and Embryonic Stem Cells Genome editing

In oncogenic alteration, the embryos or embryonic stem cell editing gives a highly efficient editing outcome and wide experimental scope in animals. Specifically, genetically engineered mouse model with germ line alteration has been efficient for testing in vivo therapies. For in vivo genetic modification, RNA-guided Cas9 nuclease (Cas9sp) from *S. pyogenes* is widely applied in a wide range of cells and organisms such as bacteria, yeast, primates, and human cell lines. Direct modification of the zygote genome can save cost and time. Crispr-cas9 may be utilized as an intermediary, which is a huge benefit over traditional gene targeting strategies. By expressing Cas9 in the germ line, the Cas9 mouse provides a simpler model for somatic genome editing, permitting constitutive and Cre-inducible Cas9 expression in organs of interest. Cas9 mRNA and sgRNA Co-

injection into zygotes in the presence of a single-strand oligonucleotide template allows for precision genome editing, including knock-out, knock-in, and gene repair. CRISPR/Cas9 technology have three main phases for embryonic genome editing: (i) separation of zygotes from super-ovulated females, (ii) sgRNA and Cas9 mRNA delivery to the zygote, and (iii) embryo transfer into pseudo-pregnant animals to establish a viable F0 generation (Jin et al., 2016).

For the administration of editing reagents, a variety of approaches have been described, including pronuclear microinjection of the sgRNA/Cas9 plasmid to generate transgenic mice, or direct injection of Cas9 mRNA or protein into the cytoplasm. In different forms such as sgRNAs, plasmid or ssRNA, Cas9 nuclease, mRNA, or protein, editing reagents are supplied into the cell (Horii et al., 2014; Mashiko et al., 2013). Team Wang generated the first gene knock-out mouse utilizing CRISPR/Cas9 in 2013. The researchers administered Tet1 and Tet2 sgRNA into zygotes together with Cas9 mRNA, resulting in animals with up to 80% mutations in both genes. This in vivo application of CRISPR/Cas9 has pioneered the door for methodical genome-engineered animals, allowing the scientists to create mice with various mutations in regions suspected of playing a role in the initiation of multigenic disorders (Haoyi Wang et al., 2013).

3.1.5. In vivo oncogenic alteration modeling: Adult Animals

In vivo application of CRISPR technology is faster in testing single or combined gene in various targeted tissue. CRISPR-cas9 has opened the door to employ adult animals in in vivo modeling. In vivo CRISPR/Cas9 requires efficient delivery system to reach the target site. Efficient delivery system such as adeno-associated virus or adenoviruses used to carry out the process in vivo. AAV containing sgRNA and Cas9 can target a single or multiple gene which can help to study reverse brain function with cancer in adult mouse model (Mou et al., 2015). Using a plasmid for temporary Cas9 and sgRNA synthesis administered through hydrodynamic tail vein injection, Xue and colleagues induced loss-of-function mutations in Pten, p53, or both genes. This proved CRISPR is a powerful technique for simulating carcinogenic alterations with great potency (Bleiziffer & Kneser, 2009).

Notable diseases that are used in modeling by using CRISPR technology through in vivo application have been summarized in table-2.

Table 2: List of in vivo cancer modeling by CRISPR-Cas9 technology (Torres-Ruiz & Rodriguez-Perales, 2015).

Approach	Alteration	Disease	Target cell	Gene	Delivery	Reference
In vivo	Directed mutation and loss-of-function	Lung adenocarcinoma	Neurons, immune and endothelial cells (mouse)	*Kras*, *p53*, *Lkb1*	AAV, LV and particle-mediated delivery	(Platt et al., 2014)
In vivo	Loss-of-function	Non-small-cell lung cancer (NSCLC)	Cell line (mouse)	GeCKO library	LV	(Chiou et al., 2015)
In vivo	Loss-of-function	Pancreatic ductal adenocarcinoma	Somatic pancreatic cells (mouse)	*Lkb1*	LV and AdV	(Chiou et al., 2015)
In vivo	Chromosomal rearrangement	Non-small-cell lung cancer (NSCLC)	Lung cells (mouse)	*Eml-Alk*	AdV	(Maddalo et al., 2014)

Another approach using CRISPR is based on lentivirus delivery, for producing Cre and Cas elements for rapid gene functionality testing (Sanchez-Rivera et al., 2014). Sanchez-Rivera *et al.* used genetically altered mice models of lung cancer to investigate the effect of inducing loss-of-function mutations in Nkx2.1, Pten, and Apc TSGs followed by intratracheal injection of lentiviral vectors. The findings revealed that the approach is extremely successful in vivo, with step-specific modifications in lung tumors after ablation of each of the three TSGs (Platt et al., 2014). Using a lentiviral CRISPR/Cas9 vector, a particular chromosomal rearrangement involving the genes Eml4-Alk, which is present in around 5–7% of malignant malignancies, was accomplished. Furthermore, in vivo use of CRISPR/Cas9 system has demonstrates that deactivation of genetic material in pancreatic cancer may be achieved without the need of extra animal alleles (Maddalo

et al., 2014).

3.2. CRISPR/Cas9 delivery to cancer cells

CRISPR/Cas9 have to be delivered to its targeted cell to be activated and deal with the cancer cells. In general, three delivery pathways are present: in vitro, ex vivo, and in vivo, for CRISPR/Cas systems delivery. Again, two major categories are present for delivery which are cargo and delivery vehicle. Further division in CRISPR/Cas9 cargo are in three part: (i) Cas9 protein with guide RNA encoded by DNA plasmid,(ii) mRNA for Cas9 translation with a distinct gRNA, and (ii) RNP complex (Cas9 protein with guide RNA). The delivery vehicles frequently determines about the packaging of cargo and their possibility to employ in vitro or in vivo. Physical approaches, viral vectors, and non-viral vectors have been used as CRISPR/Cas9 delivery vehicle (Hong-xia Wang et al., 2016). Adenovirus (Ad), AAV, retrovirus, lentivirus, Epstein-Barr virus (EBV), herpes simplex virus (HSV), and bacteriophages are the molecular tools for gene transportation into human (Kotterman et al., 2015). Furthermore, non-viral vectors for CRISPR/Cas delivery have recently been studied where lipid nanoparticles, polymer and polymeric hydrogel nanoparticles, gold-based hybrid nanomaterials, and other nanomaterials are included (Wan et al., 2019).

- In vitro delivery:

In physical method, electroporation is a high-efficient approach that electro-transfer CRISPR/Cas systems into cancerous cells by using an electrical field, resulting in enhanced cell membrane permeability through breaking down the plasma membrane's lipid bilayers (Han et al., 2015). Cas9-engineered human intestinal organoids were used to produce colorectal cancer models utilizing electroporation to transfer plasmid DNA expressing both Cas9 and mRNA (Matano et al., 2015). Additionally, it can create a human cell model of early-onset Alzheimer's disease and correct mutations that cause DMD. On the other hand, another CRISPR delivery system is microinjection which is account as ‘gold standard’ with 100% efficiency. This system can deliver CRISPR component directly to the targeted site by breaking the barrier of extracellular matrices, cell membranes, and cytoplasmic element (Lino et al., 2018). New methods are continuously being invented, among those recently a method called LAN (Lance array nanoinjection) has been invented. LAN have a substantially greater survival rate of cells (78–91%) than standard electroporation (Sessions et al., 2016). Here, intracellular delivery of acoustic nanomotors loaded

with the Cas9/sgRNA combination, knocked out 80 percent of the GFP (Green fluorescent protein) and gives an additional way of complex transduction into tumor cells in vitro.

The viral vector AAV has been widely used because of its high safety in numerous clinical studies. AAV has been successfully infected into Human reticular microvascular endothelial cell (HRECs), Human Umbilical Vein Endothelial Cells (HUVECs), and human primary retinal pigment epithelial cells (hPRPE) (Huang et al., n.d.). According to Mizukami and colleagues, human cervical cancer cell lines including HeLa, HCS-2, and SKGI can be treated in vitro by combination of Cas9 plasmid and AAV-sgE6 resulting effective growth inhibition and cell death (Yoshiba et al., 2019). In vitro applicability is expended through the non-viral vector delivery of the CRISPR/Cas systems for simple and easy-to-find commercial transfection of chemicals. RNAiMAX (a cationic lipid nucleic acid transfection reagent) is able to deliver Cas9/sgRNA complex with an 80% genome editing efficiency into growing U2OS cells (Zuris et al., 2014). Rotello and colleagues were able to make nanoassemblies of cationic arginine-decorated gold nanoparticles that delivered Cas9/sgRNA complex to HeLa cells in vitro with excellent efficiency (Mout et al., 2017).

- In vivo delivery:

Despite having different delivery systems, in vivo vector delivery is one of the crucial systems for CRISPR/Cas systems. One of the most reliable physical ways for CRISPR/Cas-based gene editing in vivo is hydrodynamic injection (Hong-xia Wang et al., 2016). It holds a lot of promise as a treatment for HBV-induced hepatocellular carcinoma in human (Habib et al., n.d.). Another study of Liu and colleagues employed a lentivirus-based CRISPR/ Cas9 system combined with sgRNA-721 to knock down HIF-1 (Hypoxia-inducible factor 1-alpha) for the treatment of human HCC (hepatocellular carcinoma) (Song et al., 2020). Further ,another vector, adenovirus (Ad) can transport CRISPR/Cas9 systems into a larger range of proliferating and non- proliferating cells in vitro and in vivo than lentiviral and retroviral vectors (Xu et al., 2019). Ad is an excellent choice for delivering the full CRISPR/Cas9 system in a single vector. A combination of Ad/Cas9 and Ad/sgEGFR which is EGFR (epidermal growth factor receptor) mutation-specific, can work on Non-small cell lung cancer (NSCLC) by editing the EGFR mutant allele which showed high efficiency (Song et al., 2020).

Additionally, non-viral vectors represent a vast diversity compared to viral vectors in the CRISPR/Cas delivery system. Nano sized preparation, Cell-penetrating peptides (CPPs)-

mediated, Receptor-mediated, Multi-modal nano-preparations delivery systems all are different non-viral carriers or delivery systems (Tong et al., n.d.).

3.3. Targeting tumor cells and therapeutic application of CRISPR technology

The CRISPR/Cas9 system has revolutionized molecular biology and gene therapy. From common types of solid tumors to non-solid tumors such as leukemia are being targeted and treated by Crispr-cas9. Decision-making processes for treatment, different and unique treatment strategies, and clinical trials can be possible and prediction of different outcomes and patient survival are revealed through CRISPR/Cas9 strategy (Ahmad & Amiji, 2018). A growing number of pre-clinical and clinical studies employing CRISPR-cas9 have led to the use of genetically modified T cells for cancer immunotherapy rather than targeting a single gene in malignant cells. As a result, CRISPR/Cas9 has been used vastly to identify prospective therapeutic targets, as well as pre-clinical and clinical trials of malignant cells in lung, breast, brain, liver, and colorectal cancer. Some mentionable diseases that have made to the Phase I and II of clinical through CRISPR technology has been showed in table-3.

Table 3: Clinical studies employing CRISPR/Cas technology in phases I and II (B. Liu et al., 2019)**.**

Cancer cells	**Target**	**Phase**	**Intervention**	**status**
B cell leukemia, B cell lymphoma	CD19 and CD 20 or CD22 CAR-T	I/II	Duel specificity of CD19 and CD 20 or CD22 CAR-T	Active
Solid tumor, adult	PD-1 and TCR	I	Anti- mesothelin CAR-T cell	Active
Synovial sarcoma, liposarcoma, melanoma	TCRendo and PD-1	I	Drug: Cyclophosphamide, NY-ESO-1	Active
Human papilliloma virus- related malignant neoplasm	HPV-related survical	I	TALEN, CRISPR/Cas9	-

3.3.1. Lung cancer

Nowadays, the mortality rate of lung cancer is one of the most concerning issue worldwide. Lung cancer treatment is now moved to targeted drug therapy, which affects multiple genes such as EGFR, ALK, and KRAS. The increasing problem of drug resistance can be reduced by editing targeted gene. CRISPR/Cas9 can reduce tumor resistance, molecularly targeted medicines or inhibitors, which is beneficial for lung cancer patients. Figure 6 illustrated the ways by which CRISPR/Cas9 works to treat lung cancer.

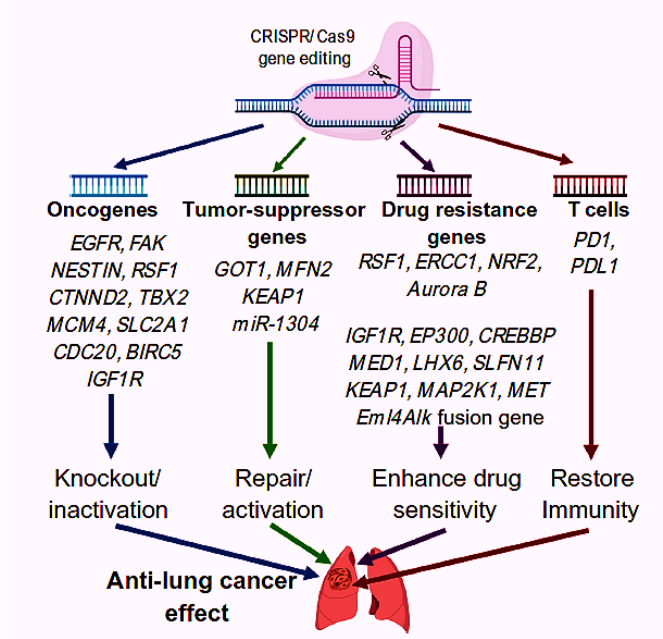

Figure 6: Illustration of the applications of CRISPR/Cas9 in studies on lung cancer treatment (Jiang et al., 2019).

Currently, several molecular targets for NSCLC therapy are accessible in clinical trials and this NSCLC subtypes responsible for approximately 85% of lung cancer (Saber et al., 2015). CRISPR/Cas9 delivery through adenovirus (AdV) vector can selectively abolish EGFR mutant alleles (L858R) in an NSCLC cell line (H1975). This use of AdV successfully proved significant aspects of using Crispr-cas9 such as decreased tumor growth in vivo, identifying healthy cell from mutant tumor cells and death of cancer cells (B. Liu et al., 2019). Recently, first-in-human trial examined the influence and safety of using CRISPR/Cas to treat last stage lung cancer through patient-derived T cell immunotherapy. The researcher used PD-1-ablated T cells in patients with advanced NSCLC. The study was designed by isolating Peripheral blood mononuclear cell.

PBMCs and electroporated with plasmids encoding Cas9 and a pair of gRNAs targeting the second exon of PD-1 gene. Figure 7 shows the diagram of PD-1 gene engineering by CRISPR/Cas9 system.

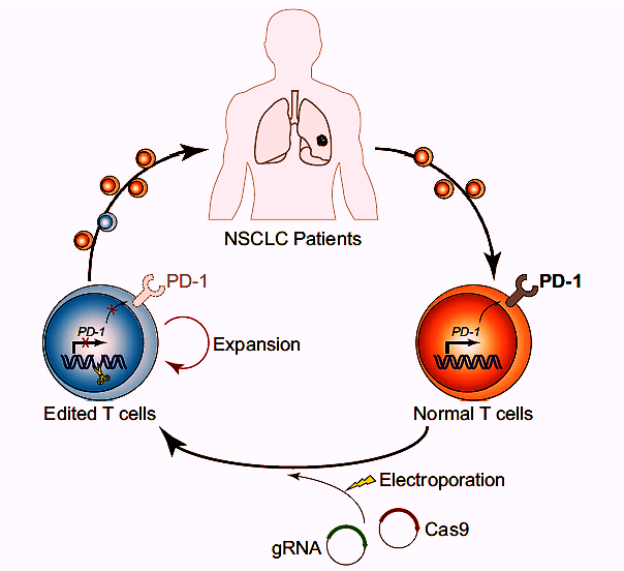

Figure 7: Illustration of PD-1 editing in T-cell. CRISPR delivery occurs by electroporation by in vivo. Later, edited T-cell infused back to human body to observe the outcome (S. He, 2020).

The research result showed that highest editing efficiency of PD-1 have longer perseverance of edited T cells in vivo. This suggests that clinical outcomes can be improved if higher editing efficiency is ensured (S. He, 2020).

3.3.2. Liver cancer

Primary liver cancer is the second most common cause of cancer-related mortality worldwide, affecting more men than women. The two most common kinds of primary liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) (Saadatmand et al., 2014). In HCC cells, genetic deletion of Nuclear receptor coactivator 5 (NCOA5, co-regulator for ERs) drastically reduced cell proliferation and migration. NCOA5 protein absence significantly reduce EMT (Epithelial-mesenchymal transition**)**, which in several cancer types is a prevalent treatment resistance process (J. He et al., 2018). The CRISPR/Cas9 system might be used to prevent HBV-derived (Hepatitis B virus) liver cancer by deleting viral DNA, which is one of the technique's potential therapeutic uses. Figure 8 shows how CRISPR modifies HBV in liver cancer.

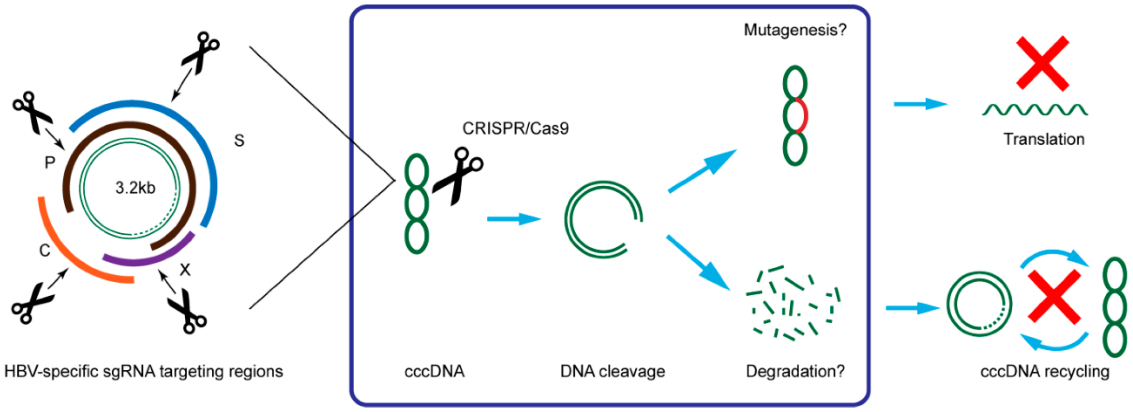

Figure 8: Schematic representation of HBV targeting technique and possible CRISPR/Cas9 mechanism in HBV suppression. Here, S: surface; P: polymerase; C: core and X: HBx protein. CRISPR targets cccDNA by sgRNA targeting specific HBA site. It cleaves cccDNA stops translation and cccDNA recycling to suppress mutagenesis (G. Lin et al., 2015).

Many preclinical studies have shown that targeting the HBV as a risk factor for liver cancer and elimination of cccDNA template can cure both HBV and HCC occurring chances. Lin and colleagues used a gRNA with CRISPR/Cas9 technology to reduce EBV surface and core protein production in vitro and in vivo, lower rcDNA and cccDNA levels which resulted in suppressing a unique genomic region of HBV (S. R. Lin et al., 2014).

3.3.3. Breast cancer

Breast cancer, which comes in several of subtypes and morphologies, is one of the leading causes of cancer-related mortality in women across the world. Now, CRISPR-mediated research has identified potential treatment targets in several of breast cancer subtypes, recurrence that may help patients live longer and have a better quality of life.

In breast cancer cell lines, CRISPR-based disruption of the MASTL kinase decreased cell growth. Increased MASTL expression is connected to a poorer prognosis and higher histological grades, making it crucial for breast cancer growth (Álvarez-Fernández et al., 2018). Inhibiting MASTL might thereby limit cancer growth in elevated breast tumors. Disrupting SHCBP1 (Src homology member and collagen homolog family) lowers cell reproduction and increases cell death in tumorous breast cell lines, and overexpression of it induces more than 60% of breast cancers, which are linked with a worse chance of survival (Feng et al., 2016). Consequently, suppression of these protein improves survival rate. Another research carried out by Zheng and team, showed that CRISPR screening for RNA-binding proteins (RBP) is implicated in breast cancer and a

crucial splicing factor, finger protein 5A (PHF5A) knocking down in breast cancer cells suppresses cell proliferation, tumor development, and cell migration in in vitro (Yang et al., 2018).

As a result of these discoveries, preclinical research, genetic inhibition of critical signaling pathways in malignant cells can create a promising therapeutic strategy for breast cancer (B. Liu et al., 2019).

Chapter 4

Duchenne Muscular Dystrophy (DMD) and CRISPR

Duchenne muscular dystrophy (DMD) is an inherited genetic disease characterized by progressive muscle degeneration and weakness caused by mutations in the dystrophin protein. Dystrophin which is a scaffolding complex protein, plays a crucial role in protecting the integrity of striated muscle, and its mutation causes development of DMD in the body. The onset of DMD starts from an early age, between 3 to 5 years and at the age of 12, many children starts to use wheelchair. DMD is located on only a single X-linked dystrophin chromosome which makes it more accessible to the male than female as men have only one copy of X-chromosome. Female have double X-chromosomes can be only be the carrier of DMD without any symptoms. So, the global prevalence of the condition is estimated to be 1 in every 35,00 males (Min et al., 2019).

Children start to show symptoms of muscle weakness, particularly of the lower external muscles which then spreads to the upper external muscles (Brison et al., 2019). Feeble proximal and distal limb muscles, enlargement of the calves associated with muscle weakness, muscle atrophy, spasm, muscle hypertonia, and myalgia are also the primary symptoms of DMD. Later, progressive weakness and scoliosis affect the heart leading to heart failure and impaired pulmonary muscle function eventually lead to acute respiratory failure. Dystrophin which is the largest gene of human body, generally absorbs shock, strengthen muscle fiber, and reduces mechanical stress induced by muscle contraction. It acts as an anchor between cytoskeleton and extracellular matrix. The dystrophin protein connects the sarcomere and sarcolemma as being the primary component of the dystrophin-glycoprotein complex, encoded by the X-chromosome consisting 79 exons. Mutation in dystrophin leads to the disruption of the open reading frame (ORF) that results in abbreviated, defective, and unstable protein production (Chemello et al., 2020). Consequently, mutations are concentrated in DMD's hotspot regions, which include the actin-binding domain-1 (ABD-1) and the central rod domain (Kyrychenko et al., 2017). In four type of dystrophinopathies, DMD is one of the fourth conditions and other three included in mild Becker Muscular dystrophy (BMD) (Brison et al., 2019). As a key therapeutic objective, to minimize the severeness of DMD, converting DMD to BMD by genome substitution or genome editing has been a major clinical trials goal.

Since the discovery of the dystrophin gene research efforts have been underway to find drugs to reduce progression of the disease and enhance muscle activity in DMD. Several treatments have been developed and divided into three classes: i) disease-modifying therapies, (ii) gene expression therapies, and (iii) gene replacement therapies, but none of them can fully recover muscular dystrophy (Chemello et al., 2020). For example, patients on corticosteroid treatment demonstrated prolonged ambulation but developed significant health problems because of the side effects of steroids. Apart from these, additional therapies are also given to improve muscle mass, reduce pain, and delay dystrophy such as myostatin inhibitors (Cohen et al., 2014). In recent years, advancement has occurred by different pharmacological approaches such as, myoblast and stem cell transfer, oligonucleotide-based exon skipping to bypass mutant exons, gene replacement and various clinical trials (Salmaninejad et al., 2018). However, majority of the aforementioned therapies could not restore long-term expression of dystrophin but CRISPR offered the opportunity to reestablish expression of dystrophin by efficient result in human cells and mouse models.

DMD was one of the first monogenic diseases to be studied from the perspective of CRISPR-mediated repair of causal gene mutations. Certain characteristics of DMD such as, large gene size made it complicated to provide long-term treatment of DMD and also slowing down the deterioration of muscles. These led the scientists to turn to gene modification as a therapeutic approach. Firstly, dystrophin's modular rod domain, allows mutant exons in this regions of genes to be removed and restore the open reading frame restored (ORF). Secondly, here is no chance of mistakenly harming a wild-type copy of the gene since only the mutant X chromosome of dystrophin has to be repaired. Finally, just a small portion of original dystrophin expression must be restored to provide a therapeutic effect (Long et al., 2014). So, the diversity, precision, and combinatorial abilities of the CRISPR/Cas9 system enable it to repair gene defects in certain genetic disorders that were previously unachievable.

4.1. Gene Editing Strategies of DMD by CRISPR

In animal models, CRISPR/Cas-mediated gene editing has been proven to completely fix DMD mutations and restore dystrophin function again (Ousterout et al., 2015). In both germ line and postnatal gene editing of mdx mice by CRISPR/Cas9 systems, reinstated dystrophin production and improved muscular function. Later, utilizing CRISPR, exon skipping or HDR were used to repair DMD abnormalities in both mdx mice and human-derived iPSCs (Zhang et al., 2017).

CRISPR/Cas9 adenine base editors were also employed to fix a DMD defective mouse using single-nucleotide adeninetoguanine replacements (Ryu et al., 2018). Beneficial results like these created potential importance to build up different strategies to correct DMD mutations by CRISPR technology.

4.1.1. Myoediting Strategies of DMD

CRISPR-mediated gene modifying in muscle is known as myoediting which is used to permanently repair the genetic alterations found in DMD and restore muscular function (Zhang et al., 2018). Figure 9 below illustrates how myoediting can be utilized to modify DMD.

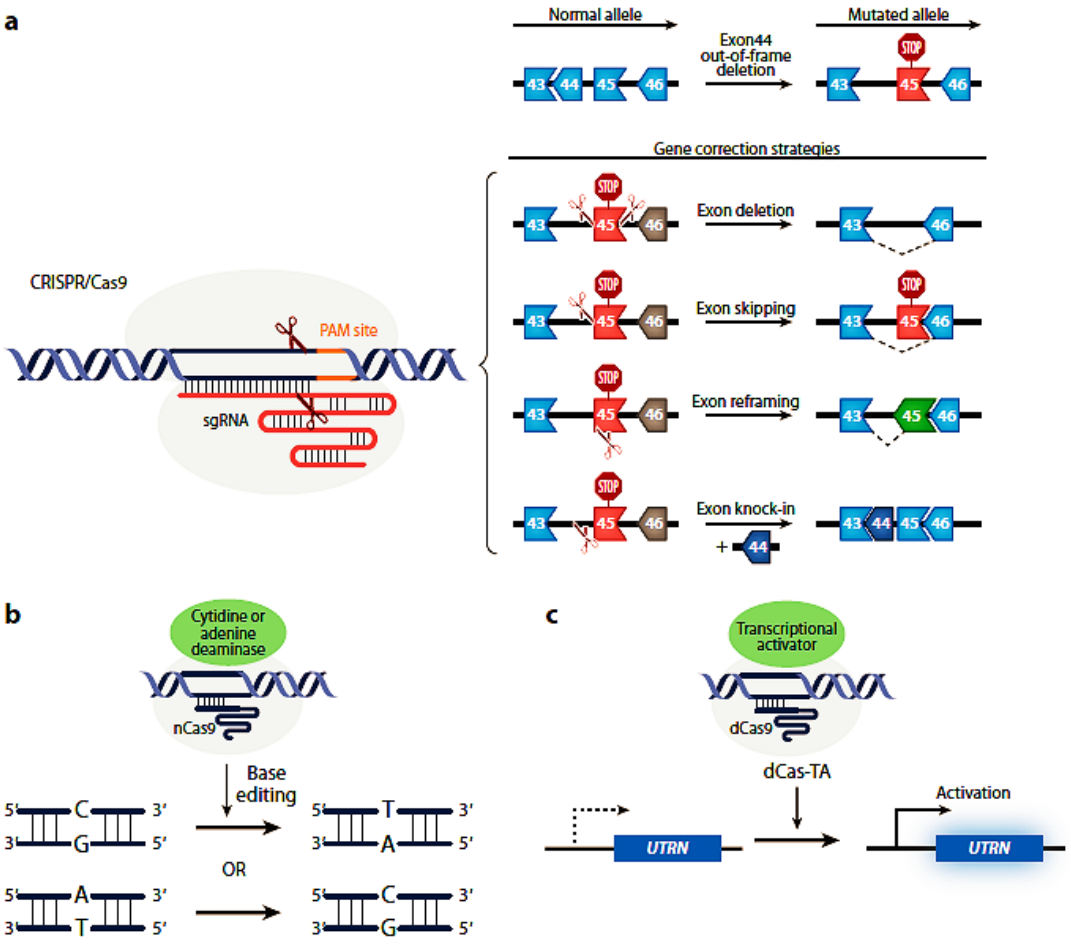

Figure 9: Correction of DMD using CRISPR-mediated editing methods. (a) Exon deletion, exon skipping, exon reframing, and exon knock-in and role of DSB (b) Attaching a cytidine or adenine deaminase to CRISPR/nCas9 results in a C:G to T:A or A:T to C:G base substitution. (c) CRISPR/dCas9 gene regulation. Its combination with a transcriptional activator can upregulate UTRN which codes for utrophin, a dystrophin compensatory (Min et al., 2019).

I. Deletion of Exon: The goal of utilizing the CRISPR/Cas9 system in DMD is to regain dystrophin expression of genes by elimination of the dystrophin mutation in muscle cells and inhibit muscle atrophy (Min et al., 2019). Two types of working strategies are included in it.

First, myoediting requires two sgRNAs, brink of DMD exon that is mutated which is sliced in the presence of Cas and deletion of exon which is known as double-cut myoediting (Young et al., 2016). Using two sgRNAs, researchers were able to eliminate multi-exonic genomic areas surrounding a mutational hotspot area (exons 45–55) in DMD. This resulted in abolition of a section of the core rod domain and the generation of a shorter but functional dystrophin (Ousterout et al., 2015). However, specific attention must be paid in case of multiple exon elimination by double cut myoediting so that dystrophin dysfunction such as, unanticipated genomic alterations, incorrect splicing do not occur and that the actin or dystroglycan remain intact (Chemello et al., 2020). A schematic diagram of Cas9 deleting exon 23 has showed in figure 10.

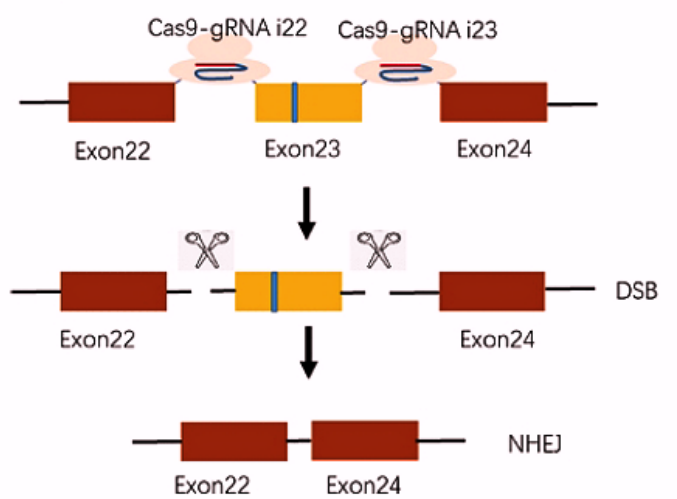

Figure 10: Exon 23 deletions by CRISPR/Cas9 through schematic diagram representation. Two gRNAs targeted intron 22 and 23 are targeted by using Cas9 nuclease. Cas9's (DSBs) cause the mutant exon 23 removal. Non-homologous end joining (NHEJ) fixes the distal ends, allowing the dystrophin gene's reading frame to be restored (Jin et al., 2019).

To correct exon duplication mutations, 5% of DMD patients can go through the exon deletion strategies. Single cut myoediting involving Cas9 where a single sgRNA targets the intron region adjacent to the out-of-frame exon. As a consequence, a single sgRNA generates two incisions and eliminates one of the duplicated exons. Renewal of the DMD open reading frame (OFR) takes place because of the removal of the duplicated exons and generates full-length dystrophin protein

that is almost identical to normal dystrophin (Long et al., 2018). The advantage of using single cut sgRNA is, it need lowest modification of genome because of the reduced chance of off-target mutation by one single cut. So, the single-cut sgRNA technique, which provides a potential option for restoring dystrophin expression in DMD treatment, is used by around 70% of DMD patients (Fletcher et al., 2010).

II. Skipping of Exon: Approximately, in 83% of DMD patients, exon skipping strategy targets to eliminate the dystrophin gene by omitting one or more exon and reestablishing the dystrophin ORF (Kole & Krieg, 2015). This strategy is mostly used to convert severe DMD to a lesser form of BMD. In this technique, a mutant exon is skipped making the exon shortened and producing semi-functional dystrophin protein (Min et al., 2019). One DSB sequence is induced by sgRNA around the intron-exon region of the out-of-frame exon. Then, at the DSB location, NHEJ damage the splicing consensus series and trigger exon skipping to regain the dystrophin ORF by adding indels (Chemello et al., 2020). Aside from CRISPR, the US Food and Drug Administration (FDA) has approved an exon skipping treatment based on Antisense oligonucleotide (AON) for exon 51 since 2016 (Min et al., 2019). Over 20 years ago, it was revealed that directing an oligo-nucleotide to the splicing sites in the exon or intron can result in pre-mRNA splicing alteration and restore a damaged reading frame. Splicing modification and exon skipping have been investigated in a number of illnesses, first in thalassemia, and then in DMD (Muntoni & Wood, 2011). The fact that AON-based exon skipping impacts just dystrophin mRNA while leaving the underlying alteration in the dystrophin gene unaffected is a drawback. Taking monthly drug by DMA patient can make his or her life better by this approach. CRISPR gene editing minimizes the loss of genomic DNA by using a single sgRNA rather than using two sgRNAs flanking mutant exons. This single sgRNA instructs Cas9 to cut the sequence at the exon junction via NHEJ to eliminate the out-of-frame exon's splice acceptor or donor region. The outcome of this process indicates the chance of splicing the next accessible exon and bypassing the out-of-frame exon. Splicing of exon 49 into exon 51 in mice, breaks the dystrophin ORF, therefore a single sgRNA targets exon 51 splice acceptor site, bypassing exon 51 and repairing out-of-frame mutations (Amoasii et al., 2017). As a result, dystrophin ORF is restored and the dystrophin expression and muscle function are also reestablished (Amoasii et al., 2018).

III. Reframing of Exon: Exon reframing is an successful NHEJ-based strategy to restore the dystrophin ORF (Li et al., 2015). Exon reframing preserves consensus splice sequence using single

sgRNA and generates minor indels during repair. It efficiently keeps the maximum dystrophin genomic sequence while avoiding the DMD mutation. Theoretically, using of a sgRNA to promote NHEJ in an out-of-frame exon, a chance of one-in-three dystrophin frame restoration occurs due to the leading of produced indels to a targeted frame-shift (Min et al., 2019) . It has also been observed that the fourth nucleotide upstream of the PAM sequence can be used to predict NHEJ repair success (Chakrabarti et al., 2019). Notably, the DNA is still recognized by the sgRNA if the indels restore the original unprocessed sequence of DNA, allowing Cas to cut until the PAM site or DNA target sequence is no longer present.

IV. Knock-in of Exon: The exon knock-in strategy works through a DNA donor template integrated into HDR which is CRISPR induced. It has the ability to repair the full-length dystrophin protein. However, the DNA donor template length is restricted for certain delivery channels, which limits its use in big deletion mutations of dystrophin (Gee et al., 2017). HITI enables targeted gene implantation in non-proliferating cells, as well as techniques to employ NHEJ to precisely knock in absent exons at a specific locus without HDR (Suzuki et al., 2016).

V. Base editing of DMD: A new editing technology named base editing associated with the CRISPR to treat point mutation that approximately 25–35% of DMD patients are suffering from (Aartsma-Rus et al., 2006). There are two types of base editing tools: cytosine base editors which convert a C:G base pair to a T:A pair, and adenine base editors which convert an A:T pair to a G:C pair (Gaudelli et al., 2017). A catalytically defective Cas9 (dCas9) or Cas9 nickase (nCas9) coupled with RNA-guided nucleotide-specific base editors. As a consequence, unlike Cas9, they don’t produce DNA double-strand breaks and don’t employ the NHEJ pathway to repair them. This technique has been used to damage splicing acceptor sites, and induce exon skipping by disrupting premature stop codons (Ryu et al., 2018). Following BEs' efforts to produce innovative BEs that can limit off-target editing activity of both RNA and DNA, another novel designed approach is devised named primary editing (PE) (Pastoret & Sebille, 1995). With similar functionalities, PEs allow all expected base conversions at a certain place of the genome. Moreover, PEs can theoretically be used to do both exon skipping and exon reframing by mutating the splicing consensus series or introducing a certain amount of nucleotides to reinstate the dystrophin ORF (Chemello et al., 2020).

4.2. In vivo mutation modification and animal models of DMD by CRISPR/CAS9

More than 60 examples of modified animal model of DMD have been recorded up to this point (McGreevy et al., 2015). The most widely used DMD mouse model is the mdx animal, which has a spontaneous nonsense point mutation in exon 23 (cytosine-to-thymine transition) that causes dystrophin expression loss (Sicinski et al., 1986). Four chemical variations of Mdx mice (mdx2cv, mdx3cv, mdx4cv, and mdx5cv) exhibit point mutations that result in whole-length dystrophin deficiency and distinct dystrophin isoforms expression. These isoforms are involved in the DMD of mutant mice and their importance in making animal models is unavoidable (Chapman et al., 1989). AAV delivery of CRISPR/Cas9 system to these mdx mouse can delete mutated exon 23 from dystriphin gene resulting in modified dystrophin gene expression, partial recovery of functional dystrophin protein in skeletal myofibers and cardiac muscle. Again, mdx mice exhibit decreased fibrosis and inflammatory cell infiltration in skeletal and cardiac muscle, but their diaphragm has significant histological alterations that are similar to human DMD patients (Stedman et al., 1991). The process of AAV delivery and exon editing of mdx mouse having DMD is presented in figure 11.

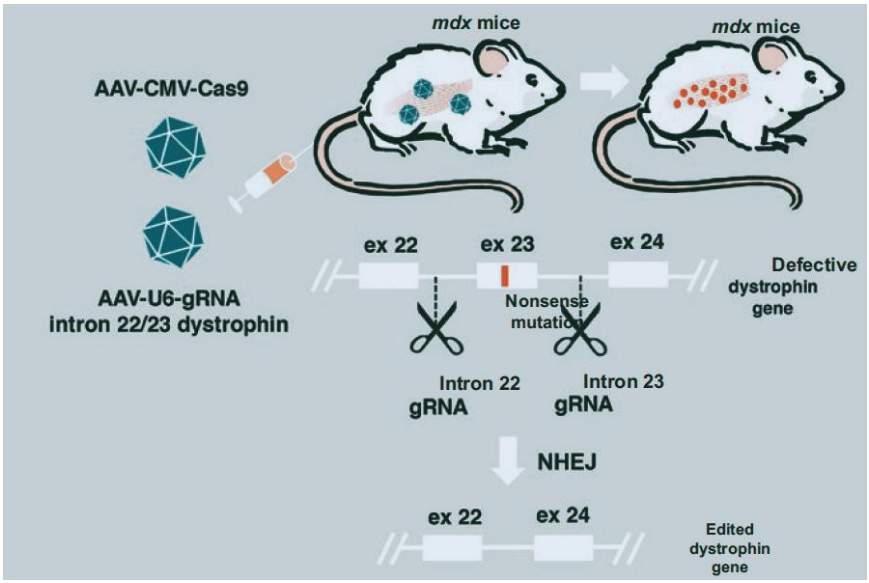

Figure 11: CRISPR/Cas9 in in vivo somatic muscle-directed editing. The Cas9 and gRNA complex with AAV vector, exhibits developed myotropic and cardiotropic properties. Cytomegalovirus (CMV) promoter expressed Cas9 and U6 pol III promoter expressed gRNAs. Systemic or local injection of vector into the muscle in adult or neonate mdx mice occurs and lastly NHEJ (Van Den Driessche & Chuah, 2016).

Another example is, exon 50 deletion which is the most frequent mutation in DMD patients, was also created in a mouse model using CRISPR/Cas9 (Amoasii et al., 2017). Also, to reproduce the

dystrophic phenotype in human DMD, many double-knockout mouse models, eliminating telomerase RNA in mdx mice have been developed (Rooney et al., 2006). Additionally, several reports about dogs (i.e. Corgi) clinical similarities with human DMD patients such as in limb weakness and cardiac defects, limb muscle atrophy, fibrosis, joint rigidity, hypersalivation, and the expected lifespan are also seen (Smith et al., 2011). So, DMD dogs are also a suitable model for preclinical gene therapy experiments because of their clinically comparable diseases.

4.3. In vitro engineering of DMD mutation in human iPSCs and modeling

In vitro disease modeling has been revolutionized through the discovery of induced pluripotent stem cells (iPSCs) that changed the way of thinking about disease modeling. The ability of human iPSCs of self-renew indefinitely and their pluripotency make them ideal for modeling illnesses. Patient-derived iPSCs, in contrast to animal models of disease, provided an expanding and limitless opportunity of testing possible therapeutics or researching underlying medical processes. Disease-specific iPSCs can be used to create surrogate models of human pathologies, scope for drug development and stem cell-based cell replacement therapies. Additionally, skin fibroblasts, peripheral blood mononuclear cells, and urine sample cells are converted to pluripotent cells and derivatives, essentially reproducing the patient's condition and genetic background in a culture plate (Landrum et al., 2016). CRISPR/Cas9- mediated deletion in hiPSCs can restore DMD reading frame and this is works as a therapeutic strategy for 60% of DMD patients.

Figure 12 schematically shows the process of modification of hiPSCs by CRISPR/Cas9.

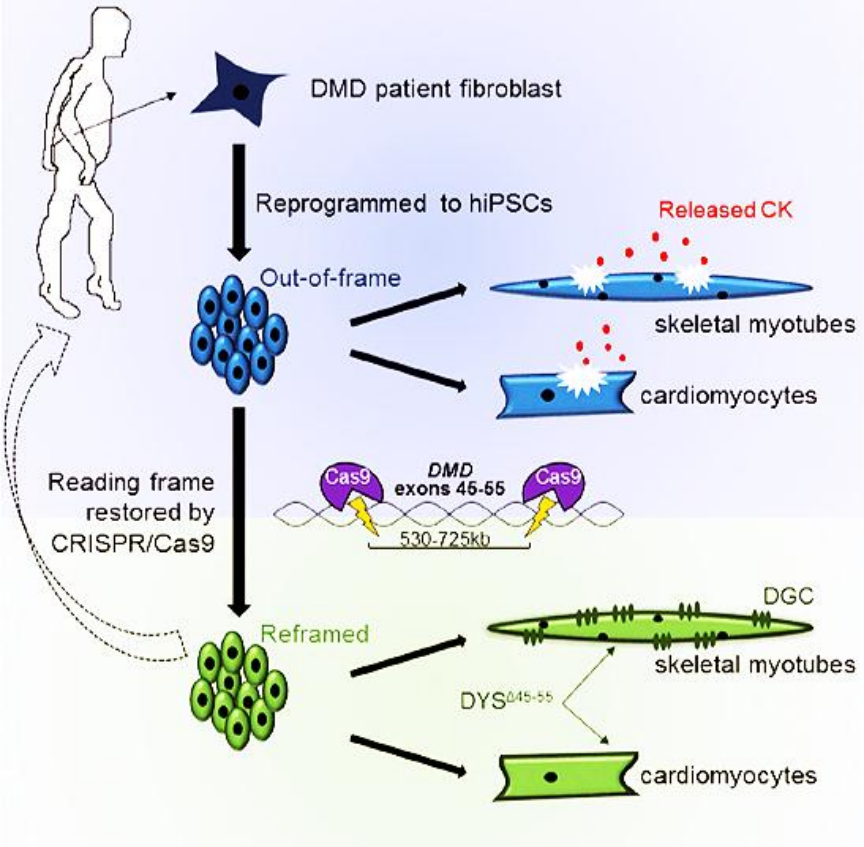

Figure 12: Schematically representation of CRISPR/Cas9-based reading frame restoration of hiPSCs. Reframed hiPSC restored dystrophin reading frame and expressed in skeletal muscle myotubes and Cardiomyocytes (Young et al., 2016).

DMD patient-derived iPSCs can be used to study disease-related systems and reproduce functional improvements. CRISPR can be used to modify and produce DMD-associated mutations in iPSCs derived from healthy subjects rather than from DMD patients. This procedure generates induced DMD iPSCs (iDMD), which result in an isogenic iPSC control line that varies from the iDMD iPSC line exclusively at the DMD locus, eliminating inherent variances across iPSC lines (Long et al., 2018). Calcium transient testing and 3D constructed heart muscle contraction experiments were also used to assess function of reestablished dystrophin in rectified DMD and iDMD iPSCs (Kyrychenko et al., 2017).

Table-4 summarizes methods, delivery and results of using CRISPR in human and mouse iPSC model.

Table 4: Corrections of DMD mutations in human iPSC and mice models(Chemello et al.,2020)**.**

Strategy		**DMD mutations**	**Correction outcomes**	**CRISPR/Cas system and Delivery method**
Human iPSC model	Double-cut myoediting	Δ Ex44	Knock-in	*Sp*Cas9, Electroporation
		Δ Ex46–51	Exons deletion	*Sp*Cas9, Electroporation
	Single-cut sgRNA myoediting	Δ Ex44	Exon skipping /reframing	*Sp*Cas9, Nucleofection
		Δ Ex48–50	Exon skipping /reframing	*Sp*Cas9, Nucleofection
	Nucleotide myoediting	Δ Ex51	Exon skipping	*Sp*Cas9-CBE, Lipotransfection
Mouse model	Double-cut myoediting	Ex53 mutation	Exons deletion	*Sp*Cas9 or *Sa*Cas9 ssAAV6
		Ex23 mutation	Exons deletion	*Sp*Cas9, Electroporation
	Single-cut sgRNA myoediting	Δ Ex50	Exon skipping /reframing	*Sp*Cas9, ssAAV9
	Nucleotide myoediting	Ex20 mutation	Base editing	*Sp*Cas9-ABE, Trans-splicing AAV9

Additionally, human iPSCs have been offered as a resource for cell-based replacement transplantation therapy in disorders including age-related muscular degeneration. Furthermore, to mimic disease-related gene expression, investigate mutation-dependent variability, and test potential DMD therapeutic possibilities, researchers used a number of patient-derived iPSC lines with diverse kinds of mutations (I. Y. Choi et al., 2016). As a result, in regenerative medicine and disease modeling in humans, this genome editing technology in iPSCs has become a major research strategy.

Chapter 5

Limitations and probable solution

From basic science to biomedical research and precision cancer therapy, CRISPR technology has revolutionized genome engineering and made it a powerful tool in the study of a variety of cancers. Despite the tremendous developments in CRISPR, which include better precision, target-specificity, ease of use, and multi-potential, there are still some limitations which limit its use. These limitations must be overcome in order to improve the performance of CRISPR/Cas systems and expand their applications in research.

Off-target mutagenesis effects are a serious issue when using programmable nucleases for genome editing, especially when used in vivo for therapeutic purposes (Zischewski et al., 2017). The development of a well-optimized and customized CRISPR technology can significantly decrease off-target consequences but not fully prevent it. For instant, improvements in high-throughput genome-wide next-generation sequencing, enhancing nuclease cleavage selectivity, or reducing the time period of functional activity of these techniques can reduce off-target effect (Zischewski et al., 2017). Also, a modified version of Cas9, known as the Cas-9 nickase creates only one DNA strand breakage rather than a double-stranded break to reduce off-target effect. Furthermore, using in silico methods to forecast potential off-target sites during the gRNA construction has been suggested to avoid off-targeting as much as possible (Lim et al., 2018). The lack of PAM sequences in the intended gene loci is one of the fundamental difficulties with genome editing techniques. However, Cas-nuclease variants such as SpCas9 and Cas12a are now accessible, which reduce PAM limitation (Kleinstiver et al., 2015). Moreover, base editors have recently made it feasible to accurately correct desired specific point mutations without using donor DNA templates, DSBs, or independence of HDR (Gaudelli et al., 2017).

Immunogenic toxicity is another issue raised by technological limits. In CRISPR-Cas9, in vitro–transcribed gRNAs stimulate RNA-sensing innate immune responses, resulting in cytotoxicity and cell death. This issue can be prevented by phosphatase treatment of gRNAs where 5′-triphosphate group is removed (Kim et al., 2018). According to Charlesworth and team, Anti-Cas9 antibodies against the most often used bacterial orthologs (SaCas9 and SpCas9) were discovered in human participants which induce immunogenic response in human (Charlesworth et al., 2019). In the future, further Cas9 immune-orthogonal orthologs may be identified, allowing for safe, repeated

gene therapy. Apart from all these, CRISPR/Cas is still a debatable and challenging ethical issue to be used in pluripotent stem cells of human.

Delivery systems creates obstacles in efficient delivery of CRISPR Cas9. Viral vector AAV raises concern which is often employed in DMD gene therapy due to its great efficacy in targeting muscle tissue (Zhang et al., 2020). AAV vector can cause immune response because of the presence of preexisting antibodies against AAV. This viral vector is limited to packing capacities and poor safety profile of CRISPR. So, to overcome the problem, multiple AAV vectors or a smaller Cas9 protein can be used to distribute individual components of the CRISPR/Cas9 system (Erkut & Yokota, 2022). Although the AAVs are frequently employed in DMD gene therapy due to its great efficacy in targeting muscle tissue, reduced pathogenicity, low immunogenicity, yet some studies in rhesus monkeys and piglets, systemic administration of a high dosage of AAV vector causes systemic and sensory neuronal damage. AAV capsid modification can make it even less immunogenic, allowing it to have a greater therapeutic impact (E. Choi & Koo, 2021).

On the other hand, addressing the difficulty of long-term maintenance of the edited DMD gene, Bengtsson et al. co-delivered a microdystrophin transgene together with CRISPR elements to stabilize the myofibers, slowing down the degeneration-regeneration cycle and preserving dystrophin expression in a mouse model. This process occurs against the persisted dystrophin in cardiomyocytes (Bengtsson et al., 2017). Furthermore, double-cut myoediting has a low editing effectiveness, which is most likely related to the requirement of both sgRNAs slicing at the same time across huge genomic lengths. Single-cut myoediting also have some limitations. Both Exon skipping and reframing are needed to get effective results and should be done using one sgRNA in some cases. For example, by using only one sgRNA to target exon 44, both exon skipping and reframing can be achieved culminating in recovery of dystrophin in Ex45 DMD mouse model. In contrast, the Ex43 DMD mouse model, when treated with the exact same sgRNA, restores dystrophin less efficiently because only exon skipping occurs in this repair mechanism (Min et al., 2020).

Conclusion

In recent times, CRISPR/Cas systems have revolutionized the field of genome editing. It has dramatically transformed research and treatment strategies in the field of cancer and DMD. CRISPR/Cas provides a number of benefits over traditional genome engineering tools, including

simplicity of design, ease of use, cost effectiveness, and high rate of successful outcomes. Thus, CRISPR/Cas system provide comparatively better results than ZFN and TALEN. As a result, this has become a key approach for cancer treatment (Song et al., 2020). The rational arrangement of delivery systems integrated with CRISPR/Cas tools has shown effective results in gene therapy for both cancer and DMD patients (Kaushik et al., 2019). Starting from animal models to human iPSC models, in vivo and in vitro experiments using a variety of vectors, CRISPR/Cas has a wide range of applications in both modeling and therapeutics of genetic disorders. It has been estimated that approximately 80% DMD patients can be cured through CRISPR/Cas-mediated treatment. Apart from lung, liver and breast cancer, CRISPR has also shown significant success in curing colorectal, pancreatic and blood cancer. Despite the numerous benefits of using CRISPR in genetic disorders, further studies, both in vitro and in animal models are required to improve the therapeutic safety and efficacy of CRISPR/Cas9-based therapies before administering them to cancer and DMD patients (Lim et al., 2018). Once the existing limitations of CRISPR are overcome, CRISPR-based treatments could be successfully used to permanently correct genetic mutations or mitigate their effects, offering the potential to transform cancer and DMD therapy in the near future (E. Choi & Koo, 2021).

Bibliography:

Aartsma-Rus, A., Van Deutekom, J. C. T., Fokkema, I. F., Van Ommen, G. J. B., & Den Dunnen, J. T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: An overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle and Nerve*, *34*(2), 135–144. https://doi.org/10.1002/mus.20586

Ahmad, G., & Amiji, M. (2018). Use of CRISPR / Cas9 gene-editing tools for developing models in drug discovery. *Drug Discovery Today*, *00*(00). https://doi.org/10.1016/j.drudis.2018.01.014

Álvarez-Fernández, M., Sanz-Flores, M., Sanz-Castillo, B., Salazar-Roa, M., Partida, D., Zapatero-Solana, E., Ali, H. R., Manchado, E., Lowe, S., Vanarsdale, T., Shields, D., Caldas, C., Quintela-Fandino, M., & Malumbres, M. (2018). Therapeutic relevance of the PP2A-B55 inhibitory kinase MASTL/Greatwall in breast cancer. *Cell Death and Differentiation*, *25*(5), 828–840. https://doi.org/10.1038/s41418-017-0024-0

Amoasii, L., Hildyard, J. C. W., Li, H., Sanchez-ortiz, E., Mireault, A., Caballero, D., Harron, R., Stathopoulou, T., Massey, C., Shelton, J. M., Bassel-duby, R., Piercy, R. J., & Olson, E. N. (2018). *Gene editing restores dystrophin expression in a canine*. *1549*, 1–10.

Amoasii, L., Long, C., Li, H., Mireault, A. A., Shelton, J. M., Sanchez-Ortiz, E., McAnally, J. R., Bhattacharyya, S., Schmidt, F., Grimm, D., Hauschka, S. D., Bassel-Duby, R., & Olson, E. N. (2017). Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy. *Science Translational Medicine*, *9*(418), 1–12. https://doi.org/10.1126/scitranslmed.aan8081

Antal, C. E., Hudson, A. M., Kang, E., Zanca, C., Wirth, C., Stephenson, N. L., Trotter, E. W., Gallegos, L. L., Miller, C. J., Furnari, F. B., Hunter, T., Brognard, J., & Newton, A. C. (2015). Cancer-associated protein kinase C mutations reveal kinase's role as tumor suppressor. *Cell*, *160*(3), 489–502. https://doi.org/10.1016/j.cell.2015.01.001

Arora, N., Pienkos, P. T., Pruthi, V., Poluri, K. M., & Guarnieri, M. T. (2018). Leveraging algal omics to reveal potential targets for augmenting TAG accumulation. *Biotechnology Advances*, *36*(4), 1274–1292. https://doi.org/10.1016/j.biotechadv.2018.04.005

Barrangou, R., & Horvath, P. (2017). A decade of discovery: CRISPR functions and applications. *Nature Microbiology*, *2*(June), 1–9. https://doi.org/10.1038/nmicrobiol.2017.92

Bengtsson, N. E., Hall, J. K., Odom, G. L., Phelps, M. P., Andrus, C. R., Hawkins, R. D.,

Hauschka, S. D., Chamberlain, J. R., & Chamberlain, J. S. (2017). Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nature Communications*, *8*, 1–9. https://doi.org/10.1038/ncomms14454

Bleiziffer, O., & Kneser, U. (2009). *Translating tissue engineering technology platforms into cancer research*. *13*(8), 1417–1427. https://doi.org/10.1111/j.1582-4934.2009.00853.x

Brabetz, O., Alla, V., Angenendt, L., Schliemann, C., Berdel, W. E., Arteaga, M. F., & Mikesch, J. H. (2017). RNA-Guided CRISPR-Cas9 System-Mediated Engineering of Acute Myeloid Leukemia Mutations. *Molecular Therapy - Nucleic Acids*, *6*(March), 243–248. https://doi.org/10.1016/j.omtn.2016.12.012

Brierley, A. S., Fernandes, P. G., Brandon, M. A., Armstrong, F., Millard, N. W., McPhail, S. D., Stevenson, P., Pebody, M., Perrett, J., Squires, M., Bone, D. G., & Griffiths, G. (2002). Antarctic krill under sea ice: Elevated abundance in a narrow band just south of ice edge. *Science*, *295*(5561), 1890–1892. https://doi.org/10.1126/science.1068574

Brison, N., Storms, J., Villela, D., Claeys, K. G., Dehaspe, L., de Ravel, T., De Waele, L., Goemans, N., Legius, E., Peeters, H., Van Esch, H., Race, V., Robert Vermeesch, J., Devriendt, K., & Van Den Bogaert, K. (2019). Maternal copy-number variations in the DMD gene as secondary findings in noninvasive prenatal screening. *Genetics in Medicine*, *21*(12), 2774–2780. https://doi.org/10.1038/s41436-019-0564-4

Brouns, S. J. J., Jore, M. M., Lundgren, M., Westra, E. R., Slijkhuis, R. J. H., Snijders, A. P. L., Dickman, M. J., Makarova, K. S., Koonin, E. V., & Van Der Oost, J. (2008). Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*, *321*(5891), 960–964. https://doi.org/10.1126/science.1159689

Carte, J., Christopher, R. T., Smith, J. T., Olson, S., Barrangou, R., Moineau, S., Glover, C. V. C., Graveley, B. R., Terns, R. M., & Terns, M. P. (2014). The three major types of CRISPR-Cas systems function independently in CRISPR RNA biogenesis in Streptococcus thermophilus. *Molecular Microbiology*, *93*(1), 98–112. https://doi.org/10.1111/mmi.12644

Chakrabarti, A. M., Henser-Brownhill, T., Monserrat, J., Poetsch, A. R., Luscombe, N. M., & Scaffidi, P. (2019). Target-Specific Precision of CRISPR-Mediated Genome Editing. *Molecular Cell*, *73*(4), 699-713.e6. https://doi.org/10.1016/j.molcel.2018.11.031

Chapman, V. M., Miller, D. R., Armstrong, D., & Caskey, C. T. (1989). Recovery of induced

mutations for X chromosome-linked muscular dystrophy in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(4), 1292–1296. https://doi.org/10.1073/pnas.86.4.1292

Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., Bode, N. M., Behlke, M. A., Dejene, B., Cieniewicz, B., Romano, R., Lesch, B. J., Gomez-Ospina, N., Mantri, S., Pavel-Dinu, M., Weinberg, K. I., & Porteus, M. H. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature Medicine*, *25*(2), 249–254. https://doi.org/10.1038/s41591-018-0326-x

Chemello, F., Bassel-Duby, R., & Olson, E. N. (2020). Correction of muscular dystrophies by CRISPR gene editing. *Journal of Clinical Investigation*, *130*(6), 2766–2776. https://doi.org/10.1172/JCI136873

Chiou, S. H., Winters, I. P., Wang, J., Naranjo, S., Dudgeon, C., Tamburini, F. B., Brady, J. J., Yang, D., Grüner, B. M., Chuang, C. H., Caswell, D. R., Zeng, H., Chu, P., Kim, G. E., Carpizo, D. R., Kim, S. K., & Winslow, M. M. (2015). Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing. *Genes and Development*, *29*(14), 1576–1585. https://doi.org/10.1101/gad.264861.115

Choi, E., & Koo, T. (2021). CRISPR technologies for the treatment of Duchenne muscular dystrophy. *Molecular Therapy*, *29*(11), 3179–3191. https://doi.org/10.1016/j.ymthe.2021.04.002

Choi, I. Y., Lim, H. T., Estrellas, K., Mula, J., Cohen, T. V., Zhang, Y., Donnelly, C. J., Richard, J. P., Kim, Y. J., Kim, H., Kazuki, Y., Oshimura, M., Li, H. L., Hotta, A., Rothstein, J., Maragakis, N., Wagner, K. R., & Lee, G. (2016). Concordant but Varied Phenotypes among Duchenne Muscular Dystrophy Patient-Specific Myoblasts Derived using a Human iPSC-Based Model. *Cell Reports*, *15*(10), 2301–2312. https://doi.org/10.1016/j.celrep.2016.05.016

Choi, P. S., & Meyerson, M. (2014). Targeted genomic rearrangements using CRISPR/Cas technology. *Nature Communications*, *5*, 1–6. https://doi.org/10.1038/ncomms4728

Cohen, S., Nathan, J. A., & Goldberg, A. L. (2014). Muscle wasting in disease: Molecular mechanisms and promising therapies. *Nature Reviews Drug Discovery*, *14*(1), 58–74. https://doi.org/10.1038/nrd4467

Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.;

Marraffini, L. A.; Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science Express*, *339*(6121), 819–823. https://doi.org/10.1038/nbt1319

Das Thakur, M., Pryer, N. K., & Singh, M. (2014). Mouse tumour models to guide drug development and identify resistance mechanisms. *Journal of Pathology*, *232*(2), 103–111. https://doi.org/10.1002/path.4285

Drost, J., Van Jaarsveld, R. H., Ponsioen, B., Zimberlin, C., Van Boxtel, R., Buijs, A., Sachs, N., Overmeer, R. M., Offerhaus, G. J., Begthel, H., Korving, J., Van De Wetering, M., Schwank, G., Logtenberg, M., Cuppen, E., Snippert, H. J., Medema, J. P., Kops, G. J. P. L., & Clevers, H. (2015). Sequential cancer mutations in cultured human intestinal stem cells. *Nature*, *521*(7550), 43–47. https://doi.org/10.1038/nature14415

Erkut, E., & Yokota, T. (2022). CRISPR Therapeutics for Duchenne Muscular Dystrophy. *International Journal of Molecular Sciences*, *23*(3). https://doi.org/10.3390/ijms23031832

Feng, W., Li, H. C., Xu, K., Chen, Y. F., Pan, L. Y., Mei, Y., Cai, H., Jiang, Y. M., Chen, T., & Feng, D. X. (2016). SHCBP1 is over-expressed in breast cancer and is important in the proliferation and apoptosis of the human malignant breast cancer cell line. *Gene*, *587*(1), 91–97. https://doi.org/10.1016/j.gene.2016.04.046

Fletcher, S., Adams, A. M., Johnsen, R. D., Greer, K., Moulton, H. M., & Wilton, S. D. (2010). Dystrophin isoform induction in vivo by antisense-mediated alternative splicing. *Molecular Therapy*, *18*(6), 1218–1223. https://doi.org/10.1038/mt.2010.45

Floc'h, N., Martin, M. J., Riess, J. W., Orme, J. P., Staniszewska, A. D., Menard, L., Cuomo, M. E., O'Neill, D. J., Ward, R. A., Finlay, R. M. V., McKerrecher, D., Cheng, M., Vang, D. P., Burich, R. A., Keck, J. G., Gandara, D. R., Mack, P. C., & Cross, D. A. E. (2018). Antitumor activity of osimertinib, an irreversible mutant-selective EGFR tyrosine kinase inhibitor, in NSCLC harboring EGFR exon 20 insertions. *Molecular Cancer Therapeutics*, *17*(5), 885–896. https://doi.org/10.1158/1535-7163.MCT-17-0758

Gaudelli, N. M., Komor, A. C., Rees, H. A., Packer, M. S., Badran, A. H., Bryson, D. I., & Liu, D. R. (2017). Programmable base editing of T to G C in genomic DNA without DNA cleavage. *Nature*, *551*(7681), 464–471. https://doi.org/10.1038/nature24644

Gee, P., Xu, H., & Hotta, A. (2017). Cellular Reprogramming, Genome Editing, and Alternative CRISPR Cas9 Technologies for Precise Gene Therapy of Duchenne Muscular Dystrophy. *Stem Cells International*, *2017*(DMD). https://doi.org/10.1155/2017/8765154

Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S., & Qi, L. S. (2013). XCRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*, *154*(2), 442. https://doi.org/10.1016/j.cell.2013.06.044

Habib, N., Havlík, R., Jiao, L., Kiri, A., Nicholls, J., Sarraf, C., Davies, A., Slavík, L., Wood, C., Tiraby, M., Drocourt, D., Reynes, J., & Tiraby, J. G. (n.d.). *whilst elongation factor promoter prolongs its duration Combination of Optison ™ with ultrasound and electroporation increases albumin and thrompoietin transgene expression whilst elongation factor promoter prolongs its duration*.

Han, X., Liu, Z., Jo, M., Zhang, K., Li, Y., Zeng, Z., Li, N., Zu, Y., & Qin, L. (2015). *CRISPR-Cas9 delivery to hard-to-transfect cells via membrane deformation*. *August*, 1–9.

He, C., Han, S., Chang, Y., Wu, M., Zhao, Y., Chen, C., & Chu, X. (2021). CRISPR screen in cancer: status quo and future perspectives. *American Journal of Cancer Research*, *11*(4), 1031–1050. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8085856/#:~:text=The development of CRISPR screen,vitro or in transplant models.

He, J., Zhang, W., Li, A., Chen, F., & Luo, R. (2018). Knockout of NCOA5 impairs proliferation and migration of hepatocellular carcinoma cells by suppressing epithelial-to-mesenchymal transition. *Biochemical and Biophysical Research Communications*, *500*(2), 177–183. https://doi.org/10.1016/j.bbrc.2018.04.017

He, S. (2020). The first human trial of CRISPR-based cell therapy clears safety concerns as new treatment for late-stage lung cancer. *Signal Transduction and Targeted Therapy*, *5*(1), 1–2. https://doi.org/10.1038/s41392-020-00283-8

Horii, T., Arai, Y., Yamazaki, M., Morita, S., Kimura, M., Itoh, M., Abe, Y., & Hatada, I. (2014). Validation of microinjection methods for generating knockout mice by CRISPR/Cas-mediated genome engineering. *Scientific Reports*, *4*(2), 1–6. https://doi.org/10.1038/srep04513

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, *157*(6), 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010

Huang, X., Zhou, G., Wu, W., Duan, Y., Ma, G., Song, J., Xiao, R., Vandenberghe, L., Zhang, F., Amore, P. A. D., & Lei, H. (n.d.). Genome editing abrogates angiogenesis in vivo. *Nature Communications*, 4–11. https://doi.org/10.1038/s41467-017-00140-3

Ishino, Y., Krupovic, M., & Forterre, P. (2018). History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology*, *200*(7). https://doi.org/10.1128/JB.00580-17

Jiang, C., Lin, X., & Zhao, Z. (2019). Applications of CRISPR/Cas9 Technology in the Treatment of Lung Cancer. *Trends in Molecular Medicine*, *25*(11), 1039–1049. https://doi.org/10.1016/j.molmed.2019.07.007

Jin, L. F., & Li, J. S. (2016). Generation of genetically modified mice using CRISPR/Cas9 and haploid embryonic stem cell systems. *Dong Wu Xue Yan Jiu = Zoological Research*, *37*(4), 205–213. https://doi.org/10.13918/j.issn.2095-8137.2016.4.205

Jin, Y., Shen, Y., Su, X., Weintraub, N., & Tang, Y. (2019). CRISPR/Cas9 technology in restoring dystrophin expression in iPSC-derived muscle progenitors. *Journal of Visualized Experiments*, *2019*(151), 1–9. https://doi.org/10.3791/59432

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). *A Programmable Dual-RNA – Guided*. *337*(August), 816–822.

Kaushik, I., Ramachandran, S., & Srivastava, S. K. (2019). CRISPR-Cas9: A multifaceted therapeutic strategy for cancer treatment. *Seminars in Cell and Developmental Biology*, *96*(April), 4–12. https://doi.org/10.1016/j.semcdb.2019.04.018

Kim, S., Koo, T., Jee, H. G., Cho, H. Y., Lee, G., Lim, D. G., Shin, H. S., & Kim, J. S. (2018). CRISPR RNAs trigger innate immune responses in human cells. *Genome Research*, *28*(3), 367–373. https://doi.org/10.1101/gr.231936.117

Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V, Nguyen, N. T., Zheng, Z., Gonzales, A. P. W., Li, Z., Peterson, R. T., Yeh, J. J., Aryee, M. J., & Joung, J. K. (2015). *Engineered CRISPR-Cas9 nucleases with altered PAM specificities*. https://doi.org/10.1038/nature14592

Kole, R., & Krieg, A. M. (2015). Exon skipping therapy for Duchenne muscular dystrophy. *Advanced Drug Delivery Reviews*, *87*, 104–107. https://doi.org/10.1016/j.addr.2015.05.008

Konermann, S., Brigham, M. D., Trevino, A. E., Joung, J., Abudayyeh, O. O., Barcena, C., Hsu, P. D., Habib, N., Gootenberg, J. S., Nishimasu, H., Nureki, O., & Zhang, F. (2015). Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*, *517*(7536), 583–588. https://doi.org/10.1038/nature14136

Kotterman, M. A., Chalberg, T. W., & Schaffer, D. V. (2015). *Viral Vectors for Gene Therapy : Translational and Clinical Outlook*. 63–89. https://doi.org/10.1146/annurev-bioeng-071813-

104938

Kozubek, J. (2016). Crispr, Cas and Capitalists. In *Modern Prometheus*. https://doi.org/10.1017/cbo9781316771440.002

Kyrychenko, V., Kyrychenko, S., Tiburcy, M., Shelton, J. M., Long, C., Schneider, J. W., Zimmermann, W. H., Bassel-Duby, R., & Olson, E. N. (2017). Functional correction of dystrophin actin binding domain mutations by genome editing. *JCI Insight*, *2*(18), 1–16. https://doi.org/10.1172/JCI.INSIGHT.95918

Landrum, M. J., Lee, J. M., Benson, M., Brown, G., Chao, C., Chitipiralla, S., Gu, B., Hart, J., Hoffman, D., Hoover, J., Jang, W., Katz, K., Ovetsky, M., Riley, G., Sethi, A., Tully, R., Villamarin-Salomon, R., Rubinstein, W., & Maglott, D. R. (2016). ClinVar: Public archive of interpretations of clinically relevant variants. *Nucleic Acids Research*, *44*(D1), D862–D868. https://doi.org/10.1093/nar/gkv1222

Li, H. L., Fujimoto, N., Sasakawa, N., Shirai, S., Ohkame, T., Sakuma, T., Tanaka, M., Amano, N., Watanabe, A., Sakurai, H., Yamamoto, T., Yamanaka, S., & Hotta, A. (2015). Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports*, *4*(1), 143–154. https://doi.org/10.1016/j.stemcr.2014.10.013

Lim, K. R. Q., Yoon, C., & Yokota, T. (2018). Applications of CRISPR/Cas9 for the treatment of duchenne muscular dystrophy. *Journal of Personalized Medicine*, *8*(4). https://doi.org/10.3390/jpm8040038

Lin, G., Zhang, K., & Li, J. (2015). Application of CRISPR/Cas9 technology to HBV. *International Journal of Molecular Sciences*, *16*(11), 26077–26086. https://doi.org/10.3390/ijms161125950

Lin, S. R., Yang, H. C., Kuo, Y. T., Liu, C. J., Yang, T. Y., Sung, K. C., Lin, Y. Y., Wang, H. Y., Wang, C. C., Shen, Y. C., Wu, F. Y., Kao, J. H., Chen, D. S., & Chen, P. J. (2014). The CRISPR/Cas9 system facilitates clearance of the intrahepatic HBV templates in vivo. *Molecular Therapy - Nucleic Acids*, *3*(August), e186. https://doi.org/10.1038/mtna.2014.38

Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering crispr: A review of the challenges and approaches. *Drug Delivery*, *25*(1), 1234–1257. https://doi.org/10.1080/10717544.2018.1474964

Liu, B., Saber, A., & Haisma, H. J. (2019). PT SC. *Drug Discovery Today*.

https://doi.org/10.1016/j.drudis.2019.02.011

Liu, Z., Dong, H., Cui, Y., Cong, L., & Zhang, D. (2020). Application of different types of CRISPR/Cas-based systems in bacteria. *Microbial Cell Factories*, *19*(1), 1–14. https://doi.org/10.1186/s12934-020-01431-z

Long, C., Li, H., Tiburcy, M., Rodriguez-Caycedo, C., Kyrychenko, V., Zhou, H., Zhang, Y., Min, Y. L., Shelton, J. M., Mammen, P. P. A., Liaw, N. Y., Zimmermann, W. H., Bassel-Duby, R., Schneider, J. W., & Olson, E. N. (2018). Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. *Science Advances*, *4*(1), 1–12. https://doi.org/10.1126/sciadv.aap9004

Long, C., McAnally, J. R., Shelton, J. M., Mireault, A. A., Bassel-Duby, R., & Olson, E. N. (2014). Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science*, *6201*, 1184–1188. https://doi.org/10.1126/science.1254445

Maddalo, D., Manchado, E., Concepcion, C. P., Bonetti, C., Vidigal, J. A., Han, Y., Ogrodowski, P., Crippa, A., Rekhtman, N., Stanchina, E. De, Lowe, S. W., & Ventura, A. (2014). rearrangements with the CRISPR / Cas9 system. *Nature*. https://doi.org/10.1038/nature13902

Manguso, R. T., Pope, H. W., Zimmer, M. D., Brown, F. D., Yates, K. B., Miller, B. C., Collins, N. B., Bi, K., La Fleur, M. W., Juneja, V. R., Weiss, S. A., Lo, J., Fisher, D. E., Miao, D., Van Allen, E., Root, D. E., Sharpe, A. H., Doench, J. G., & Haining, W. N. (2017). In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature*, *547*(7664), 413–418. https://doi.org/10.1038/nature23270

Mashiko, D., Fujihara, Y., Satouh, Y., Miyata, H., Isotani, A., & Ikawa, M. (2013). Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Scientific Reports*, *3*, 1–6. https://doi.org/10.1038/srep03355

Matano, M., Date, S., Shimokawa, M., Takano, A., Fujii, M., Ohta, Y., Watanabe, T., Kanai, T., & Sato, T. (2015). Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nature Medicine*, *21*(3), 256–262. https://doi.org/10.1038/nm.3802

McGreevy, J. W., Hakim, C. H., McIntosh, M. A., & Duan, D. (2015). Animal models of Duchenne muscular dystrophy: From basic mechanisms to gene therapy. *DMM Disease Models and Mechanisms*, *8*(3), 195–213. https://doi.org/10.1242/dmm.018424

Min, Y. L., Bassel-Duby, R., & Olson, E. N. (2019). CRISPR correction of duchenne muscular

dystrophy. *Annual Review of Medicine*, *70*(October 2018), 239–255. https://doi.org/10.1146/annurev-med-081117-010451

Min, Y. L., Chemello, F., Li, H., Rodriguez-Caycedo, C., Sanchez-Ortiz, E., Mireault, A. A., McAnally, J. R., Shelton, J. M., Zhang, Y., Bassel-Duby, R., & Olson, E. N. (2020). Correction of Three Prominent Mutations in Mouse and Human Models of Duchenne Muscular Dystrophy by Single-Cut Genome Editing. *Molecular Therapy*, *28*(9), 2044–2055. https://doi.org/10.1016/j.ymthe.2020.05.024

Mojica, F. J. M., & Rodriguez-Valera, F. (2016). The discovery of CRISPR in archaea and bacteria. *FEBS Journal*, 3162–3169. https://doi.org/10.1111/febs.13766

Mou, H., Kennedy, Z., Anderson, D. G., Yin, H., & Xue, W. (2015). Precision cancer mouse models through genome editing with CRISPR-Cas9. *Genome Medicine*, *7*(1), 1–11. https://doi.org/10.1186/s13073-015-0178-7

Mout, R., Ray, M., Lee, W., Tay, T., Sasaki, K., & Rotello, V. M. (2017). *Direct Cytosolic Delivery of CRISPR / Cas9- Ribonucleoprotein for Efficient Gene Editing*. https://doi.org/10.1021/acsnano.6b07600

Muntoni, F., & Wood, M. J. A. (2011). Targeting RNA to treat neuromuscular disease. *Nature Reviews Drug Discovery*, *10*(8), 621–637. https://doi.org/10.1038/nrd3459

Nidhi, S., Anand, U., Oleksak, P., Tripathi, P., Lal, J. A., Thomas, G., Kuca, K., & Tripathi, V. (2021). Novel crispr–cas systems: An updated review of the current achievements, applications, and future research perspectives. *International Journal of Molecular Sciences*, *22*(7), 1–42. https://doi.org/10.3390/ijms22073327

Ousterout, D. G., Kabadi, A. M., Thakore, P. I., Majoros, W. H., Reddy, T. E., & Gersbach, C. A. (2015). Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause duchenne muscular dystrophy. *Nature Communications*, *6*, 1–13. https://doi.org/10.1038/ncomms7244

Pastoret, C., & Sebille, A. (1995). Mdx Mice Show Progressive Weakness and Muscle Deterioration With Age. *Journal of the Neurological Sciences*, *129*(2), 97–105. https://doi.org/10.1016/0022-510X(94)00276-T

Platt, R. J., Chen, S., Zhou, Y., Yim, M. J., Swiech, L., Kempton, H. R., Dahlman, J. E., Parnas, O., Eisenhaure, T. M., Jovanovic, M., Graham, D. B., Jhunjhunwala, S., Heidenreich, M., Xavier, R. J., Langer, R., & Anderson, D. G. (2014). Resource CRISPR-Cas9 Knockin Mice

for Genome Editing and Cancer Modeling. *Cell*, 1–16. https://doi.org/10.1016/j.cell.2014.09.014

Pourcel, C., Salvignol, G., & Vergnaud, G. (2005). CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology*, *151*(3), 653–663. https://doi.org/10.1099/mic.0.27437-0

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, *8*(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Ratan, Z. A., Son, Y. J., Haidere, M. F., Uddin, B. M. M., Yusuf, M. A., Zaman, S. Bin, Kim, J. H., Banu, L. A., & Cho, J. Y. (2018). CRISPR-Cas9: A promising genetic engineering approach in cancer research. *Therapeutic Advances in Medical Oncology*, *10*, 1–15. https://doi.org/10.1177/1758834018755089

Riordan, S. M., Heruth, D. P., Zhang, L. Q., & Ye, S. Q. (2015). Application of CRISPR/Cas9 for biomedical discoveries. *Cell and Bioscience*, *5*(1), 1–11. https://doi.org/10.1186/s13578-015-0027-9

Rooney, J. E., Welser, J. V., Dechert, M. A., Flintoff-Dye, N. L., Kaufman, S. J., & Burkin, D. J. (2006). Severe muscular dystrophy in mice that lack dystrophin and α7 integrin. *Journal of Cell Science*, *119*(11), 2185–2195. https://doi.org/10.1242/jcs.02952

Ryu, S. M., Koo, T., Kim, K., Lim, K., Baek, G., Kim, S. T., Kim, H. S., Kim, D. E., Lee, H., Chung, E., & Kim, J. S. (2018). Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. *Nature Biotechnology*, *36*(6), 536–539. https://doi.org/10.1038/nbt.4148

Saadatmand, S., Vos, J. R., Hooning, M. J., Oosterwijk, J. C., Koppert, L. B., Bock, G. H. De, Ausems, M. G., Asperen, C. J. Van, Aalfs, C. M., Gómez, E. B., Wanna, A. G. B., Noble, J. H., Carlson, M. L., Gifford, H., Dietrich, M. S., Haynes, D. S., Dawant, B. M., Saadatmand, S., Vos, J. R., … Chester, J. D. (2014). This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please c. *The Laryngoscope*, *2*, 2–31.

Saber, A., Wekken, A. Van Der, Hiltermann, T. J. N., Kok, K., Berg, A. Van Den, & Groen, H. J. M. (2015). Genomic aberrations guiding treatment of non-small cell lung cancer patients.

Cancer Treatment Communications, *4*, 23–33. https://doi.org/10.1016/j.ctrc.2015.03.005

Salmaninejad, A., Valilou, S. F., Bayat, H., Ebadi, N., Daraei, A., Yousefi, M., Nesaei, A., & Mojarrad, M. (2018). Duchenne muscular dystrophy: an updated review of common available therapies. *International Journal of Neuroscience*, *128*(9), 854–864. https://doi.org/10.1080/00207454.2018.1430694

Sanchez-Rivera, F. J., Papagiannakopoulos, T., Romero, R., Tammela, T., Bauer, M. R., Bhutkar, A., Joshi, N. S., Subbaraj, L., Bronson, R. T., Xue, W., & Jacks, T. (2014). Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature*, *516*(7531), 428–431. https://doi.org/10.1038/nature13906

Sessions, J. W., Skousen, C. S., Price, K. D., Hanks, B. W., Hope, S., Alder, J. K., & Jensen, B. D. (2016). CRISPR‐Cas9 directed knock‐out of a constitutively expressed gene using lance array nanoinjection. *SpringerPlus*. https://doi.org/10.1186/s40064-016-3037-0

Sharan, S. K., Thomason, L. C., Kuznetsov, S. G., & Court, D. L. (2009). Recombineering: A homologous recombination-based method of genetic engineering. *Nature Protocols*, *4*(2), 206–223. https://doi.org/10.1038/nprot.2008.227

Sicinski, P., Geng, Y. A. N., Ryder-cook, A. S., Barnard, E. A., Darlison, M. G., & Barnardt, P. J. (1986). *WRONG CITATION Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., & Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science (New York, N.Y.), 244(4912), 1578–1580. https://doi.o*. *244*(21), 14–16.

Smith, B. F., Yue, Y., Woods, P. R., Kornegay, J. N., Shin, J. H., Williams, R. R., & Duan, D. (2011). An intronic LINE-1 element insertion in the dystrophin gene aborts dystrophin expression and results in Duchenne-like muscular dystrophy in the corgi breed. *Laboratory Investigation*, *91*(2), 216–231. https://doi.org/10.1038/labinvest.2010.146

Song, X., Liu, C., Wang, N., Huang, H., He, S., Gong, C., & Wei, Y. (2020). Delivery of CRISPR / Cas systems for cancer gene therapy and immunotherapy. *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2020.04.010

Stedman, H. H., Sweeney, H. L., Shrager, J. B., Maguire, H. C., Panettieri, R. A., Petrof, B., Narusawa, M., Leferovich, J. M., Sladky, J. T., & Kelly, A. M. (1991). The mdx mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. In *Nature* (Vol. 352, Issue 6335, pp. 536–539). https://doi.org/10.1038/352536a0

Suzuki, K., Tsunekawa, Y., Hernandez-Benitez, R., Wu, J., Zhu, J., Kim, E. J., Hatanaka, F., Yamamoto, M., Araoka, T., Li, Z., Kurita, M., Hishida, T., Li, M., Aizawa, E., Guo, S., Chen, S., Goebl, A., Soligalla, R. D., Qu, J., … Belmonte, J. C. I. (2016). In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*, *540*(7631), 144–149. https://doi.org/10.1038/nature20565

Tong, S., Moyo, B., Lee, C. M., Leong, K., & Bao, G. (n.d.). Engineered materials for in vivo delivery of genome-editing machinery. *Nature Reviews Materials*. https://doi.org/10.1038/s41578-019-0145-9

Torres-Ruiz, R., & Rodriguez-Perales, S. (2015). CRISPR-Cas9: A revolutionary tool for cancer modelling. *International Journal of Molecular Sciences*, *16*(9), 22151–22168. https://doi.org/10.3390/ijms160922151

Torres, R., Martin, M. C., Garcia, A., Cigudosa, J. C., Ramirez, J. C., & Rodriguez-Perales, S. (2014). Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. *Nature Communications*, *5*, 1–8. https://doi.org/10.1038/ncomms4964

Van Den Driessche, T., & Chuah, M. K. (2016). CRISPR/Cas9 flexes its muscles: In vivo somatic gene editing for muscular dystrophy. *Molecular Therapy*, *24*(3), 414–416. https://doi.org/10.1038/mt.2016.29

Vasquez, K. M., Marburger, K., Intody, Z., & Wilson, J. H. (2001). Manipulating the mammalian genome by homologous recombination. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(15), 8403–8410. https://doi.org/10.1073/pnas.111009698

Wan, T., Niu, D., Wu, C., Xu, F., & Church, G. (2019). Material solutions for delivery of CRISPR / Cas-based genome editing tools : Current status and future outlook. *Materials Today*, *xxx*(xx). https://doi.org/10.1016/j.mattod.2018.12.003

Wang, Haoyi, Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/cas-mediated genome engineering. *Cell*, *153*(4), 910–918. https://doi.org/10.1016/j.cell.2013.04.025

Wang, Hong-xia, Li, M., Lee, C. M., Chakraborty, S., Kim, H., Bao, G., & Leong, K. W. (2016). *CRISPR / Cas9-Based Genome Editing for Disease Modeling and Therapy : Challenges and Opportunities for Nonviral Delivery*. https://doi.org/10.1021/acs.chemrev.6b00799

Wong, N., Liu, W., & Wang, X. (2015). WU-CRISPR: Characteristics of functional guide RNAs for the CRISPR/Cas9 system. *Genome Biology*, *16*(1), 1–8. https://doi.org/10.1186/s13059-015-0784-0

Xu, C. L., Ruan, M. Z. C., Mahajan, V. B., & Tsang, S. H. (2019). Viral delivery systems for crispr. *Viruses*, *11*(1), 1–12. https://doi.org/10.3390/v11010028

Yang, Y., Zhu, J., Zhang, T., Liu, J., Li, Y., Zhu, Y., Xu, L., Wang, R., Su, F., Ou, Y., & Wu, Q. (2018). PHD-finger domain protein 5A functions as a novel oncoprotein in lung adenocarcinoma. *Journal of Experimental and Clinical Cancer Research*, *37*(1), 1–14. https://doi.org/10.1186/s13046-018-0736-0

Yoshiba, T., Saga, Y., Urabe, M., & Uchibori, R. (2019). *CRISPR / Cas9 ‐ mediated cervical cancer treatment targeting human papillomavirus E6*. 2197–2206. https://doi.org/10.3892/ol.2018.9815

Young, C. S., Hicks, M. R., Ermolova, N. V., Nakano, H., Jan, M., Younesi, S., Karumbayaram, S., Kumagai-Cresse, C., Wang, D., Zack, J. A., Kohn, D. B., Nakano, A., Nelson, S. F., Miceli, M. C., Spencer, M. J., & Pyle, A. D. (2016). A Single CRISPR-Cas9 Deletion Strategy that Targets the Majority of DMD Patients Restores Dystrophin Function in hiPSC-Derived Muscle Cells. *Cell Stem Cell*, *18*(4), 533–540. https://doi.org/10.1016/j.stem.2016.01.021

Zhang, Y., Li, H., Min, Y. L., Sanchez-Ortiz, E., Huang, J., Mireault, A. A., Shelton, J. M., Kim, J., Mammen, P. P. A., Bassel-Duby, R., & Olson, E. N. (2020). Enhanced CRISPR-Cas9 correction of Duchenne muscular dystrophy in mice by a self-complementary AAV delivery system. *Science Advances*, *6*(8), 1–12. https://doi.org/10.1126/sciadv.aay6812

Zhang, Y., Long, C., Bassel-Duby, R., & Olson, E. N. (2018). Myoediting: Toward prevention of muscular dystrophy by therapeutic genome editing. *Physiological Reviews*, *98*(3), 1205–1240. https://doi.org/10.1152/physrev.00046.2017

Zhang, Y., Long, C., Li, H., McAnally, J. R., Baskin, K. K., Shelton, J. M., Bassel-Duby, R., & Olson, E. N. (2017). CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice. *Science Advances*, *3*(4). https://doi.org/10.1126/sciadv.1602814

Zischewski, J., Fischer, R., & Bortesi, L. (2017). Detection of on-target and off-target mutations generated by CRISPR/Cas9 and other sequence-specific nucleases. *Biotechnology Advances*, *35*(1), 95–104. https://doi.org/10.1016/j.biotechadv.2016.12.003

Zuris, J. A., Thompson, D. B., Shu, Y., Guilinger, J. P., Bessen, J. L., Hu, J. H., Maeder, M. L.,

Joung, J. K., Chen, Z., & Liu, D. R. (2014). A RT I C L E S Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nature Publishing Group*, *October*, 1–10. https://doi.org/10.1038/nbt.3081