CRISPR/Cas9 System: A Versatile Gene Editing Tool in Disease Prevention and Treatment

By

Amatul Maria
14246006

A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy

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Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
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.
4. I/We have acknowledged all main sources of help.

Student's Full Name & Signature:

Amatul Maria
14246006

Approval

The thesis/project titled "CRISPR/Cas9 System: A Versatile Gene Editing Tool in Disease Prevention and Treatment" submitted by Amatul Maria (14246006) of Spring 2015, has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy on October, 2019.

Examining Committee:

Supervisor:
(Member)

_____

Dr. Afrina Afrose
Assistant Professor, Department of Pharmacy
Brac University

Program Coordinator:
(Member)

_____

Dr. Hasina Yasmin
Professor, Department of Pharmacy
Brac University

Departmental Head:
(Chair)

_____

Professor Dr. Eva Rahman Kabir
Chairperson, Department of Pharmacy
Brac University

Abstract

With the development of genome editing as a promising mechanism for disease prevention and treatment, clustered regularly interspaced short palindromic repeats (CRISPR) is a robust and multiplexed tool that is able to address the challenges faced by its predecessors such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nuclease (TALENs). The findings indicate that CRISPR-Cas9 is more precise, convenient and cost effective in comparison with its forerunners in terms of DNA cleavage. Recent advancements of this tool have enabled researchers and scientists to adopt treatment options for diseases like cancer, AIDS, muscular dystrophy, blood disorders, cystic fibrosis, Huntington's disease etc. Despite massive potential of this tool to detect and modify genes responsible for various diseases, human trials remain the most controversial factor in scientific community and academia. Hence, more research is needed to explore more possibilities and fill the gaps in information that can prove to be crucial for achieving desired outcomes in disease prevention and treatment.

Keywords:

CRISPR-Cas9; genome editing; screening; disease modeling; ethical concerns

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I would like to emphasize at the end that the limitations of this thesis are entirely mine.

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List of Acronyms

AAVs	Adeno-Associated Virus
BCL2	B-cell lymphoma 2
CCR5	C-C Chemokine Receptor Type 5
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
DMD	Duchenne muscular dystrophy
GABA	Gamma-Aminobutyric Acid
GeCKO	Genome-Scale CRISPR-Cas9 Knockout
HIV	Human Immunodeficiency Viruses
iPSC	Induced Pluripotent Stem Cell
JAK3	Janus Kinase 3
MYH6	Myosin Heavy Chain 6
PAMs	Protospacer Adjacent Motifs
RNAi	RNA Interface
sgRNAs	Single Guide RNA
TALE	Transcription Activator-like Effector

TALENs	Transcription Activator-like Effector Nuclease
tracrRNA	trans-activating crRNA
ZFNs	Zinc-Finger Nucleases

Chapter 1

Introduction

1.1 Background

With the advancement of technology, disease prevention and treatment has become more precise and accessible around the world. Although significant improvement has been experienced in drug discovery and treatment of ailments, the prospects of genome editing are yet to be fully discovered. Identifying the mutations that cause diseases has become easier with microarray technologies and next generation sequencing (Guernet & Grumolato, 2017). Nonetheless, next generation sequencing creates a greater thread of candidates of disease mutation in the data mining process which makes the process more complex. The current potential of gene editing techniques, which allows modifying genome at the single nucleotide level, has changed the way of mutation screening (Driehuis & Clevers, 2017). In disease therapy, some revolutionary and essential genome editing technologies have been developed so far which include RNA Interface (RNAi), Zinc-Finger Nucleases (ZFNs) and Transcription Activator-like Effector Nuclease (TALENs), CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) etc. ZFNs and TALENs are fusions of non-specific cleavage domain of DNA and binding domains of sequence-specific DNA as well as Transcription Activator-Like Effector (TALE) proteins (Savić & Schwank, 2016). For each new target site, ZFNs and TALENs require recoding of proteins. This process is very expensive and highly time consuming (Hryhorowicz, Lipiński, Zeyland, & Słomski, 2017). CRISPR/Cas9 technology was able to address this issue and mitigate the problems substantially.

CRISPR-Cas9 is the latest step into developing a unique genome editing tool. It is better than ZFNs and TALENs because CRISPR/Cas9 only requires suitable single guide RNA (sgRNA) to be designed in order to target a new site. That is because in all cases, the Cas9 protein remains

the same. Furthermore, for simultaneous editing of several targets in the genome of mammalian species, the expression of Cas9 and multiple guide RNAs can be used (Cong et al., 2013). Hence, this system is evidently easier, less cytotoxic and efficient compared with the traditional genome editing technology (Khan et al., 2016). It has shown to correct mutations that cause particular diseases and promises to be a promising therapy for protecting patients at a genetic level. The CRISPR-Cas9 system mainly works as a molecular scissor that is used in sectors such as drug discovery, cancer research, mental disease treatment, plant applications etc. In this study, a review of existing research on advancements in CRISPR-Cas9 technology in research, diagnosis and therapeutics on human diseases and future prospects of this technology have been focused.

1.2 Objectives of the Study

For this study, the broad objective is to contemplate the advancements and future prospects of CRISPR-Cas9 technology in disease mutation identification, prevention and treatment. To achieve this broad objective, some specific objectives have been focused in this study. The specific objectives of the study are as follows:

1. To understand how CRISPR-Cas9 works as a genome editing tool for humans
2. To discover the recent developments in CRISPR-Cas9 technology to detect and treat humans diseases
3. To extrapolate future possibilities of CRISPR-Cas9 technology in disease prevention and treatment
4. To delineate the ethical concerns of using CRISPR-Cas9 technology for disease prevention on humans

1.3 Rationale of the Study

Genome editing is one of the most exciting scientific discoveries in modern era. From retaining desirable traits in descendants of species to offering prevention and treatment options, gene editing has become widely popular in the scientific community. So far, the most widely used gene editing tools are ZFNs, TALENs and CRISPR-Cas9. Each of these genome editing tools has its own benefits and challenges which allow the scientists to address problems that can be solved with genetic modification. Through an in-depth analysis of the advantages and drawbacks of these tools, the most effective and efficient gene editing tool for disease prevention and treatment can be identified. In this review study, the effectiveness of CRISPR-Cas9 as a versatile gene editing tool in disease prevention and treatment is analyzed. In comparison with ZFNs and TALENs, the precision and convenience of CRISPR-Cas9 can be properly depicted. This study also explores the potential ethical concerns of using CRISPR-Cas9 on humans to prevent or treat various kinds of diseases. Since the future of biotechnology in medical research is driven by genome editing, this study to discover the most effective and efficient tool will be highly beneficial.

1.4 CRISPR

What is CRISPR?

CRISPR technology is a highly effective and powerful tool for genome editing which enables the researchers to modify gene function and alter DNA sequences. It has several potential applications ranging from preventing diseases from spreading, treating diseases, correcting of genetic defects, improving crop quality and so on (Heidi Ledford, 2016). It is basically a family of sequences of DNA that is found in the genomes of prokaryotic organisms. When viruses attack bacteria more than once, these sequences are derived from the DNA fragments that they have. Then, using subsequent infections, they are able to identify and incapacitate DNA that

belongs to same kind of viruses. Two distinct characteristics of DNA (i.e. repeats and spacers) constitute the specialized region of CRISPR. Throughout the CRISPR region, the repeated sequences of nucleotides are distributed whereas spacers are interspaced among the repeated sequences of DNA. The spacers primarily function as bank of memories allowing bacteria to recognize viruses that attacked before and fight them off. In archaea and bacteria, an adaptive RNA-mediated defense system is constituted that invades the plasmids and phages which are known as CRISPR RNAs (crRNAs). When the virus attacks again and the spacer is incorporated, transcribing and processing of a portion of the CRISPR is initiated intocrRNA (Aquino-Jarquin, 2017). In order to produce a complementary sequence of a single stranded-RNA, a template can be discovered in the nucleotide sequence of the CRISPR.

CRISPR-Cas9

Clustered regularly-interspaced short palindromic repeats (CRISPR) are a revolutionary invention in genome editing field due to its affinity and efficiency in molecular biology and gene editing precision. CRISPR has become so popular within a short period of time because it requires less training and low laboratory cost compared to the other gene-editing techniques (Shen et al , 2017). This criterion also makes it more accessible than the other. CRISPR-associated protein (Cas9) is primarily an enzyme that cuts foreign DNA. It primarily binds to two RNA molecules that are crRNA (CRISPR RNA) and trans-activating crRNA (tracrRNA). Cas9 is guided to the target site where the cut will be made. Consequently, a 20-nucleotide stretch of crRNA complements this expanse of DNA. Both strands of DNA double helix are cut by Cas9 by using two separate domains or regions on its structure. In order to ensure that Cas9 does not cut anywhere else in a genome, a built-in safety mechanism is ensured (Schwank et al., 2013). In this process, a short DNA sequence is used which is known as PAMs (Protospacer Adjacent Motifs). These PAMS sit adjacent to the targeted sequence of DNA and

they serve as tags. The Cas9 complex will not cut its targeted DNA sequence if is not acquainted with a PAM.

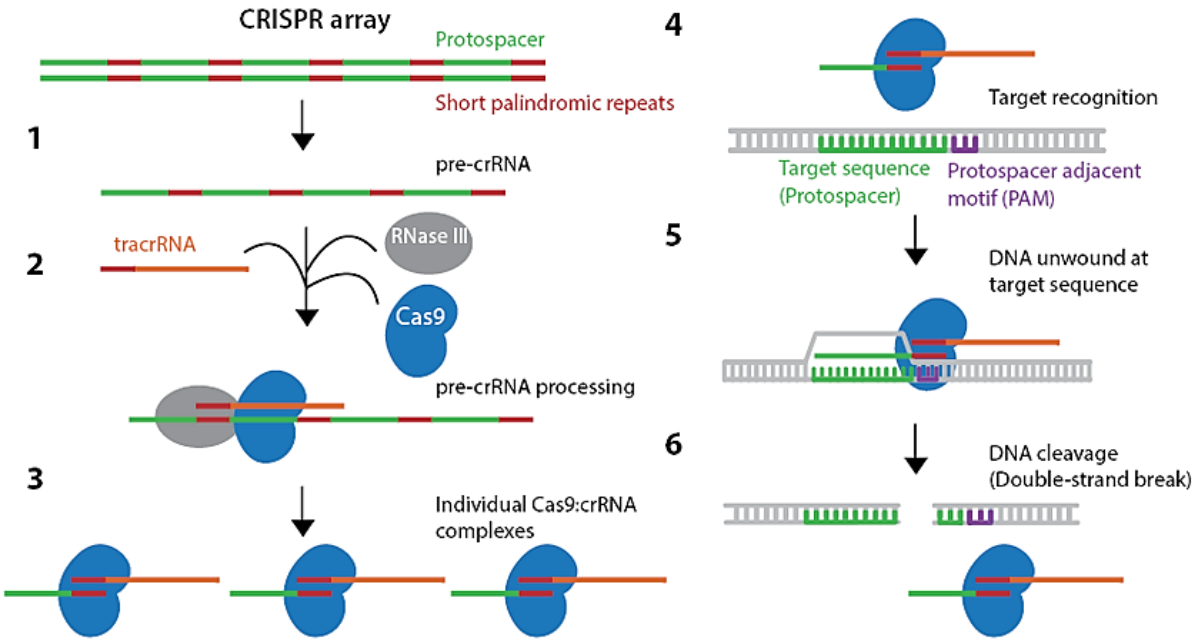

Figure 1: DNA cutting using CRISPR-Cas9 (Shen et al., 2017)

Chapter 2

CRISPR-Cas9 as a Genome-Editing Tool

With the process of genome editing, the genomic DNA can be modified and genetic information can be changed artificially. The discovery of double strand breaks (DSBs) in DNA was the basis of the development of this technology (Takata et al., 1998). The endogenous DNA repair machinery can be simulated by these DSBs. In the present time, three major types of gene editing tools are prominent in genetic engineering which are Zinc Finger Nuclease (ZFN), Transcription Activator-Like Effector Nuclease (TALEN) and CRISPR. ZFN was primarily applied in 1990s for gene editing that was site specific. DNA cleavage domain as well as DNA binding domain was primarily used by ZFNs (Wei et al., 2018). Primarily, an array of Cys_2His_2 ZFs is contained by the DNA binding domain. Here, a bacterial type IIS restriction endonuclease can be found that consist of N-terminal DNA-binding domain which is known as FokI. This FokI endonuclease can be guided in the DNA cleavage domain for achieving effective genome editing process (Salsman & Dellaire, 2016). Nonetheless, commercial synthesis of the editing system is needed in this process which makes it highly difficult to use. That is why, other systems eventually replaced ZFNs in gene editing.

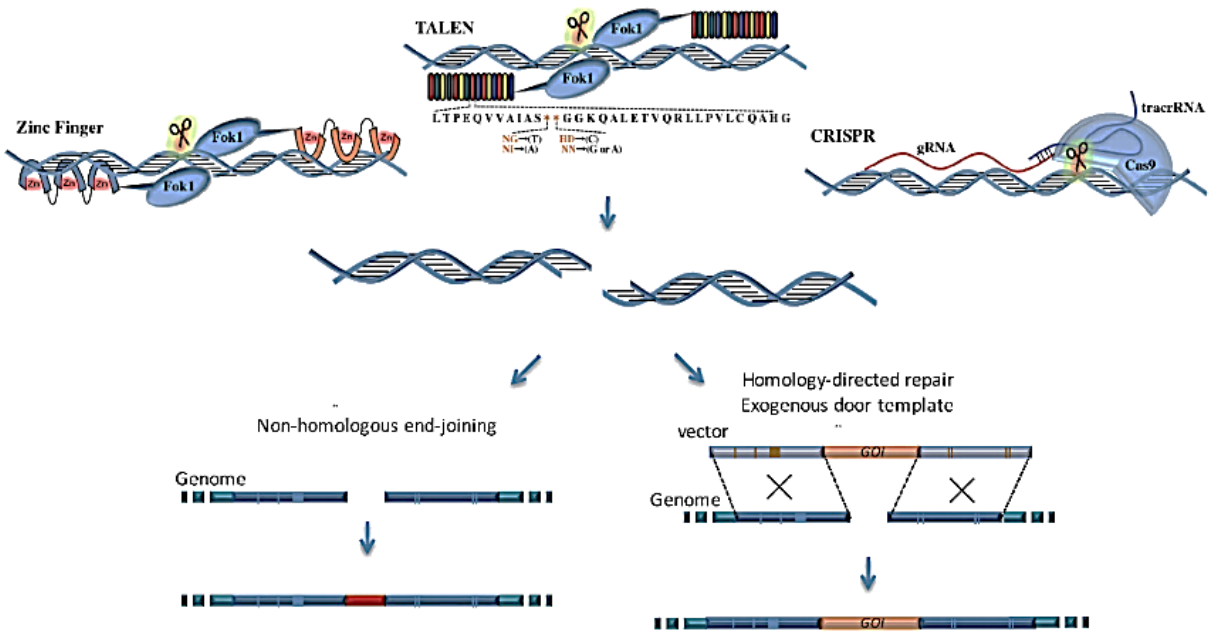

Figure 2: Comparison of working mechanism in ZFNs, TALENs & CRISPR-Cas9 (Gaj, Gersbach, & Barbas, 2013)

Another major genome editing tool is TALEN which was able to address some of the key challenges shown by ZFNs. Much like ZFNs, DNA-binding domain and DNA cleavage domain is possessed in TALENs. In order to cut the target DNA, the FokI endonuclease works as a dimer with its cleavage ability. This DNA-binding protein was discovered in *Xanthomonas* bacteria and a tandem array of 15 to 19 modules are included in the process. Specific sequences could be targeted by this enzyme with a high frequency by ranging the modules in various ways according to need. Nonetheless, the TALEN gene editing tool faced its most significant challenge to clone the large modules in a particular series. The capacity to join the modules in designated order by ligase in an efficient manner was affected. On top of that, low screening efficiency is another major technical barrier. When these factors are combined in the recent times, they can significantly limit its application in genome editing. Especially, with the development of CRISPR-Cas9, the use of these tools in gene editing was significantly affected.

Discovery of CRISPR repeats were made in *Escherichia coli* genome as a repeat locus that is unusual. When the investigators were able to identify similarities of phage sequences with the

spacer sequences, they primarily acknowledged its significance in the overall structure (Mahmoudian-sani et al., 2018). Normally, a series of messages and instructions are encoded by the genomes of various organisms within their DNA sequences. Changing those sequences, thereby changing the messages is the major focus of genome editing. In order to do so, a cut or break is inserted in DNA which tricks the natural DNA repair mechanisms of the cell to introduce the changes that are wanted. CRISPR-Cas9 has opened the doorway to genome editing in this way. Cas9 can be used to cut any directed region of the DNA. By simply changing the nucleotide sequence of the crRNA, this region can be cut since a complementary DNA target is bound with it.

This process was further simplified by fusing tracrRNA and crRNA for creating a 'guide RNA'. In a nutshell, only two components are required for genome editing: the Cas9 protein and a guide RNA (Chen, Sun, Miao, & Deng, 2016). In this process, a stretch of 20 nucleotide base pairs have to be designed to edit a particular gene. Simultaneously, an RNA molecule is constructed that functions as complementary to those 20 base pairs. There is also an added focus on the importance of making sure that the target gene is the only place where the nucleotide sequence can be found instead of any other place in the genome (Canver et al., 2015). In this way, the cut will take place by the Cas9 protein and the RNA. The cells natural repair mechanisms will kick in once cut takes place in the DNA. Likewise, it will work to introduce any kind of mutation or desired changes to the genome (Guernet & Grumolato, 2017).

The following comparison between ZFN, TALEN and CRISPR-Cas9 is crucial to understand their major differences:

Table 1: Comparison between ZFN, TALEN & CRISPR-Cas9

Gene Editing Process	First introduction	Recognition pattern of DNA	Validation Time	Relative Efficiency	Relative Specificity	Clinical Development
ZFN	2000	ZF protein	8 weeks	Medium	Low	Phase 1/2
TALEN	2011	TAL protein	8 weeks	Medium	Medium	Phase 1
CRISPR-Cas9	2013	Guide RNA, single strand	2-4 weeks	High	High	Preclinical

According to the difference between the structure and the sequence of Cas proteins, there are three major types of adaptive immunity in the systems of CRISPR-Cas9. Greater complexity is observed in the type I and III immunity mechanisms which is why they are overlooked by the scientists in the gene editing process in the recent years. Consequently, the simplest CRISPR/Cas system is type II where a single multi-functional Cas9 protein is required to interfere with invading genetic elements (Makarova et al., 2011). The type II CRISPR nuclease is primarily a simple tool for genome editing as it requires on three components (crRNA, tracrRNA & Cas9). Because of this trait, the scientists are able to adopt this tool as per their convenience. Using this type II system, small fragments of invading DNA from viruses or plasmids are cut and incorporated amidst a series of short repeats into a CRISPR locus. After this, the loci are transcribed. In order to generate small RNAs (crRNA), the transcripts are processed. In order to guide the effector endonuclease targeting invading DNA, these RNAs are used on the basis of complementary sequence (Figure 1).

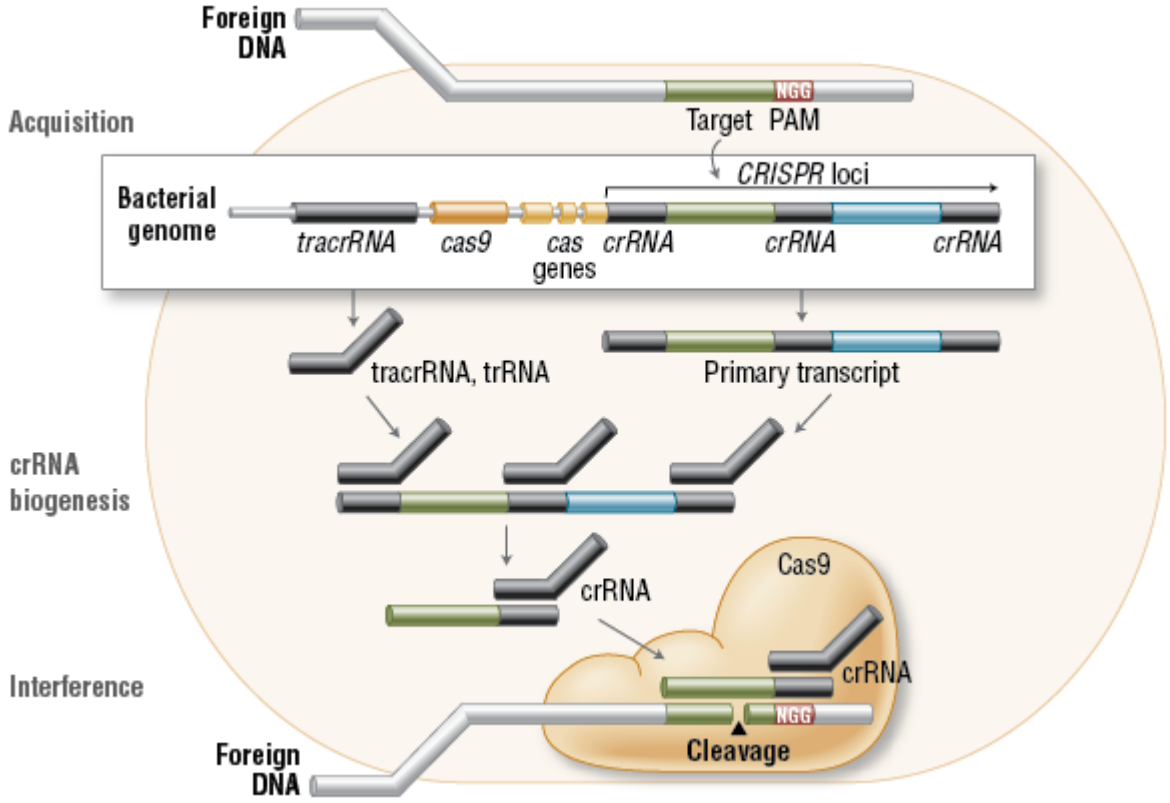

Figure 3: Genome editing in vivo with CRISPR-Cas9 (Pineda, Moghadam, Ebrahimkhani, & Kiani, 2017)

At the CRISPR loci, the bacterial genome incorporates the foreign DNA in the acquisition phase. During biogenesis of crRNA, the CRISPR loci is transcribed and processed. This genome editing tool is also able to achieve greater popularity it comprises a higher targeting efficiency. It is also one of the most important parameters of genome editing tools. CRISPR-Cas9 has a greater targeting efficiency (>70%) compared to ZFNs and TALENs (1%-50%) (Miller, et al., 2011). In zebra fish and plants, CRISPR-Cas9 has been able to demonstrate the greatest efficiency in targeting (Hwang, et al., 2013). This genome editing mechanism using CRISPR-Cas9 has been widely utilized since its establishment. In order to reach the current state, several modifications to the CRISPR-Cas9 genome editing technique took place. At present, genome editing using CRISPR-Cas9 mainly depends on the utilization of programmable features in the biding of the nuclease Cas9 to DNA target.

Chapter 3 Advancements in CRISPR-Cas9 Technology

CRISPR-Cas9 is routinely used for transcriptomic and genomic engineering for inducing epigenetic effects in non-human studies. The researchers are shifting their focus on studies with greater direct clinical translation although the current non-patient experiments generate major platform in the process of understanding of the major components of CRISPR-Cas9 (You et al., 2019). In order to fix the pathological mutations that cause genetic diseases, so far the researches that have been conducted on cells and small animals were able to provide evidence of therapeutic efficiency of employing CRISPR-Cas9 genome editing technology. The findings of the existing technology direct towards a crucial step toward clinical application. Nonetheless, there has to be significant consideration of the long term efficiency of the process and the safety of the patients in the longer run (Song, 2017). Symptoms of some hereditary diseases can be attenuated with the existing treatments using CRISPR-Cas9. However, having a mutation associated conditions can bring about psychological or social pressures for the patient. Scientists have been able to achieve major progress in rectifying pathogenic gene mutations in the embryos of humans, even though they must address the regulatory and ethical considerations associated with the process of treatment.

3.1 Application in Disease Discovery

With CRISPR technology, DNA of species can be cut in a directed and precise manner which makes it a powerful gene editing tool. The CRISPR screening process enables assessments of functions of genes as well as their modification in a single experiment. This state of the art tool significantly helps to pinpoint and corroborate precise targets for drugs (Qi et al., 2017). Furthermore, the underlying causes of disease can also be identified in this process. In the process of CRISPR screening, the CRISPR-Cas9 enzyme is used to guide the RNA to edit the genome at a precise location.

There are basically three mechanisms that are used in the process. Among those three, the most commonly used process is CRISPR KO (Knockout). Primarily, gene knockout (KO) is a genetic technique where one of the genes of the organism is made inoperative as in 'knocked out' of the organism. CRISPR KO completely inactivates a gene in the process of screening. In loss of function mutation studies, frequent utilization of CRISPR-Cas9 system is experienced. Discovery of novel tumor suppressors in somatic cell lines in humans has been possible in various outstanding studies that used CRISPR-Cas9 system. The genome-scale CRISPR-Cas9 knockout (GeCKO) system was established in 2014 by Zhang Lab (Chen, Sun, Miao, & Deng, 2016). This system is used for screening of the candidate gene that responds a therapeutic RAF inhibitor in a melanoma model indicated as vemurafenib. With this study, scientists can get library plasmids that are cheap and accessible. The other two mechanisms of CRISPR screening are CRISPRa (CRISPR activation) and CRISPRi (CRISPR interference). CRISPRa activates and CRISPRi inhibits the expression of a gene without altering the DNA.

CRISPR screening produces a great specificity to its targeted genome and it is able to generate a cleaner signal in the process which provides it with a major advantage over the older technologies such as RNA interference. Moreover, the effects of a complete gene knockout can be viewed in CRISPR screening compared to a partial reduction in other technologies (Evers et al., 2016). With this process, the expression of the gene can be edited at a cellular state. Hence, a more biological context can be derived from the process. Subsequently, CRISPR screening has helped the scientists to identify particular genes relating to a particular pathway very quickly. In this way, the compounds that will be successful in clinical trials for diseases can be identified more conveniently. In this way, they can also identify the ideal candidates for a particular drug or identify patients who to exclude from human trials. It can significantly reduce the risks of trial failure in the late state of medicine or treatment procedures.

3.2 Disease Modelling with CRISPR-Cas9

3.2.1 Cardiovascular Disease Modeling

The generation of transgenic cardiac specific Cas9 mouse is the recent advancement in the cardiovascular disease modeling process (Martinez-Lage, Torres-Ruiz, & Rodriguez-Perales, 2017). When a Cas9 plasmid is injected by Myosin Heavy Chain 6 (MYH6) promoter into the mouse zygotes, the Cas9 mice expresses high levels in the heart cardiomyocytes (Carroll, Makarewich, & McAnally, 2016). Also, a cardiac specific genome editing was demonstrated using Adeno-Associated Virus (AAVs) to deliver Single Guide RNA (sgRNAs) against MYH6 by the authors. Furthermore, a targeted loss of functions mutations was also introduced using adenovirus delivered Cas9. A high rate of mutagenesis was reported by the authors and also analyzed their pathophysiological effects (Ding , Strong, & Patel , 2014). They observed reduction in blood cholesterol level.

A few approaches that allowed the targeting of gene in animals existed before the latest gene editing techniques were developed. One of the examples was the generation of ‘knockout’ mice. Nonetheless, it was evident that these techniques consumed more time and they demonstrated various challenges and inefficiencies in the process(German, Mitalipov, Mishra, & Kaul, 2019). Gene targeting for treatment of cardiovascular diseases was revolutionized by CRISPR because it creates the opportunity to produce double-standard DNA breaks at user-directed and precise locations that is present within the genome. This process helps to edit a particular and selected segment of DNA efficiently.

3.2.2 Infectious Disease Modeling

Specifically targeting of HIV in latently infected T-cell line in the cellular reservoirs has been possible with CRISPR. Latently infected T-cell lines can also be targeted using CRISPR in the cellular reservoir of HIV. This process can provide a long term resistance to HIV-1. In order

to target the regulatory genes of HIV-1, an RNA-guided CRISPR-Cas9 was designed (Ophinni, Inoue, Kotaki, & Kameoka, 2018). These guided RNAs (gRNA) are chosen based on CRISPR specificity from each gene. The sequence conservation was also a factor in targeting HIV-1 genes across its six major subtypes. Furthermore, CRISPR-Cas9 genome engineering tool can also target and cleave conserved regions in the virus genome of chronic hepatitis B. It results in strong suppression of viral gene expression and replication. (Ramanan , Shlomai, & Cox, 2015).

3.2.3 Neurological Disease Modeling

The generation of neurological models can be described by several high quality studies. Study conducted by Liu et al. (2016) primarily incorporated the use of CRISPR-Cas9 mediated genome engineering in an Induced Pluripotent Stem Cell (iPSC) based model which helped significantly to explore the underlying mechanism of epilepsy aggravated by loss-of-function mutations. The basis for this model was a knock-in strategy that distinguished Gamma-Aminobutyric Acid (GABAergic) subtype neurons. It can be seen from this approach that Nav1.1 was expressed primarily on GABAergic neurons. On the other hand, for editing the genome of mouse muscle and muscle stem cells directly, Tabebordbar et al. (2016) developed an in-vivo approach. The deletion of exon in the mutated DMD (Duchenne muscular dystrophy) gene is induced by targeting both ends of EXON23. In this way, a truncated by functional protein can be produced in the process (Araki and Tetsuya, 2014). Using this process, muscle functional deficiencies can be partially resolved.

3.2.4 Immunodeficiency Disease Modeling

Researchers observed that, iPSCs that are deficient for Janus Kinase 3 (JAK3) perished when the disease is in initial stage in the prospect of curing human severe immunodeficiency. It happens because of the low B-cell lymphoma 2 (BCL2) expression pattern. Nevertheless, iPSC

can differentiate into fully functional T cells that includes their full receptor spectrum through the correction of JAK3 mutation with approach based on CRISPR (Baliou et al., 2018). Moreover, three promising therapies have been postulated so far as per the therapeutic strategies against Human Immunodeficiency Virus (HIV). The first one is remodeling of immune response by enhancing awareness of HIV-infected cells. In order to ensure that T cells are able to boost the response of other immunological populations, a moderate number of T cells are recruited in the process (Chang et al., 2015). The second approach is to increase the endurance and action of T cells and the third is triggering the development of memory cells by the immune system. At specific epitopes, HIV is able to escape the immune mechanism through mutagenesis or viral quasispecies (vQS). The use of multiplex CRISPR can generate various types of immunodeficiency strains in mouse when microinjection of embryo is conducted (Bengtsson et al., 2017).

3.2.5 Cancer Modeling

In order to generate effective in vivo and in vitro cancer models, there are numerous excellent studies carried out. In a study conducted by Maresch et al. (2016), complex chromosomal arrangements are modeled by the author which is a hallmark of pancreatic cancer. The results of this study indicated the generation of pancreatic cancer by setting multiple gene networks using transfection-based multiplex delivery of the components of CRISPR-Cas9 to the adult mice. Conversely, an approach for functional characterization of candidate genes in mouse model was generated in a separate study conducted by Sanchez-Rivera et al. (2014). In this research, the authors used Kras (G12D)-driven lung cancer model. In order to edit the genome of tumor suppressor genes in human lung cancer with known loss-of-function alterations, the authors used CRISPR-Cas9 which resulted in the generation of lung adenocarcinomas in mice.

3.2.6 Autism Modeling

With the recent advancements in the process of gene modification, the scientists have been able to create genetically engineered non-human models for primates. These models are able to approximate the neural as well as behavioral phenotypes of autism spectrum disorder (ASD). With the use of CRISPR-Cas9, the generation of germline-transmissible mutations of SH3 and Multiple Ankyrin Repeat Domains 3 (SHANK3) (Williams et al., 2016). The SHANK3 gene is primarily regarded as a major risk factor for the autism spectrum disorder. According to the results of experiment by Zhou et al. (2019), the altered global and local patterns of connectivity were significantly indicative of the abnormalities in circuit. Major traits of the mutations include motor deficits, sleep disturbances, learning impairments, repetitive behaviors etc. Zebrafish has been used to model ASD as it is able to demonstrate a greater level of efficiency while going through genetic manipulation (C. X. Liu et al., 2018). In this experiment, CRISPR-Cas9 was used in loss of function mutation along with a series of behavioral tests, morphological measurements and molecular analysis which systematically characterized the molecular and behavioral changes of the mutant Zebrafish. This versatile model of Zebrafish will play a significant role in conducting drug screening and neurodevelopment in future studies of human ASD as well as SHANK3 function (Baker et al., 2019).

3.3 Application in Disease Prevention & Treatment

From the past till the present time, the preclinical models only validated the practical prospects of CRISPR approaching disease prevention and treatment. However, scientists and researchers hope that this approach can be translated into clinical practice quite soon (Redman, King, Watson, & King, 2016). The potential of CRISPR/Cas9 to treat genetic disorders caused by single gene mutations is one of its most exciting applications in disease prevention and treatment. The fact that CRISPR-Cas9 provides a much faster, easier and cheaper gene editing technology has enabled it to gain massive popularity in genetic research. The use of CRISPR-

Cas9 in disease prevention and therapy is just at the beginning stage (German et al., 2019). Some diseases are already being tackled with the revolutionary technology of CRISPR-Cas9 at present which are discussed as follows:

3.3.1 Cancer

One of the first and most advanced clinical trials of CRISPR is running in China at present. The potential of using this gene editing tool for treatment of patients of advanced esophagus cancer is being discovered (Fernandez, 2019). This research is ongoing in Hangzhou Cancer Hospital and it is directed towards extracting immune T cells from patients. These cells are modified so that the gene encoding Programmed Cell Death Protein-1 (PD-1) can be removed. This protein is able to bind with some tumors on the immune cell surface and instruct them not to attack those cells. The capacity of these immune cells can be modified to increase so that cancer cells can be attacked more efficiently. This treatment has been proven effective for at least 86 people in China (Rana, Marcus, & Fan, 2018). In April 2019, CRISPR technology was used by scientists at University of Pennsylvania to remove PD-1 from cells. They also attempted to change an immune cell's surface molecule for finding and attacking tumors (Stein, 2019).

3.3.2 AIDS

In order to combat Acquired Immunodeficiency Syndrome (AIDS), CRISPR technology can be used in various ways. One of the primary uses of CRISPR involve cut the DNA of HIV virus in the immune cell DNA out of the place of its hiding. Using this approach, the virus can be attacked in its inactive and hidden condition (Ophinni, Inoue, Kotaki, & Kameoka, 2018). Other treatments are unable to achieve this which makes it impossible to get rid of the HIV in a complete manner. Conversely, the resistance to HIV infections can be increased by another approach using CRISPR. Due to the mutation in a gene named C-C Chemokine Receptor Type 5 (CCR5), some people have a natural resistance to HIV. In this process, the structure of the

protein is changed by the mutation which takes away the capacity of HIV to bind to it (Azvolinsky, 2017). However, this approach proved to be much controversial in China as scientists in there used CRISPR-Cas9 to edit embryos of humans for increasing their resistance from being infected by HIV. Outrage was sparked among scientific community as some studies argued that the experimented babies would face a higher risk of premature death (Wei & Nielsen, 2019).The scientific community then came to a consensus that more research is needed on this approach before implementing it on humans.

3.3.3 Muscular Dystrophy

*A*nother disease which has been investigated by CRISPR/Cas9 is Duchenne's muscular dystrophy (DMD) where AAV were used in the delivery of CRISPR/Cas9 endonucleases. Recovery of dystrophin expression in mouse model of DMD was carried out by deletion of exon where the original mutation was contained (Pandupuspitasari et al., 2016). In this way, a truncated, but still functional protein is produced. The muscle functional deficiencies were partially recovered in the treated mice and the mature muscle tissue was replenished by dystrophin gene that was edited in muscle stem cells (Doetschman & Georgieva, 2017). Primarily, DMD gene is responsible for Duchenne's muscular dystrophy. This gene encodes a protein that is important for muscle contraction. Progressive muscle degeneration is faced by the children who are born with this disease. Apart from palliative care, there is currently no options of treatment available (Nelson et al., 2017). According to recent research, the genetic mutations causing this disease can be fixed using CRISPR technology which has demonstrated positive results on mice. With the use of CRISPR, a group of researchers in USA revealed an innovative method. They used CRISPR to cut 12 strategic mutation hotspots in approximately 3,000 different mutations causing the disease is muscles (Long et al., 2018). In the ongoing research, scientists are focusing on removing the whole sections of mutated protein instead of fixing mutations using CRISPR (Dever et al., 2016).

3.3.4 Blood Disorders

Treatment of haemoglobinopathies has also been accelerated by using CRISPR/Cas9. Fetal haemoglobin can be induced by CRISPR/Cas9 through BCL11A enhancer disruption in both mice and primary erythroblast cells of humans. Fetal haemoglobin can be expressed in patients with abnormal adult haemoglobin with this approach in the future. The first patient in the first CRISPR trial was enrolled in Europe and US in February 2018 (Offord, 2018). This treatment approach is directed towards sickle cell disease and beta-thalassemia. The oxygen transport system in the blood is primarily affected by these two disorders. This therapy involves harvesting bone marrow stem cells from patients. After that, CRISPR technology is used to produce fetal hemoglobin from those cells. The investigation of treatment of CF using CRISPR/Cas9 was conducted by using adult intestinal stem cells (Fan, 2019). These cells were obtained from two patients who were diagnosed with CF in their intestinal organoids. This study revealed that the function of CF transmembrane conductor receptor was restored once the mutation had been corrected. On top of this, a novel therapeutic strategy can also be derived through this process for the patients possessing diseases such as thalassemia or sickle cell disease (Weintraub, 2019).

3.3.5 Cystic Fibrosis

Primarily, cystic fibrosis is a genetic disease that is able to cause severe respiratory problems. The life expectancy of the persons is as low as 40 years, although there are some treatments available for dealing with the symptoms. With the use of CRISPR technology, the mutation that is responsible for the origin of the cystic fibrosis can be detected and edited (Schneider-Futschik, 2019). The mutation is primarily located in a gene called CFTR. It is proven by the researchers that CRISPR can be used in the human lung cells that are responsible for the mutation. After identifying the lung cells that contain the mutation behind the disease, CRISPR technology can be used to fix the mutation. Nonetheless, multiple mutations are responsible

for the genesis of cystic fibrosis which is why development of different CRISPR therapies is essential for fixing different genetic defects. This process is yet to be tested on humans (Johnson, 2019).

3.3.6 Huntington's disease

Huntington's disease has a strong genetic component as part of its being a neurodegenerative condition. The abnormal repetition of particular DNA sequence can cause this disease. This disease can be manifested as early as the number of copies increase (Tzelepis et al., 2016). Scientists have faced challenges to ensure treatment of Huntington's disease with CRISPR because the brain can face detrimental consequences if there is any off-target effect (Dunbar, et al., 2019). That is why, the scientists are looking for ways to bring about changes in the gene editing to so that it becomes safer for use. Hence, a new version of CRISPR-Cas9 has been developed by US researchers which is called KamiCas9 involving a 'self-destruct' sequence (Eisenstein, 2018). Also, for making the gene editing process more precise, a team of Polish researchers tried to make the gene editing process more precise by pairing an enzyme called Nickase with CRISPR-Cas9.

Chapter 4

Future Prospects of CRISPR-Cas9 on Disease Prevention and Therapy for Humans

Future research directives of CRISPR-Cas9 will emphasize on improving the accuracy of the predictive models which will incorporate the additional features of the tool. The sequence of the target site is the sole basis of the current methods of predicting the specificity and efficiency of the diseases. Nonetheless, it is accepted that CRISPR-Cas9 activity can be influenced by chromatin environment (Chari et al., 2015). Later studies also indicated support for the fact that high activity target sites were enriched for histone modification. That is because these modifications are associated with open-chromatin environment (Horlbeck et al., 2016). In the year 2016, a direct link was also drawn between CRISPR-Cas9 and chromatin. The presence of nucleosomes physically blocked access of CRISPR-Cas9 at reduced the overall activity at the target site (Isaac et al., 2016).

The fact that different activities can be displaced across cell lines by the same CRISPR-Cas9 target site can be explained by the difference in chromatin environment. The off-target predictions would likely to be improved by the incorporation of chromatin environments (Rossidis et al., 2018). They are considered to be more susceptible to chromatin accessibility. The inclusion of variant information should also be a major focus of future off-target pipelines beside chromatin information. Furthermore, recent studies have also discovered that the off-target landscape is significantly affected by the variance between individuals (Lessard et al., 2017). Not taking the unique genome of humans into account can bring about detrimental side effects in the treatment procedure. In almost all fields, this information is crucial for the application of CRISPR technology (Scott and Zhang, 2017).

The future models of CRISPR-Cas9 editing will not only be able to predict its success, but it will also be able to anticipate its outcome. Moreover, specific DNA segments can be deleted by the researchers by selecting and exploiting the sites and repair pathways of microhomology. It will contribute significantly to control the outcome of the CRISPR-Cas9 editing (Yao et al., 2017). Another recent study discovered that the repair of CRISPR-Cas9 induces non-random mutations which are determined by the target sequence (van Overbeek et al., 2016). According to this finding, it can be quite presumably said that mutational outcome of CRISPR-Cas9 editing can be predicted which can support the researchers to conduct gene editing more precisely without concerning about knock-ins (Gaj et al., 2017). Some models in future can also contribute to predicting the success factors of other CRISPR-Cas9 applications (i.e. knock-ins). They can involve the repair of double strand break that incorporates the use of supplied template. Conversely, it may also be of significant use for base-editing where one base is converted into another by Cas9 fusion protein without need for a cleavage (Gaudelli et al., 2017).

Chapter 5

Ethical Concerns of Using CRISPR-Cas9 on Humans for Disease Prevention and Treatment

In genome editing process, CRISPR-Cas9 is the most versatile gene editing tool which can be used in disease prevention and treatment. The scientific community is both excited and alarmed by its capacity to edit various gene types with great precision and ease. Although it has gained significant public support in its therapeutic applications, major concerns about its ethical and safety issues is present (Ma, Zhang, & Huang, 2014). The Napa Valley meeting held in 2015 initiated discussions where developers, scientists, ethicists and researchers associated with CRISPR-Cas9 sat together to analyze the legal, biomedical and ethical issues of this tool (Baltimore, et al., 2015). The participants of the meeting looked to deliberate when, where and how the technology may be used. In this regard, the Chinese Academy of Sciences and UK's Royal Society were invited by US National Academies of Sciences, Engineering and Medicine (NASEM) to participate in the International Summit on Human Gene Editing (Brokowski & Adli, 2019). The following factors were emphasized which are still worth considering while conducting research and experiments with CRISPR-Cas9 on humans:

5.1 Long Term Effects

Since CRISPR-Cas9 is able to alter gene of humans and those humans have to live with the effects for the rest of their life along with their offspring, it raises significant moral and ethical questions. Concerns regarding power and technical limitations of CRISPR technology has been raised due to its limited editing efficiency on-target as well as incomplete editing (Baylis & McLeod, 2017). CRISPR experiments that involve human cell lines and animals demonstrate these issues more vividly. However, CRISPR technology has been evolving at a rapid rate which may enable it to address these challenges more effectively and they may become

obsolete in the near future (Redman et al., 2016). Subsequently, limitation in the existing scientific research is evident on the issue whether the modified organisms will be affected in the long term or whether these adverse effects will be carried by future generations and face unexpected consequences (Hirsch, Iphofen, & Koporc, 2019). Without concrete evidence addressing the complexities and technical limitations of the process, it may be very difficult, if not impossible, to assess the risks and benefits of CRISPR technology in the long term.

5.2 Complexities in Gene Editing Factors

Furthermore, the scientific community as well as the general populace is skeptical regarding the fact that the complex relationship present between biological phenotypes and genetic information is uncertain even when the gene editing is completed as expected and desired output is achieved at the time (Pineda et al., 2017). Hence, it is very difficult to predict the biological consequences of editing a gene as various complex regulatory actions of genes primarily determine several biological traits. Therefore, editing a single gene can trigger unforeseen impact on various biological outcomes since various genes contribute in shaping a complex biological trait on an organism (Tian et al., 2019). So, it is highly difficult to understand the potential risks and benefits considering the uncertainty on what kind of biological outcomes can be influenced by gene expression and modification.

5.3 Most Recent Example of Gene Editing by He Jiankui:

In the late November 2018, He Jiankui, an associated professor at the Southern University of Science and Technology in Shenzhen, China, presented his findings in International Summit on Human Genome Editing on two baby girls born from CRISPR-Cas9 edited embryos. He used *in vitro* fertilization (IVF) pregnancy process to achieve the objective of his experiment (Begley, 2019). Several moral and ethical issues were raised with this experiment regarding its methods, procedures, timing and announcement in which he violated various several ethical

norms. His attempt desecrated national regulations, international consensus guidelines and a number of well-established bioethics. The scientific community was also keen on pointing out the fact that he is not a medical doctor, but rather he completed his doctorate in biophysics and post doctorate in gene sequencing (Krimsky, 2019). Hence, he is not properly equipped to conduct such experiments as he does not possess training in bioethics.

Furthermore, a statement released by International Summit on Human Gene Editing in 2015 stated that without addressing the relevant safety and efficiency issues addressing the associated risks and potential benefits as well as greater consensus in scientific community on its appropriateness, it will be irresponsible to proceed with any kind of clinical use of germline editing (Cyranoski, 2019). These conditions have not been met with this experiment. On top of it, any kind of prior studies on CRISPR editing on mice, primates or human embryos have not been reported by him. Hence, a serious question arises regarding his capacity to properly use this tool addressing the underlying risks and unforeseen outcomes. It was also claimed that he did not comply with China's national ethical guidelines for embryo research (Dickenson & Darnovsky, 2019). That is why; China's Clinical Trial Registry removed his clinical trial information for not issuing sufficient data on validity of his work as well as safety and precautionary measures taken in the process. Consequently, the Southern University of Science and Technology withdrew any association with He's clinical trials as well as any ethical approval for his experiments of embryo editing because he was unable to meet the guidelines of the university's ethical framework (Lei, Zhai, Zhu, & Qiu, 2019). Considering all of these facts, this instance serves among the most egregious acts of violation of ethical guidelines and as a lesson for future human experiment.

Chapter 6

Discussion

6.1 CRISPR-Cas9 as a Genome Editing Tool

Genome editing technologies provide scientists with the capacity to change the DNA of the organism to add or remove any trait which provides them greater immunity from various diseases and ailments. CRISPR-Cas9 is a comparatively new gene editing tool and its effectiveness can be contemplated when it is compared with other predominant gene editing tools such as RNAi, ZFNs, TALENs etc. The discussion is provided as follows:

The effectiveness and versatile characteristics of CRISPR-Cas9 can be contemplated when it is compared with the other prevailing gene editing mechanisms. Compared with the other predominant gene editing technologies such as RNAi, ZFNs, TALENs etc., CRISPR-Cas9 has proven its effectiveness in several experiments in the recent years. As ZFNs contain a Cys_2-Hys_2 DNA-binding domain as well as DNA cleavage domain, it incorporates a complex procedure and its effectiveness is compromised as a result. Subsequently, another gene editing tool such as TALEN is primarily derived from a natural protein from *Xanthomonas* which is a plant pathogenic bacterium. It is able to be programmed for targeting specific DNA sequence by repeating motifs of amino acid recognition. Compared to ZFNs and TALENs, CRISPR-Cas9 is dependent on sequence-specific cleavage for RNA and only programmable RNA is required for generation of this sequence. This makes it capable of convenient development and application.

Moreover, a series of conserved repeated sequences that are interspaced by non-repetitive sequences primarily comprise the CRISPR locus. The Cas nuclease processes the invading foreign DNA in the ideal CRISPR-Cas9 system. These DNA fragments are incorporated into the host genome's CRISPR loci which are called spacers. In order to produce CRISPR RNA

(crRNA), the spacers are primarily used as transcriptional templates. So far, the scientists have discovered 40 different types of Cas protein families play a significant role in spacer incorporation, cleavage of invading DNA as well as biogenesis. The advantage of CRISPR-Cas9 is evident in the experiments of gene modification in comparison with ZFNs and TALENs. The protein guided DNA cleavage that is used in ZFNs and TALENs make the process more time-consuming and complex. On the other hand, only a short programmable gRNA is needed in CRISPR-Cas9 genome modification which is convenient, cheap and easy to design. Furthermore, at multiple independent sites, CRISPR-Cas9 is able to induce genomic modifications concurrently. Hence, it is possible to disrupt multiple genes or accelerate generation of multiple gene mutations in transgenic animals in order to investigate gene function.

From the existing literature and research findings, it can be safely said that CRISPR-Cas9 is the most precise, cost-effective and convenient gene editing tool compared to the others. It is also becoming very popular among the scientists in the biotechnology field for disease prevention and treatment. Commencing from plant, agriculture and animal gene modification, CRISPR-Cas9 technology is now getting ready for human trials. Although clinical applications of CRISPR-Cas9 looks promising, scientists and physicians should do their best to fully grasp its effects and potential risks.

6.2 Recent Developments in Disease Prevention & Treatment

The use of CRISPR-Cas9 is crucial in genetic and biotechnology research as it is a convenient and efficient tool. Significant developments have taken place so far since its discovery and it will continue to develop in the upcoming days. In this section, the recent developments of CRISPR-Cas9 gene editing in disease prevention and treatment will be discussed.

Recently, scientists are focusing on using CRISPR-Cas9 for clinical application on humans as it has great potential to treat or prevent certain diseases at a genetic level. Nonetheless, the accuracy and effectiveness of treatments provided with CRISPR-Cas9 is still a major issue for the scientific community. Through CRISPR screening process, certain diseases can be easily identified which leads to an effective clinical trial reducing the potential risks. Disease modeling using CRISPR-Cas9 is one of the major steps towards addressing the key factors that aggravate the disease. The use of CRISPR-Cas9 demonstrated effective and efficient results compared to AAVs and sgRNAs against MYH6 in mice. In the case of HIV, the use of RNA-guided CRISPR-Cas9 is used in targeting of HIV-1 genes which demonstrated positive results. Moreover, CRISPR-Cas9 incorporated use of iPSC in order to detect the underlying mechanism of epilepsy as a neurological disease. Consequently, in cancer modeling process, the scientists engaged in the experiment used CRISPR-Cas9 to create a transfection-based multiplex delivery system. Hence, it can be opined that the use of CRISPR-Cas9 in disease modeling is evolving at a decent pace and more developments can be experienced in the future.

Application of CRISPR-Cas9 in cancer treatment has taken major steps in China where the scientists are trying to extract the immune T-cells and removing PD-1 which is responsible for aggravating esophagus cancer. On the other hand, CRISPR-Cas9 has been used to cut DNA of HIV out of its hiding place which will make it easier to attack the viruses in their hiding conditions. Clinical trials are still under process in China and the ethical and compliance issues are being emphasized as they proceeded to edit human embryos with the use of CRISPR-Cas9 to provide immunity from HIV. In the treatment process of Duchene's muscular dystrophy, CRISPR-Cas9 was used in editing dystrophin gene in muscle stem cells which was proved to be crucial in the replenishment of mature muscle tissues and treatment of mice containing the disease. Scientists are trying for transition in clinical trials on humans learning from positive results in mice. In the case of sickle cell disease and beta-thalassemia, CRISPR-Cas9 is used

to harvest and edit bone marrow stem cells of patients and produce fetal hemoglobin. CRISPR-Cas9 is also used to identify and fix mutated cells containing cystic fibrosis and Huntington's disease.

To sum up, it is evident that CRISPR-Cas9 has paved the way for significant advancements in disease treatment and prevention. Nonetheless, the experiments only demonstrated positive results in mice trials. In order to ensure effective transition to human clinical trials, more result-based evidence is needed. Scientists are contemplating ethical and moral ramifications of human clinical trials or gene editing of human embryos in the process. All in all, CRISPR-Cas9 demonstrates massive potential as per the recent advancements.

6.3 Future Possibilities in Disease Prevention & Treatment

CRISPR-Cas9 is being improved for disease prevention and treatment in a decent pace where new discoveries are made frequently. Scientists have found positive results in trials on mice. However, human application is still a major way forward as most parts of CRISPR-Cas9 gene editing consequences are unknown. In this section, the future possibilities of this great gene editing tool are discussed.

The major focus of future research on the application of CRISPR-Cas9 in clinical trials is to improve its accuracy of predictive models and identification of the long term effects of the intervention. Predicting the specificity and efficiency of CRISPR-Cas9 in the sequence of the target site is the primary basis of future research. Some researchers believe that the off-target predictions of CRISPR-Cas9 can be improved by incorporating chromatin environment in the process. Nonetheless, there is also a major acknowledgement to the fact that the individuals have different genetic configurations which can affect the off-target landscape of CRISPR-Cas9 gene editing process. In order to avoid the detrimental side effects of CRISPR-Cas9 gene

editing for disease prevention and treatment in the future, the unique genome of humans should be appropriately addressed by the scientists. The prediction of mutational outcome of CRISPR-Cas9 is induced by non-random mutations determined by target sequence. In the future research and application, this process can improve the precision and accuracy of gene editing with CRISPR-Cas9 without major concerns of knock-ins.

In a nutshell, the future of CRISPR-Cas9 is primarily determined by the emphasis of the researchers on improving the accuracy and precision of the results. However, the scientists face a major challenge in future to address the ethical and moral considerations of using CRISPR-Cas9 in gene editing on humans.

6.4 Ethical Concerns of CRISPR-Cas9 Application on Humans

In the process of using CRISPR-Cas9 as a prominent genome editing tool in disease prevention and treatment, the ethical and moral obligations have always been major factors to emphasize for the scientists. Due to the lack of sufficient data on long term effects of gene editing, these ethical considerations play a significant role to regulate the scope of research.

The major ethical and moral considerations of CRISPR-Cas9 have emerged from the fact that the patients that are subjected to its therapeutically applications must live with its effects for the rest of their lives. Although CRISPR-Cas9 has been able to demonstrate pivotal breakthrough in agricultural and animal gene editing procedures, human application is still in the inception phase. Organizations such as National Academies of Sciences, Engineering and Medicine (NASEM), Chinese Academy of Sciences and UK's Royal Society are still moving discussions regarding the compliance and guidelines of gene editing with CRISPR-Cas9. The repercussions of failed experiments or unforeseen results can be detrimental for the subject as well as the society on a broader scale. More research is needed in the genome editing field

before it can be applied in the regular clinical applications for disease prevention and treatment. To conclude, it is evident that CRISPR-Cas9 needs more research and precise mechanisms of addressing the ethical and moral obligations before being mainstreamed in clinical applications for disease prevention and treatment.

Chapter 7

Conclusion

As a genome editing technology, CRISPR-Cas9 has proven to be of great potential for the scientific community. This gene editing tool will soon replace RNA inhibitory approaches (i.e. RNAi, shRNA etc.) because of its specificity and low side effects. CRISPR-Cas9 is applicable to most cell types in a wide variety of plant and animal species. Moreover, compared to the FokI-based gene editing processes such as ZFNs and TALENs, CRISPR-Cas9 also demonstrates some major advantages. CRISPR-Cas9 is able to construct the necessary vectors for convenient use of each system. It only requires cloning of 19 nucleotides into a plasmid that encodes gRNA & Cas9. On the other hand, ZFNs and TALENs require cloning of larger sequences which can be coded into multiple zinc-finger motifs. CRISPR-Cas9 also demonstrates the highest efficiency in genome editing compared to both ZFNs and TALENs. This makes it the most versatile gene editing tool available in the field. The fact that it has the potential to get rid of life threatening diseases at the molecular level makes it so appealing to use in the field of medicine and biomedical engineering. However, there are lots of factors that are unknown in this area and new discoveries are still being made which are enthralling. Scientists are working relentlessly to improve its accuracy and specificity in future research and experiments. In the upcoming years, the long term effects of gene editing can be predicted accurately and the off target predictions and efficiency of research may also be improved. Along with the clinical appeal for prevention and treatment of disease at a molecular level, the scientists have to consider the repercussions of this tool. Safety, specificity, efficiency and reduction of potential risks should be the major concern for scientists or anyone who is willing to explore this field.

References

Aquino-Jarquin, G. (2017). Emerging role of CRISPR/Cas9 technology for MicroRNAs editing in cancer research. *Cancer Research*, 77(24), 6812–6817.

Araki, M., and Tetsuya I., (2014) International Regulatory Landscape and Integra- tion of Corrective Genome Editing Into in Vitro Fertilization. *Reproductive Biology and Endocrinology* 12: 108.

Azvolinsky, A. (2017, 8 3). *Resistance to HIV Engineered Via CRISPR*. Retrieved 8 22, 2019, from The Scientist: https://www.the-scientist.com/daily-news/resistance-to-hiv-engineered-via-crispr-31137.

Baliou, S., Adamaki, M., Kyriakopoulos, A. M., Spandidos, D. A., Panayiotidis, M., Christodoulou, I., & Zoumpourlis, V. (2018). CRISPR therapeutic tools for complex genetic disorders and cancer (Review). *International Journal of Oncology*, 53(2), 443–468.

Baltimore, D., Berg, P., Botchan, M., Carroll, D., Charo, R. A., Church, G., Yamamoto, K. R. (2015). Biotechnology. A prudent path forward for genomic engineering and germline gene modification. *Science,* 36-38. doi:10.1126/science.aab1028.

Baylis, F., & McLeod, M. (2017). First-in-human Phase 1 CRISPR Gene Editing Cancer Trials: Are We Ready? *Current Gene Therapy*, 17(4), 309–319. https://doi.org/10.2174/1566523217666171121165935

Begley, S. (2019). *He Jiankui tried to protect 'CRISPR babies' against HIV. But his attempted fix shortens lives, study shows*. Statnews: https://www.statnews.com/2019/06/03/crispr-babies-scientist-pushed-too-quickly/

Bengtsson, N.E., Hall, J.K., Odom, G.L., Phelps, M.P., Andrus, C.R., Hawkins, R.D., Hauschka, S.D., Chamberlain, J.R., and Chamberlain, J.S. (2017). Muscle-specific

CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. Nat. Commun. 8, 14454.

Bhandari, I., Colet, E., Parker, J., Pines, Z., Pratap, R., & Ramanujam, K. (1997, March). Advanced Scout: Data Mining and Knowledge Discovery in NBA Data. *Data Mining and Knowledge Discovery*, 121-125.

Brian, S. (2012, January 25). The Problem of Shot Selection in Basketball. *PLoS One*. Retrieved from https://doi.org/10.1371/journal.pone.0030776

Brokowski, C., & Adli, M. (2019). CRISPR Ethics : Moral Considerations for Applications of a Powerful Tool. *Journal of Molecular Biology*, 431(1), 88–101. https://doi.org/10.1016/j.jmb.2018.05.044

Canver, M. C., Smith, E. C., Sher, F., Pinello, L., Sanjana, N. E., Shalem, OBauer, D. E. (2015). BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature*, 527(7577), 192–197. https://doi.org/10.1038/nature15521

Carroll, K., Makarewich, C., & McAnally, J. (2016). A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc Natl Acad Sci* (USA), 338-43.

Chang, C. W., Lai, Y. S., Westin, E., Khodadadi-Jamayran, A., Pawlik, K. M., Lamb, L. S., Townes, T. M. (2015). Modeling Human Severe Combined Immunodeficiency and Correction by CRISPR/Cas9-Enhanced Gene Targeting. *Cell Reports*, 12(10), 1668–1677. https://doi.org/10.1016/j.celrep.2015.08.013

Chen, S., Sun, H., Miao, K., & Deng, C. X. (2016). CRISPR-Cas9: From genome editing to cancer research. *International Journal of Biological Sciences*, 12(12), 1427–1436. https://doi.org/10.7150/ijbs.17421

Choudhury, R. D., & Bhargava, P. (2007). Use of Artificial Neural Networks for Predicting the Outcome. *International Journal of Sports Science and Engineering*, 1(2), pp. 87-96.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science (New York, N.Y.)*, 339(6121), 819–823. https://doi.org/10.1126/science.1231143.Multiplex

Cyranoski, D. (2019). The CRISPR-baby scandal: what's next for human gene-editing. *Nature*, 440-442. doi:10.1038/d41586-019-00673-1

Dever, D.P., Bak, R.O., Reinisch, A., Camarena, J., Washington, G., Nicolas, C.E., Pavel-Dinu, M., Saxena, N., Wilkens, A.B., Mantri, S., et al. (2016). CRISPR/Cas9 b-globin gene targeting in human haematopoietic stem cells. Nature 539, 384–389.

Dickenson, D., & Darnovsky, M. (2019). Did a permissive scientific culture encourage the 'CRISPR babies' experiment? *Nature Biotechnology*, 355-357.

Ding , Q., Strong, A., & Patel , K. (2014). Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ Res*, 488-92.

Doetschman, T., & Georgieva, T. (2017). Gene Editing with CRISPR/Cas9 RNA-Directed Nuclease. *Circulation Research*, 120(5), 876–894. https://doi.org/10.1161/CIRCRESAHA.116.309727

Driehuis, E., & Clevers, H. (2017). CRISPR/Cas 9 genome editing and its applications in organoids. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 312(3), G257–G265. https://doi.org/10.1152/ajpgi.00410.2016

Dunbar, G. L., Koneru, S., Kolli, N., Sandstrom, M., Maiti, P., & Rossignol, J. (2019). Silencing of the Mutant Huntingtin Gene through CRISPR-Cas9 Improves the Mitochondrial

Biomarkers in an In Vitro Model of Huntington's Disease. *SAGE Journals*, 460-463. doi:https://doi.org/10.1177/0963689719840662

Eisenstein, M. (2018). CRISPR takes on Huntington's disease. *Nature,* 42-43. doi:doi: 10.1038/d41586-018-05177-y

Evers, B., Jastrzebski, K., Heijmans, J. P. M., Grernrum, W., Beijersbergen, R. L., & Bernards, R. (2016). CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nature Biotechnology*, 34(6), 631–633. https://doi.org/10.1038/nbt.3536

Fan, S. (2019). CRISPR Used in Human Trials for the First Time in the US. *Singularity Hub*. 25(2), 1–8

Fernandez, C. R. (2019). *'CRISPR Babies' Might Be at Risk of Dying Younger*. Retrieved from Labiotech: https://labiotech.eu/medical/crispr-babies-death-risk/

Fernandez, C. R. (2019, 07 23). *7 Diseases CRISPR Technology Could Cure*. Retrieved 08 23, 2019, from Labiotech: https://labiotech.eu/tops/crispr-technology-cure-disease/

Gaj, T., Ojala, D.S., Ekman, F.K., Byrne, L.C., Limsirichai, P., and Schaffer, D.V. (2017). In vivo genome editing improves motor function and extends survival in a mouse model of ALS. *Sci. Adv.* 3, eaar3952.

German, D. M., Mitalipov, S., Mishra, A., & Kaul, S. (2019). Therapeutic Genome Editing in Cardiovascular Diseases. JACC: *Basic to Translational Science*, 4(1), 122–131. https://doi.org/10.1016/j.jacbts.2018.11.004

Guernet, A., & Grumolato, L. (2017). CRISPR/Cas9 editing of the genome for cancer modeling. *Methods*, 121–122(March), 130–137. https://doi.org/10.1016/j.ymeth.2017.03.007

Heidi Ledford. (2016). CRISPR: gene editing is just the beginning. *Nature*, 531.

Hirsch, F., Iphofen, R., & Koporc, Z. (2019). Research integrity corner Ethics assessment in research proposals adopting CRISPR technology. *Nature.* 29(2), 1–8.

Hryhorowicz, M., Lipiński, D., Zeyland, J., & Słomski, R. (2017). CRISPR/Cas9 Immune System as a Tool for Genome Engineering. *Archivum Immunologiae et Therapiae Experimentalis,* 65(3), 233–240. https://doi.org/10.1007/s00005-016-0427-5

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Kaini, P., Sander, J. D., Yeh, J.-R. J. (2013). Heritable and Precise Zebrafish Genome Editing Using a CRISPR-Cas System. *PLOS.* 275(23), 5186–5193

Johnson, C. (2019). A New Approach to Cystic Fibrosis Treatment: Personalized Medicine through CRISPR/Cas9 and Organoid Models. *Stamford University Undergraduate Research Journal*, 23-31.

Krimsky, S. (2019). Ten ways in which He Jiankui violated ethics. *Nature Biotechnology*, 19-20. doi:10.1038/nbt.4337

Lei, R., Zhai, X., Zhu, W., & Qiu, R. (2019). Reboot ethics governance in China. *Nature*, 184-186. doi:10.1038/d41586-019-01408-y

Liu, J., Gao, C., Chen, W., Ma, W., Li, X., Shi, Y., Li, Z. (2016). CRISPR/Cas9 facilitates investigation of neural circuit disease using human iPSCs: Mechanism of epilepsy caused by an SCN1A loss-of-function mutation. *Translational Psychiatry*, 6 (November 2015). https://doi.org/10.1038/tp.2015.203

Long, C., Li, H., Tiburcy, M., Rodriguez-caycedo, C., Kyrychenko, V., Zhou, H., Olson, E. N. (2018). Correction of diverse muscular dystrophy mutations in human engineered heart muscle by single-site genome editing. *Science.* (January), 1–12.

Ma, Y., Zhang, L., & Huang, X. (2014). Genome modification by CRISPR/Cas9. *FEBS Journal*, 281(23), 5186–5193. https://doi.org/10.1111/febs.13110

Mahmoudian-sani, M. R., Farnoosh, G., Mahdavinezhad, A., & Saidijam, M. (2018). CRISPR genome editing and its medical applications. *Biotechnology and Biotechnological Equipment*, 32(2), 286–292. https://doi.org/10.1080/13102818.2017.1406823

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J. J., Charpentier, E., Horvath, P., Koonin, E. V. (2011). Evolution and classification of the CRISPR-Cas systems. *Nature Reviews Microbiology*, 9(6), 467–477. https://doi.org/10.1038/nrmicro2577

Maresch, R., Mueller, S., Veltkamp, C., Ollinger, R., Friedrich, M., Heid, J., Rad, R. (2016). Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. *Nature Communications*, 10770.

Martinez-Lage, M., Torres-Ruiz, R., & Rodriguez-Perales, S. (2017). CRISPR/Cas9 Technology: Applications and Human Disease Modeling. *Progress in Molecular Biology and Translational Science*, 152(June 2016), 23–48. https://doi.org/10.1016/bs.pmbts.2017.09.002

Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Rebar, E. J. (2011). A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology*, 143-148.

Nelson, C. E., Hakim, C. H., Ousterout, D. G., Thakore, P. I., Moreb, E. A., Rivera, R. M. C., Ann, F. (2017). Model of Duchenne muscular dystrophy. *BioTech*. 351(6271), 403–407. https://doi.org/10.1126/science.aad5143.In

Offord, C. (2018). *US Companies Launch CRISPR Clinical Trial*. Retrieved from The Scientist: https://www.the-scientist.com/news-opinion/us-companies-launch-crispr-clinical-trial-64746

Ophinni, Y., Inoue, M., Kotaki, T., & Kameoka, M. (2018). CRISPR/Cas9 system targeting regulatory genes of HIV-1 inhibits viral replication in infected T-cell cultures. *Scientific Reports*, 8(1), 1–12. https://doi.org/10.1038/s41598-018-26190-1

Ophinni, Y., Inoue, M., Kotaki, T., & Kameoka, M. (2018). CRISPR/Cas9 system targeting regulatory genes of HIV-1 inhibits viral replication in infected T-cell cultures. *Nature.*

Pineda, M., Moghadam, F., Ebrahimkhani, M. R., & Kiani, S. (2017). Engineered CRISPR Systems for Next Generation Gene Therapies. *ACS Synthetic Biology*, 6(9), 1614–1626. https://doi.org/10.1021/acssynbio.7b00011

Qi, X., Zhang, J., Zhao, Y., Chen, T., Xiang, Y., Hui, J., Li, G. (2017). The applications of CRISPR screen in functional genomics. *Briefings in Functional Genomics*, 16(1), 34–37. https://doi.org/10.1093/bfgp/elw020

Ramanan , V., Shlomai, A., & Cox, D. (2015). CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Sci Rep*, 10833.

Rana, P., Marcus, A. D., & Fan, W. (2018, 1 21). *China, Unhampered by Rules, Races Ahead in Gene-Editing Trials.* Retrieved 08 23, 2019, from The Wall Street Journal: https://www.wsj.com/articles/china-unhampered-by-rules-races-ahead-in-gene-editing-trials-1516562360

Redman, M., King, A., Watson, C., & King, D. (2016). What is CRISPR/Cas9? *Archives of Disease in Childhood: Education and Practice Edition*, 101(4), 213–215. https://doi.org/10.1136/archdischild-2016-310459

Rossidis, A.C., Stratigis, J.D., Chadwick, A.C., Hartman, H.A., Ahn, N.J., Li, H., Singh, K., Coons, B.E., Li, L., Lv, W., et al. (2018). In utero CRISPR-mediated therapeutic editing of metabolic genes. Nat. Med. 24, 1513–1518.

Salsman, J., & Dellaire, G. (2016). Precision genome editing in the CRISPR era. *Biochemistry and Cell Biology*, 95(2), 187–201. https://doi.org/10.1139/bcb-2016-0137

Sanchez-Rivera, F. J., Papagiannakopoulos, T., Romero, R., Tammela, T., Mauer, M. R., Bhutkar, A., Jacks, T. (2014). Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature*, 428-431. doi:10.1038/nature13906

Sankaranarayanan, V. V., Sattar, J., & Lakshmanan, L. S. (2014). Auto-play: A data mining approach to ODI cricket simulation and prediction. *SIAM International Conference on Data Mining*, (p. 1064).

Savić, N., & Schwank, G. (2016). Advances in therapeutic CRISPR/Cas9 genome editing. *Translational Research*, 168, 15–21. https://doi.org/10.1016/j.trsl.2015.09.008

Schneider-Futschik, E. K. (2019). Beyond cystic fibrosis transmembrane conductance regulator therapy: a perspective on gene therapy and small molecule treatment for cystic fibrosis. *Nature*. doi:https://doi.org/10.1038/s41434-019-0092-5

Schwank, G., Koo, B. K., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., Clevers, H. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*, 13(6), 653–658. https://doi.org/10.1016/j.stem.2013.11.002

Shen, S., Loh, T. J., Shen, H., Zheng, X., & Shen, H. (2017). CRISPR as a strong gene editing tool. *BMB Reports*, 50(1), 20–24. https://doi.org/10.5483/BMBRep.2017.50.1.128

Song, M. (2017). The CRISPR/Cas9 system: Their delivery, in vivo and ex vivo applications and clinical development by startups. *Biotechnology Progress*, 33(4), 1035–1045. https://doi.org/10.1002/btpr.2484

Stein, R. (2019, 4 16). *First U.S. Patients Treated With CRISPR As Human Gene-Editing Trials Get Underway*. Retrieved 08 22, 2019, from National Public Radio, Inc.:

https://www.npr.org/sections/health-shots/2019/04/16/712402435/first-u-s-patients-treated-with-crispr-as-gene-editing-human-trials-get-underway

Tabebordbar, M., Zhu, K., Cheng, J. K. W., Chew, W. L., Widrick, J. J., Yan, W. X., Amy, J. (2016). In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science.* 351(6271), 407–411. https://doi.org/10.1126/science.aad5177.In

Takata, M., Sasaki, M. S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Takeda, S. (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *EMBO Journal*, 17(18), 5497–5508. https://doi.org/10.1093/emboj/17.18.5497

Tian, X., Gu, T., Patel, S., Bode, A. M., Lee, M.-H., & Dong, Z. (2019). CRISPR/Cas9 – An evolving biological tool kit for cancer biology and oncology. *Npj Precision Oncology*, 3(1). https://doi.org/10.1038/s41698-019-0080-7

Tulabandhula, T., & Rudin, C. (2014). Tire Changes, Fresh Air, and Yellow Flags: Challenges in Predictive Analytics for Professional Racing. *Big data.* Retrieved from http://doi.org/10.1089/big.2014.

Wei, C., Wang, F., Liu, W., Zhao, W., Yang, Y., Li, K., Shen, J. (2018). CRISPR/Cas9 targeting of the androgen receptor suppresses the growth of LNCaP human prostate cancer cells. *Molecular Medicine Reports*, 17(2), 2901–2906. https://doi.org/10.3892/mmr.2017.8257

Wei, X., & Nielsen, R. (2019). CCR5-Δ32 is deleterious in the homozygous state in humans. *Nature*, 909-910. doi:https://doi.org/10.1038/s41591-019-0459-6

Weintraub, K. (2019). *Despite Controversy, Human Studies of CRISPR Move Forward in the U.S.* Retrieved from Scientific American: https://www.scientificamerican.com/article/despite-controversy-human-studies-of-crispr-move-forward-in-the-u-s/

You, L., Tong, R., Li, M., Liu, Y., Xue, J., & Lu, Y. (2019). Advancements and Obstacles of CRISPR-Cas9 Technology in Translational Research. Molecular Therapy: *Methods & Clinical Development*, 13(June), 359–370. https://doi.org/10.1016/j.omtm.2019.02.008