The Revolutionary Genome Editor: CRISPR-Cas9 Systems

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Abstract

Genetic engineering is the modification of an organism's genetic material to alter its traits through adding, deleting, or changing specific genes. CRISPR-Cas9 systems are groundbreaking tools for genetic engineering, in short utilizing a molecule called RNA to guide a protein called Cas9 to a specific location in DNA to add, delete, or replace genes. The history of how the CRISPR-Cas9 systems came into existence, how it was adapted from a natural defense system in bacteria, and its mechanism of action in both are explained. Its applications, both present and future, competing genetic modifiers, advantages and disadvantages, and the ethical dilemmas surrounding genetic engineering as a whole from a Christian perspective are also discussed throughout this review.

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I. INTRODUCTION

Throughout the mid-1800s, Gregor Mendel, an Australian monk, conducted a series of experiments with pea plant reproduction, ultimately discovering fundamental laws to define heredity (Sequeira, 2000). After the existence of these distinct inheritance units was established, a new biological discipline in science was created: genetics. Over time, with rapid progression of genetic research and technology and eventual development of automated DNA sequencing machines, the specific makeup of multiple species' genomes began to be understood. As complete genome sequences became available, geneticists were finally able to analyze how genetic variations led to diverse phenotypic expression and investigate possible methods to modify genes in order to change expression (Hudson, 2008). This newly emerging medical biotechnology, now known as gene editing, has the potential to reconstruct and repair malfunctioning genetic material in order to treat, and even cure, genetic diseases.

1.1 History of the CRISPR-Cas systems: The most promising genomic editing technology is an RNA-guided nuclease system known as CRISPR-Cas9. CRISPR, short for clustered regularly interspaced short palindromic repeats, is a group of repetitive DNA sequences separated by spacer regions in the genomes of prokaryotes such as archaea and bacteria (Ishino, 2018). It is paired with CRISPR-associated (Cas9) proteins, endonucleases encoded by Cas9 genes found downstream to CRISPR, to effectively locate, cut, and alter abnormal DNA sequences in the human genome (Morange, 2015).

1.2 Ishino's Initial Encounter: CRISPR was initially discovered in 1987 by Yoshizumi Ishino and his research team while studying the Escherichia coli genome. The primary purpose of the experimentation was to analyze the specific genes involved in alkaline phosphate metabolism, but an unusual and repetitive sequence of DNA was also identified in several

areas of the genome (Ishino, 2018). In the article's concluding remarks, it was noted that no DNA sequence similar to these had previously been located anywhere else in E. Coli, or any prokaryote for that matter, and the biological significance of these clusters of repetitive sequences, subsequently defined as CRISPR, was not known (Ishino, 1987).

1.3 Increased CRISPR Prevalence and Naming: Soon after, homologous patterns of DNA were found in various other bacteria (Van Emben, 1993) and archaea (Mojica et al., 1993). Throughout the rest of the 1990s and early 2000s, as technological sequencing development and capabilities increased, more and more clustered repeats of DNA were identified in microorganisms. With the increasing prevalence and identification of these genomic regions, terms such as large cluster of tandem repeats (LCTRs) (She et al., 2001), spacers interspersed direct repeats (SPIDR) (Jansen et al., 2002), and short regularly spaced repeats (SRSRs) (Mojica et al., 2000) were coined by several different researchers to describe them. Finally, clusters of regularly interspaced short palindromic repeats (CRISPR) was proposed (Jansen et al., 2002) and became acknowledged by the research community as the main name for the sequences, alleviating confusion caused by the multiple names aforementioned (Ishino, 2018).

1.4 CRISPR Functionality in Prokaryotes: Even though CRISPR sequences were repetitively found in various prokaryotes, the underlying reason for their existence was still unknown until the realization that the CRISPR regions matched portions of bacteriophage, plasmid, and prophage genomes (Mojica et al., 2005). After discovering this, Mojica and his team hypothesized that CRISPR was somehow involved in an adaptive immune response. It was later confirmed by another group who published similar findings shortly after (Pourcel et al., 2005).

At the same time as CRISPR's discovery, another set of genes was under investigation. These genes, known as clusters of orthologous groups (COGs) at the time, were initially thought to create DNA repair proteins in thermophilic archaea and bacteria (Makarova et al., 2002), but were later identified as CRISPR-associated genes and subsequently named Cas9 genes (Jansen et al., 2002). With further research and examination, it was hypothesized that the Cas9 proteins, the proteins produced from Cas9 genes, and CRISPR collaborated to form the acquired immune system in prokaryotes and defends prokaryotic organisms against plasmid and virus invasion (Markova et al., 2006).

Finally, an experiment which involved inserting phage DNA sequences into spacer regions of CRISPR of *Streptococcus thermophilus* bacterium proved that CRISPR played a role in prokaryotic acquired immunity. The research revealed that the bacterium became resistant to the phage when phage DNA sequences were integrated into its CRISPR spacer regions in its DNA. However, once the phage sequence was removed from the CRISPR spacer region, the bacterium was no longer resistant to that particular phage (Barrangou, 2007). Interestingly enough, this research was not initially conducted in hopes of identifying a genetic engineering system, but rather to stop bacteriophage attack on *Streptococcus thermophilus*. The pivotal role of the prevalent bacterial microbe in the yogurt industry stems from its ability to catalyze the conversion of lactose into lactic acid, which in turn, results in the desired thickening of yogurt. However, the intrusion of bacteriophage predators can be detrimental to Streptococcus thermophilus, leading to accelerated spoilage rates of the yogurt product. Barrangou and his team were working at Danisco, a prominent yogurt manufacturer, when they stumbled upon this remarkable discovery (Cohen, 2017).

1.5 Investigation into the CRISPR-Cas9 Mechanism: Although experimentation had demonstrated that CRISPR-Cas9 functionality was associated with successful virus resistance, the details surrounding how CRISPR-Cas9 systems were able to inhibit phage invasion was still largely unknown. The first piece of the CRISPR-Cas9 puzzle came from a research team in the Netherlands who provided evidence that spacer sequences derived from phages in *Escherichia coli* underwent transcription into RNA molecules during adaptive immune activation. These RNA molecules, coined CRISPR RNAs (crRNAs), were found to guide Cas9 proteins to their intended DNA target areas (Brouns et al., 2008). From there, it was discovered that once the CRISPR-Cas9 system finds its target DNA, it is able to break both strands of DNA at precise positions, particularly 3-4 nucleotides upstream of a protospacer adjacent motif (PAM) region which acts as a targeting component for the CRISPR-Cas9 system by distinguishing bacterial non-self-DNA from self-DNA (Garneau et al. 2010).

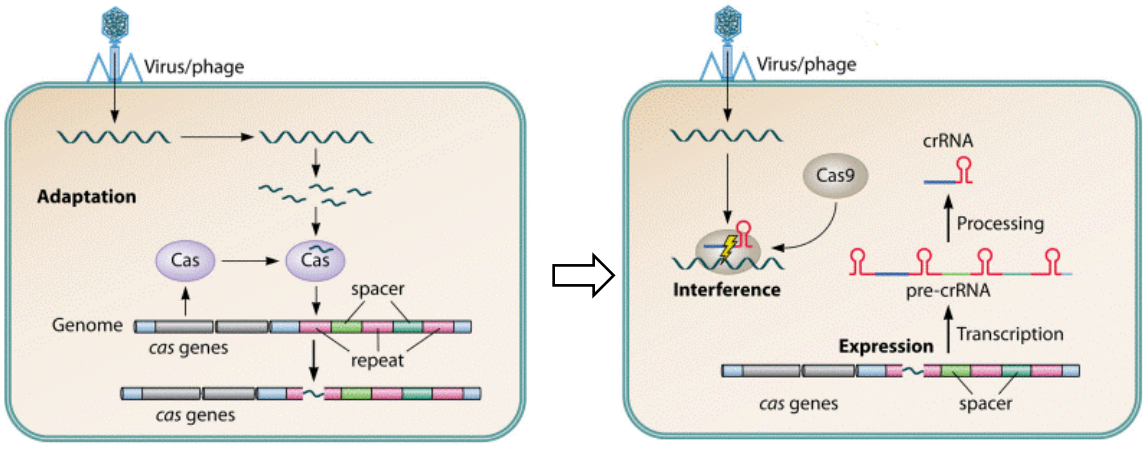

Figure 1. Cas proteins recognize the invading viral DNA, cutting and integrating it into the CRISPR region during the Adaptation phase. With the viral DNA history stored, when another virus attacks, the CRISPR DNA sequence codes for pre-crRNA. Pre-crRNA is eventually processed into small subunits known as crRNA and combines with Cas9 in the interference phase to activate nuclease activity to cleave and destroy the viral DNA (Ishino, 2018). Permission for figure use was granted by the author of the article. See appendix for more information.

In addition to the crRNA discovery, RNA sequencing also revealed another small, secondary RNA, subsequently named trans-activating CRISPR RNA (tracrRNA), that joined with crRNA to guide a Cas9 protein to its correct editing area (Deltcheva et al., 2011). Shortly after, the specific Cas9 protein that acted as the endonuclease agent in the prokaryotic adaptive immune responses was also identified as Cas9 (Barrangou, 2012) and it was finally understood how of all the components of the CRISPR-Cas9 system effectively allowed the cell to remember, recognize, and clear infections, evident through the mechanism is detailed in Fig. 1. With this knowledge, the CRISPR-Cas9 system was proven to work as an RNA-guided DNA endonuclease.

1.6 CRISPR-Cas9's Genetic Editing Potential Discovered: The most revolutionary revelation came about when scientists found a way to reprogram this RNA-guided DNA endonuclease system to target a site of their choosing by encoding a particular crRNA sequence. Purified Cas9-CRISPR RNA was now capable of cleaving target DNA in vitro (Jinek et al., 2012, Gasiunas et al., 2012). Members of one research team in UC Berkeley, Emmanuelle Charpentier and Jennifer Doudna, also found a way to fuse together tracrRNA and crRNA to form a simple, single, synthetic guide for the guide (Jinek et al., 2012). With this technology, dreams of being able to alter DNA by replacing existing sequences with a customized segment to modify an individual's genome were becoming a reality.

1.7 The CRISPR-Cas9 Race for Patent Rights: Charpentier and Doudna of UC Berkeley were not the only two scientists actively researching CRISPR-Cas9 systems. The Broad institute, a combination of Massachusetts Institute of Technology and Harvard University, also had geneticists George Church and Feng Zhang investigating the untapped potential of the RNA-guided endonuclease system (Cohen, 2017). Church and Zhang, with their respective

research teams, were able to use *Streptococcus pyogenes* CRISPR-Cas9 systems to edit the genomes of kidney cells in mice and human nerves (Cong et al., 2013, Mali et. al, 2013). It was initially thought that the best and brightest CRISPR-Cas9 researchers could work together and simplify the intellectual property process, but the attempt at unity was unsuccessful. As potential for CRISPR-Cas9 human integration and financial profit rapidly increased, the desire to form CRISPR companies and gain intellectual rights intensified (Cohen, 2017). Although Doudna and Charpentier were awarded the Nobel Prize in Chemistry in 2020 for discovering CRISPR-Cas9's genomic engineering capacities, the dispute over who deserves a patent for the technology is still ongoing (Ledford, 2022). To this day, the long-running dispute between the Broad Institute and the UC Berkeley team remains unresolved. Both organizations filed patent applications for the genetic editing technology of CRISPR-Cas9 in all eukaryotic cells in the same year (Ledford, 2022). UC Berkeley did file for their original patient a few months prior to Broad Institute, but, at that time, the United States Patent and Trademark Office awarded patents based on which group or individual was the first to invent a particular technology rather than based on who filed for the patent first (Ledford, 2022).

1.8 Most Recent CRISPR-Cas9 Activity: Although the debate over intellectual property continues, CRISPR-Cas9 system research and implementation has not ceased. With the RNA-guided endonuclease system proven functional in eukaryotic cells in vitro, experimentation in vivo and in other aspects of life such as agriculture and the environment are flourishing. The expanded capabilities and applicabilities of CRISPR-Cas9 systems will be discussed in detail in a subsequent section.

II. STRUCTURE AND MECHANISM OF ACTION

2.1 Structural Features of CRISPRs: As mentioned previously, the CRISPR-Cas9 system has two major components: the CRISPR sequences of DNA and Cas9 proteins. The two main parts of CRISPR are its repeats and spacers. Repeated sequences are short segments of DNA that maintain a constant length, dyad symmetrical, and palindromic structure, evident by the arrows facing one another in Fig. 2 (Ishino, 2018). The palindromic structure allows transcribed RNA to form hairpins. Each repeat is identical and interspaced and in combination with palindromic hairpins, allow prokaryotic organisms to find spacer regions. Spacer regions, sequences of extrachromosomal nucleotides in between repeats, are not homologous like repeat regions but constant in length (Ishino, 2018). Each spacer sequence is distinct because its DNA nucleotide arrangement matches that of a viral bacteriophage that previously attempted to invade and infect the prokaryote.

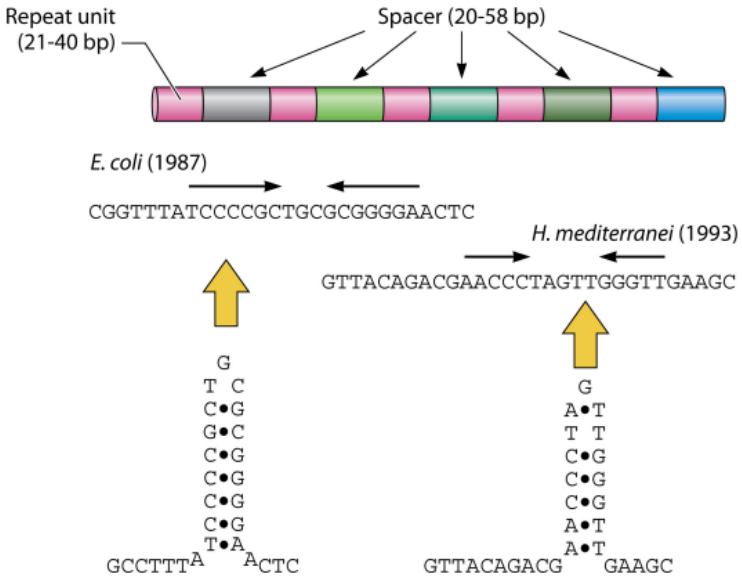

Figure 2. Structural components of CRISPR. Spacers and repeat sequences form palindromic sequences are visualized. *Escherichia coli* (bacteria) and *Haloferax mediterranei* (archaea) CRISPR regions are shown (Ishino, 2018). Permission for figure use was granted by the author of the article. See appendix for more information.

2.2 Structure of the Cas9 Protein: Additionally, the CRISPR-Cas9 system encompasses a suite of CRISPR-associated genes (CAS9 genes) that encode for CAS9 proteins. These proteins are responsible for executing the fundamental functions of the CRISPR-Cas9 system and can be broadly classified into two distinct categories, including helicases and nucleases. The helicases function as enzymes that unwind DNA, while the nucleases act as enzymes that cleave DNA (Jiang & Doudna, 2017). Despite the abundance of Cas9 proteins utilized in various CRISPR-Cas9 systems, the Cas9 protein has become the most commonly employed due to CRISPR-Cas9's exceptional versatility in genome manipulation. The widespread usage of the CRISPR-Cas9 system can be attributed to several factors, including its simplicity in design, ease of application, and remarkable efficiency (Jiang & Doudna, 2017). Consequently, the CRISPR-Cas9 system has established itself as an indispensable tool across a diverse range of organisms, where its precise genome manipulation capabilities have proven to be of immense value.

The Cas9 protein is a complex, multidomain endonuclease composed of 1,368 amino acids that functions in the snipping of double-stranded DNA 3 base pairs upstream of the PAM. This snipping is achieved through the action of two distinct nuclease domains, including an HNH-like nuclease domain that cleaves the target strand complementary to the guide RNA sequence, and an RuvC-like nuclease domain responsible for cleaving the non-target strand. Beyond its vital role in CRISPR interference, Cas9 also plays a role in the maturation of crRNA and the acquisition of spacers (Jiang & Doudna, 2017).

2.3 CRISPR-Cas9 Adaptive Immune Response Mechanism: The way in which CRISPR-Cas9 systems work within prokaryotes to protect against foreign invaders is fairly

complex. There are three stages of CRISPR-mediated adaptive immunity, namely the acquisition of foreign, viral DNA and subsequent adaptation, biogenesis of CRISPR RNA (crRNA), and target interference (Sorek et al., 2013). These basic stages appear to be present in all CRISPR-Cas9 systems, but the particular Cas9 proteins and CRISPR loci that facilitate the process differ from organism to organism.

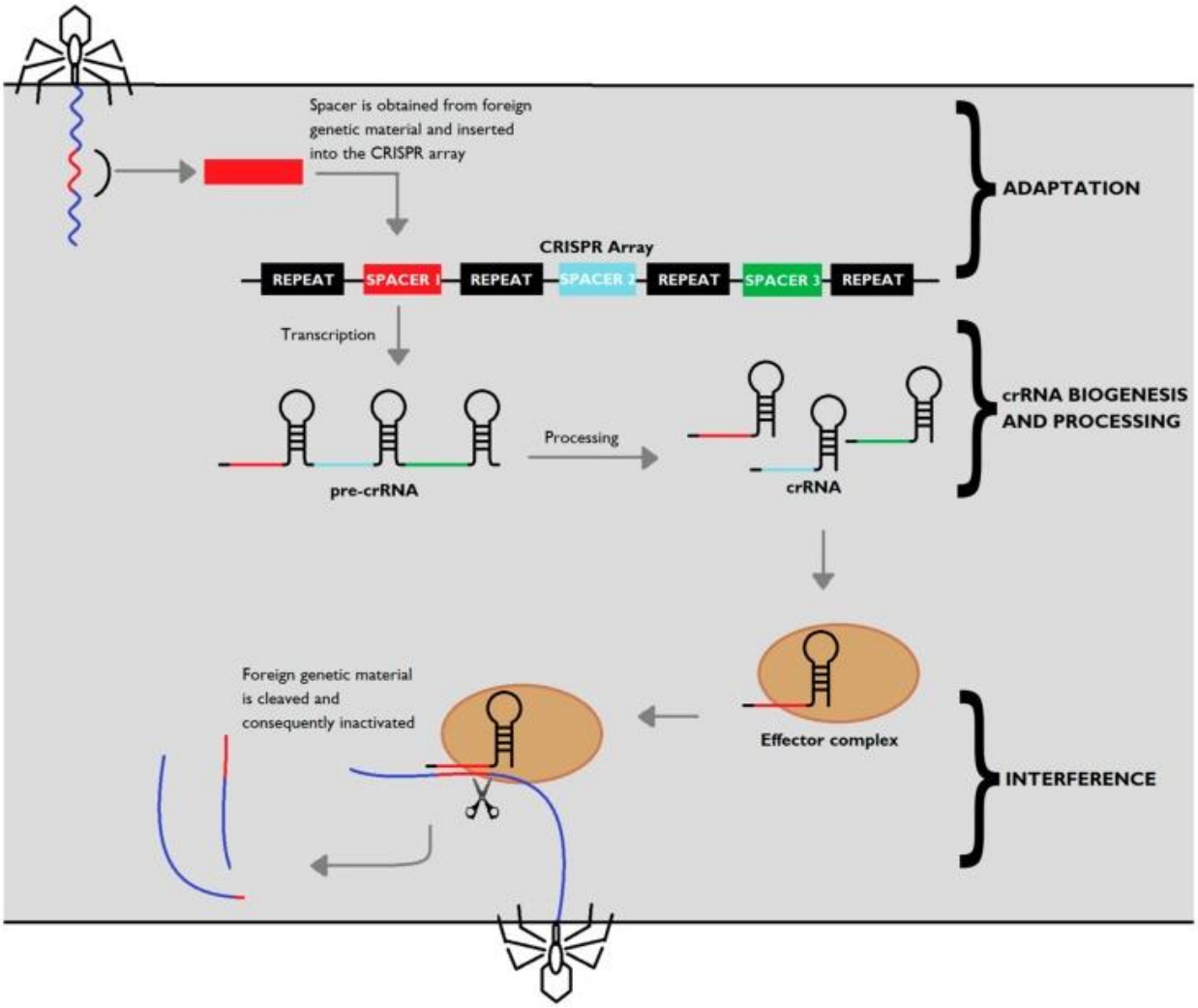

Figure 3. The detailed process of CRISPR-Cas9-mediated adaptive immune response. With injection of viral genetic material into a prokaryote, a portion of the invader's DNA sequence is cleaved and integrated into CRISPR, creating a new spacer region. Transcription of the newly formed CRISPR-array into precursor and subsequently mature crRNA occurs to eventually form complexes with Cas9 proteins (Loureiro & Silva, 2019). Permission for figure use was granted by the author of the article. See appendix for more information.

2.4 Acquisition and Adaptation: In the acquisition and adaptation phase, the invading bacteriophage's viral DNA is integrated into the spacer region of the organism's CRISPR sequence, allowing the organism to recognize and respond appropriately to future infections by that particular phage strain and demonstrated in Fig. 3 (Loureiro & Silva, 2019). The two Cas9 proteins utilized in the acquisition or adaptation step of adaptive immunity are Cas91 and Cas92, regardless of the specific type of CRISPR-Cas9 system in use (Makarova, 2006). Without the expression of both Cas9 proteins, spacer acquisition does not occur, evident by mutation experimentation in *Escherichia coli*. When Cas1 or Cas2 genes were mutated independently, viral DNA spacer acquisition was not possible. In contrast, an overexpression of Cas9 protein allowed for greater spacer integration, validating the need for a combination of both for adequate spacer acquisition (Datsenko, 2012). Once both the Cas1 and Cas2 proteins are present, they form a complex which can then select protospacer DNA, the viral DNA sequence that precedes a spacer region, modify into pre-spacer DNA, and incorporate it into its corresponding CRISPR sequence (Nuñez et al., 2014). Cas1-Cas2 complexes know which protospacer DNA to select by identifying and binding to a 2-5 nucleotide-length sequence specific to a particular bacterium and its CRISPR-Cas9 subtype that is adjacent to protospacers, subsequently named protospacer adjacent motifs (PAMs) (Loureiro & Silva, 2019).

As aforementioned, modification occurs to the protospacer region to prepare it for integration into the spacer area of CRISPR. Particularly, Cas1 subunits containing Tyrosine residues act as brackets around a portion of the viral DNA and limit its length to 23 nucleotides to match the spacer length, while Cas2 subunits stabilize its central

dsDNA (Loureiro & Silva, 2019). A 3-nucleotide overhang is also created to complement the PAM sequence of the foreign DNA, and it is eventually cleaved by Cas9 proteins to generate 2 hydroxyl groups on each 3' end. Now fully mature, the protospacer is incorporated into prokaryote's CRISPR array with the help of Cas1-Cas2's integrase activity and nucleophilic attacks from the 3' hydroxyl groups in the spacer DNA onto the 5' ends of the repeat DNA in the CRISPR sequence (Nuñez et al., 2014). Additionally, because the majority of prokaryotic organisms have both Cas91 and Cas92 proteins, it is assumed that most use the Cas1-Cas2 protein complex during the acquisition and adaptation phase of CRISPR-mediated adaptive immunity, but some CRISPR-Cas9 systems have also been observed to rely on Cas4 nuclease or reverse transcriptase activity, meriting the need for more investigation into the potential capabilities of other Cas systems (Silas et al., 2016).

2.5 Biogenesis of CRISPR RNA: After appropriate modification has occurred to insert viral DNA into the spacer region of the CRISPR array, transcription of the spacer genomic sequence begins to create small precursor-CRISPR RNA (pre-crRNA). Another round of modification and cleaving by ribonucleases and Cas9 proteins occurs to form fully mature smaller units of crRNA in between repeat regions. Within this biogenetic process, the different types of CRISPR-Cas systems develop for diverse processes in the adaptive immune response, but it is extremely hard to classify these systems into groups due to their architectural complexity. The most commonly used classification approach is the "polyethic' approach, which integrates comparative genomic, phylogenetic, and structural analysis techniques (Loureiro & Silva, 2019). The three major distinguishable groups are type I, II, and III CRISPR systems, each differentiated with a more prominent

Cas9 protein (Makarova & Koonin, 2015). Ultimately, the CRISPR-Cas9 system most heavily researched in regard to genetic engineering capabilities is CRISPR-Cas9 due to its endonuclease activity.

2.6 Target Interference: After the prokaryotic organism is infected by the same invader, the mature crRNA is now able to guide the CRISPR-Cas9 interference machinery towards the particular foreign nucleotide sequence, subsequently silencing and destroying the non-self-genetic material (Sorek et al., 2013). The interference step in Type II CRISPR-Cas9 systems relies on both Cas9 and tracrRNA. In this process, Cas9 functions as an endonuclease that is guided by two RNAs, crRNA and tracrRNA, which form a dual RNA complex (tracrRNA-crRNA) through tracrRNA's complementarity to spacer sequences in crRNA. This dual RNA structure triggers conformational changes in Cas9, causing it to become activated. Upon activation, the guide RNA-bound complex searches for the correct PAM site on foreign genetic elements, opposite to the target strand. Once found, the dsDNA is opened and crRNA binds to the target ssDNA, leading to an R-loop formation and eventually resulting in a blunt double-strand break by Cas9's catalytic sites, 3 nucleotides upstream of the PAM site, illustrated by Fig. 4 (Loureiro & Silva, 2019).

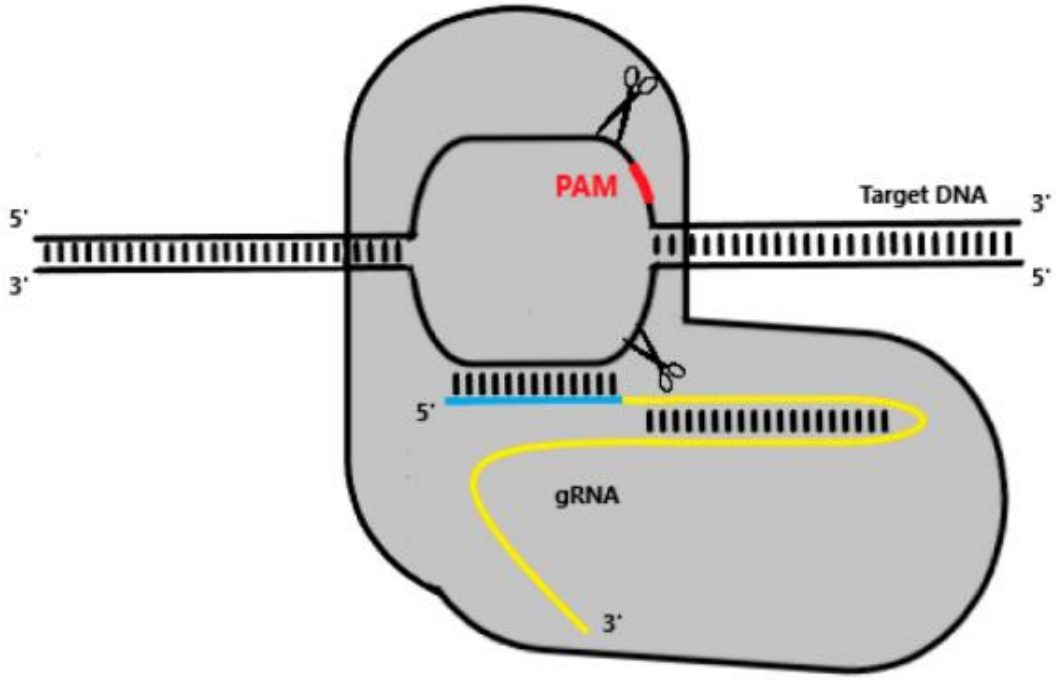

Figure 4. Gene Editing with CRISPR-Cas9: A graphical representation. When a suitable PAM site (red) is identified, the targeting sequence (blue) of the gRNA molecule (yellow) starts bonding with the target DNA in a 3′-5′ direction. If there is sufficient similarity between the strands, Cas9 undergoes a structural change that leads to a blunt double-stranded break (DSB) (Loureiro & Silva, 2019). Permission for figure use was granted by the author of the article. See appendix for more information.

2.7 CRISPR-Cas9 Implementation into Genetic Editing: As previously mentioned, Jinek et al.'s paper from 2012 laid the groundwork for a major breakthrough in genome editing and regulation of transcription. The authors proposed that in CRISPR-Cas9 systems, the dual guide RNA complex of tracrRNA-crRNA in Cas9 could be merged into a single chimeric RNA by connecting the 30 end of crRNA to the 50 end of tracrRNA (Ishino, 2018). This would allow for targeted DNA cleavage through the engineering of the chimeric RNA molecule, later referred to as sgRNA or gRNA (guide RNA). This hypothesis was confirmed with the successful design of five gRNA molecules to target the green fluorescent protein (GFP) gene, which resulted in precise and efficient cleavage of a plasmid containing the GFP gene by the programmed Cas9 for all five gRNA molecules (Ishino, 2018).

gRNA molecules consist of two parts: a scaffold sequence that binds to Cas9 and a targeting sequence that directs the system towards the target location. As Cas9-gRNA searches for a potential target, the first 8-12 base pairs closest to the PAM site of the targeting sequence, also known as the seed sequence, will start pairing with the target DNA in the 3'-5' direction if a PAM site is recognized (Liu et al., 2016). While mismatches in the seed sequence prevent pairing and decrease Cas9 cleavage activity, mismatches towards the 5'-PAM-distal end may not always affect Cas9 function. When there is a match between the gRNA and target sequence, a double-stranded break (DSB) in the DNA is created, catalyzed by the catalytic domains of Cas9 (Liu et al., 2016).

The repair of the DSB is then carried out through either non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is an active but error-prone process where random DNA fragments align with both ends of the DSB and are joined by the body's own repair machinery, provided the base pairs at both ends have some degree of complementarity (Qi et al., 2013). This pathway does not require a repair template and is the main way in which Cas9-induced DSBs are repaired. NHEJ can lead to small insertions or deletions of nucleotides (indels) in the DSB region, which can then result in a variety of insertions, deletions, or frameshift mutations (Loureiro & Silva, 2019). These mutations from Cas9-induced DSBs can be useful when trying to inactivate a targeted gene, as indels often cause premature stop codons and make the gene inoperable. However, NHEJ is a highly random and unpredictable process that is not suitable for generating single-base edits or inserting specific sequences (Qi et al., 2013).

III. PROS AND CONS OF THE CRISPR-CAS SYSTEMS

3.1 Advantages: CRISPR-Cas9 technology has seen tremendous growth in recent years and has become the mainstream genetic editor in scientific endeavors because of its precision, efficiency, versatility, simplicity, and low-cost. The precision of CRISPR-Cas9 is due to the ability of the guide RNA molecule to specifically target DNA sequences, allowing for precise and efficient gene editing. (Liang et al., 2015). The guide RNA molecule contains a 20-nucleotide sequence that is complementary to the target DNA sequence, allowing the Cas9 nuclease to bind specifically to the target site.

In contrast, other gene editing techniques, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have been shown to have higher rates of off-target effects due to their broader specificity (Hsu et al., 2013). This has been made evident by multiple studies, including one that compared the specificity of the CRISPR-Cas9 system to ZFNs and TALENs and found that the CRISPR-Cas9 system had significantly lower rates of off-target effects (Hsu et al., 2013).

Moreover, CRISPR-Cas9 boasts a significant advantage in terms of speed and simplicity when compared to other gene editing techniques. The CRISPR-Cas9 system is designed and executed faster and with less effort than alternative techniques such as zinc finger nucleases or TALENs. This is because CRISPR-Cas9 requires less time-consuming and complicated design and assembly processes (Sander & Joung, 2014). In contrast to the traditional DNA editing methods of ZFNs and TALENs, CRISPR-Cas9 uses a 20-nt guide RNA sequence for DNA recognition, instead of protein. This eliminates the time-consuming process of protein engineering for each target site to be modified and greatly enhances the practicality and ease of large-scale genomic manipulation or screening (Jiang & Doudna, 2017). With its rapid and

straightforward nature, researchers can perform gene editing experiments more quickly and efficiently.

Lastly, the cost-effectiveness of CRISPR-Cas9 is one of its most attractive advantages, particularly for researchers and institutions with limited funding. CRISPR-Cas9 is much cheaper than previous gene editing methods, such as the aforementioned ZFNs and TALENs. This lower cost makes it more accessible for smaller institutions and research groups where budgeting may not allow for expensive gene editing technologies or in low- and middle-income countries, where funding for research is often limited (Cong et al., 2013). CRISPR-Cas9 has the potential to improve the health and well-being of populations in these countries, particularly for those suffering from genetic diseases that are often neglected by the pharmaceutical industry. The cost-effectiveness of CRISPR-Cas9 has also made it more widely available for use in academic and commercial research, helping to drive the rapid pace of progress in the field. In addition to being more accessible, the lower cost of CRISPR-Cas9 has also made it easier for researchers to pursue new and innovative applications (Cong et al., 2013). For example, the technology can be used to study the function of individual genes in various organisms, including plants and animals, which can help to further our understanding of genetics and biochemistry.

3.2 Limitations: CRISPR-Cas9 is a relatively new technology, and much is still unknown about its full capabilities and limitations. Despite its numerous advantages, there are still many questions that need to be answered. The long-term effects of CRISPR-Cas9 on organisms, particularly humans, are still largely uncertain. CRISPR-Cas9 can also sometimes cause unintended mutations in genes other than the target gene, which can have unintended consequences (Hsu et al., 2014).

The reasons that Cas9 proteins cleave off-target sides in some instances are still largely uncertain (Modrzejewski, 2020). One suspected factor is the structure of the chromatin that the Cas9 protein is cleaving. Some research has shown that Cas9 cleaves open chromatin regions more efficiently than closed chromatin regions (Hinz et al., 2015). Another component that scientists have speculated plays a role in unintentional off-target cleaving is the matches of PAM regions. For unknown reasons, experiments have shown that PAM distal mismatches actually inhibit cleavage at off-target sites. Within Cas9, there is a domain called HNH that flips from being active and bound to RNA and active, to an inactive, docked state when bound to DNA, which rapidly enables the cleavage and destruction of the DNA (Modrzejewski, 2020). When distal aspects of PAM are mismatched, the transition from inactive to active is delayed and inhibited in some cases, subsequently stopping off-target site cleavage (Dagdas et al., 2017).

Additionally, the delivery of the CRISPR-Cas9 system into cells remains a significant challenge, particularly in the context of in vivo applications. The current methods of delivery are not yet efficient or straightforward, which can limit the potential use of CRISPR-Cas9 in various fields (Liu et al., 2014). Ultimately, even with optimization of CRISPR-Cas9 and elimination of these issues, there are still regulatory and ethical barriers that hinder the growth of widespread implementation of this genetic editing technology.

IV. CRISPR-CAS9 APPLICATIONS AND FUTURE USES

4.1 Genetically Modified Agriculture: CRISPR-Cas9 technology has the potential to revolutionize agriculture by enabling precise and efficient gene editing. Gene editing can be

used to improve crops in a variety of ways, such as increasing resistance to pests and diseases, enhancing nutritional value, and improving stress tolerance. In recent years, there have been numerous studies exploring the application of CRISPR-Cas9 in agriculture, which have demonstrated the feasibility and potential of this technology.

One of the most promising applications of CRISPR-Cas9 in agriculture is the development of crops with improved resistance to pests and diseases. Researchers have used CRISPR-Cas9 to introduce resistance to fungal diseases in rice, the most important staple food in many developing countries (Borrelli et al., 2018). The researchers demonstrated that the edited rice plants showed significantly increased resistance to the fungal pathogen. Another study showed that CRISPR-Cas9 could be used to introduce resistance to viral diseases in crops such as tomatoes, which are important horticultural crops worldwide (Liu et al., 2020).

Another important application of CRISPR-Cas9 in agriculture is the enhancement of the nutritional value of crops. CRISPR-Cas9 has been used to increase the iron and beta-carotene content in rice, a staple food for more than half of the world's population (Zheng et al., 2018). This has the potential to address the problem of iron and vitamin A deficiencies, which are major public health issues in many developing countries. Another study demonstrated that CRISPR-Cas9 could be used to introduce high-quality protein content in soybeans, an important source of protein for many populations (Li et al., 2019).

CRISPR-Cas9 technology has also been used to improve the stress tolerance of crops, which is important in the face of changing climates and unpredictable weather patterns. For example, researchers have used CRISPR-Cas9 to improve the drought tolerance of maize, one of the most important staple crops worldwide (Zheng et al., 2018). The study showed

that the edited maize plants exhibited increased survival rates under water-limited conditions. Another study showed that CRISPR-Cas9 could be used to improve the salt tolerance of rice, which is important for crops grown in areas with high soil salinity (Liu et al., 2020).

4.2 Disease Treatment: The use of CRISPR-Cas9 genomic editing for the treatment of various genetic diseases is becoming a real possibility. There are numerous genetic disorders that have seen positive results from the utilization of CRISPR-Cas9 genomic editing, including Cancer, Sickle Cell Anemia, and Cystic Fibrosis.

One promising applications of CRISPR in the treatment of cancer is the use of CAR-T cell therapy, which involves modifying the T cells of the immune system to make them more effective at recognizing and killing cancer cells. This approach has shown promising results in clinical trials for various cancers, including leukemia and lymphoma (Greenbaum et al., 2020). Another area of research is the use of CRISPR-Cas9 to create “suicide genes” that can be inserted into cancer cells to make them more susceptible to chemotherapy and radiation therapy (Chira et al., 2022). By introducing these genes into the cancer cells, scientists aim to increase the effectiveness of traditional cancer treatments and reduce the toxic side effects. CRISPR-Cas9 can also be used to disrupt the expression of specific genes associated with cancer growth and progression. Oncogenes, unlike normal genes, are regulated differently and can induce normal cells to become cancerous (Zhang et al., 2021). The CRISPR/Cas9 system offers a potential solution for inhibiting tumor growth by deleting, interfering with the expression, or altering the activity of these oncogenes. For example, in colon cancer cells, the knockout of CD133 using CRISPR/Cas9 resulted in reduced cell proliferation, colony formation, migration, and invasion, possibly by downregulating vimentin expression (Zhang et al., 2021). Lastly, CRISPR is currently being utilized in clinical trials for somatic cell

genome editing to address cancer. These studies involve either removing cells from the patient, editing them in tissue culture, and then reintroducing them back into the patient (ex vivo), or administering genome editors packaged within viral vectors or lipid nanoparticles intravenously to target specific tissues (in vivo) (Kan & Doudna, 2022).

Sickle cell disease (SCD) is another common genetic blood disorder caused by a single point mutation in the hemoglobin beta gene. It can cause severe pain, organ damage, and early death in severe cases (Park & Bao, 2021). SCD has limited treatment options, with only four FDA-approved drugs available to alleviate acute symptoms, and the only definitive cure being hematopoietic stem cell transplantation, which typically requires a genetically matched donor who is related to the patient. However, the use of CRISPR/Cas9 gene-editing technology to correct the SCD mutation in the HBB gene or induce fetal hemoglobin production offers a potential permanent cure for all patients, regardless of donor availability (Park & Bao, 2021). Sickle cell disease has also been treated with ex vivo therapies or transfusion-dependent β-thalassemia (TDT). At least 6 clinical trials are underway aiming to cure SCD or TDT by either increasing fetal hemoglobin expression or directly correcting the HBB variants (Kan & Doudna, 2022). A pivotal trial involving 11 patients with SCD demonstrated durable editing that substantially reduced the frequency of vaso-occlusive crises, with no significant adverse events (SAEs) observed. Additionally, clinical trials have been initiated for cancer immunotherapy using ex vivo edited T cells (Kan & Doudna, 2022).

Cystic fibrosis could also potentially be treated by using CRISPR because it is caused by mutations in a particular gene known as CFTR, which codes for a Chloride ion channel protein that helps maintain electrolyte levels and keep the linings of membranes fluid. When mutations occur in the CTFR gene, such as the delta F508 mutation, the most common

symptom is thick, mucus membranes that inhibit digestion and clog airways. Using CRISPR-Cas9, researchers have been able to correct the delta F508 in cell models of intestinal organs derived from Cystic Fibrosis patients and restored CTFR channel function (Maule et. al, 2020). Additionally, the CRISPR-Cas9 system was effective in repairing some splice mutations that cause Cystic Fibrosis in approximately 10% of cases they attempted by targeting aberrant mRNA transcripts that impair CFTR expression (Maule et. al, 2020).

4.3 Targeted Epigenome Editing: The discovery of the CRISPR-Cas9 gene-editing system has paved the way for researchers to modify the Cas9 nuclease to perform targeted epigenome editing. CRISPR-based epigenome editing has the potential to revolutionize the way we treat genetic diseases and other biological disorders by allowing for the precise and efficient modulation of gene expression. The modified Cas9, known as enzymatically dead Cas9 (dCas9), can be paired with various enzymes to alter the epigenome, including DNA demethylases, methylases, or acetyltransferases. The dCas9, guided by a specific RNA, is directed to the targeted genomic region, where instead of cutting the DNA, it modifies the epigenome through its association with the chosen enzyme (Lau & Suh, 2018). Epigenome editing can manipulate transcription, either by activating it through demethylation of DNA by enzymes such as dCas9-Tet1 or by histone modification through dCas9 linked to the histone acetyltransferase p300 enzyme. On the other hand, transcription can be repressed by methylating DNA using DNA methyltransferase, or by linking dCas9 to enzymes that recruit corepressor proteins, thereby silencing genes (Lau & Suh, 2018).

Genome-mapping projects and association studies have also revealed that non-protein-coding elements in the genome play critical roles in biological processes, but understanding their specific functions is challenging. However, CRISPR-Cas9-based epigenome editing

enables precise manipulation of regulatory elements' activity (Klann et al., 2017). In one study, researchers used CRISPR-Cas9-based epigenomic regulatory element screening (CERES) to screen for regulatory elements surrounding the β-globin and HER2 genes in human cells, identifying both known and previously unknown elements and offering a high-throughput method for functional annotation of putative regulatory elements in their native chromosomal context (Klann et al., 2017). Also applicable in cancer research, dCas9-p300 was recently used to modify chromatin structure and transcriptional activity, leading to the activation of silent tumor suppressor genes in human cancer cells (Lau & Suh, 2018).

4.4 Other Applications: The targeting capability of the Cas9 protein has been utilized by scientists to affect a range of modifications at specific loci including activation or repression, epigenetics, visualization techniques, RNA targeting, purification and tagging. By fusing dCas9 with transcriptional activators or repressors, CRISPR-Cas9 system can potentially control transcription in a reversible manner. The use of dCas9 in combination with epigenetic modifiers allows for modulation of transcription, creating heritable epigenetic marks. Fluorescently labeled dCas9 or gRNAs bound to fluorescent proteins can be used to visualize genomic loci in live cells (Lo & Qi, 2017). The Cas913 enzymes target RNA instead of DNA, although a protospacer flanking sequence may be required. While in bacteria, Cas913 targeting results in non-specific RNA cleavage, this does not occur in mammalian cells. Epitope-tagged dCas9 can also be used to purify a genomic locus and its associated proteins or RNAs through techniques such as ChIP (enChIP) or biotin-streptavidin pulldown (CAPTURE) (Lo & Qi, 2017). Quite frankly, the versatility of the CRISPR-Cas9 system has enabled numerous modifications techniques, with the potential for even more possibilities to be discovered in the future.

V. ETHICAL DILEMMAS

The rapid advancements in CRISPR-Cas9 technology have created a new era in genetic engineering. This revolutionary method of editing genes has a wide range of potential applications, from disease prevention and treatment to the enhancement of crops and animals. However, as with any new technology, there are also significant ethical concerns and dilemmas that need to be addressed.

5.1 Designer Babies: One of the primary ethical dilemmas of CRISPR is the potential for abuse and exploitation of the technology. As CRISPR becomes more accessible and affordable, there is a risk that it may be used for eugenic purposes, such as creating "designer babies" with enhanced physical and intellectual abilities. This became apparently evident when a Chinese scientist by the name of He Jiankui attempted to use CRISPR technology to disable the CCR5 gene, a gene that is known to enable the HIV infection and associated with brain function, in vitro embryos in 2018 (Raposo, 2019). Jiankui was eventually given a 3-year sentence for violating a law in China that bans experimentation in embryos over 14 days old, but it fueled debate over genetic engineering, questioning the legal limitations currently in place. In this case and many others, the potential of the CRISPR-Cas9 system to cure diseases is significant, but the distinction between necessary and unnecessary use is unclear. The deployment of eugenic applications of CRISPR-Cas9 technology has the potential to exacerbate existing disparities and further entrench the divide between individuals who possess access to the technology and those who do not. Furthermore, there is a risk that this technology may be utilized as a tool for discrimination, particularly against marginalized populations such as those with disabilities or genetic predispositions to specific illnesses. (Brokowski & Aldi, 2019).

5.2 Off-Target Effects: An additional ethical difficulty that arises in the context of CRISPR-Cas9 technology is the possibility of "off-target" effects. In order to use genome editing for therapeutic purposes, it is crucial to thoroughly evaluate any possible off-target effects. This task is challenging due to the difficulty in accurately predicting such effects and conducting unbiased genome-wide searches. As CRISPR/Cas9 technology advances towards clinical use, it is imperative to establish reliable methods for monitoring and following up with patients. Similar to medications, CRISPR/Cas9 treatments are expected to have some level of off-target edits, or side effects, which require diligent monitoring over time to prevent any unintended consequences (Park & Bao, 2021). These occur when the CRISPR system alters genes that were not intended to be modified, and the resultant unintended mutations can have profound and potentially hazardous implications. Despite advances in the field, there is currently no foolproof method to guarantee that the CRISPR system operates with specificity and precision, targeting only the intended genes (Hsu et al., 2014). This highlights the imperative for scientists and healthcare professionals to exercise due diligence and employ the technology in a manner that prioritizes safety, while also adhering to ethical considerations and obligations. Furthermore, it raises important questions regarding the responsibility of these stakeholders in ensuring that the technology is utilized in a safe and responsible manner, particularly in the realm of medical applications. The potential long-term consequences of using CRISPR to edit genes in humans and other living organisms are also fairly unknown. For example, the effects of a genetic modification made using CRISPR may not become apparent until many years after the fact, and it is possible that the consequences may not be fully understood until it is too late to reverse them (Hsu et al.,

2014). This raises questions about the wisdom of using CRISPR for genetic engineering, and the need for caution and responsible decision-making when using the technology.

VI. CHRISTIAN PERSPECTIVE

6.1 Varying Opinions: Christians may be interested in the theological implications of gene editing, including questions around the sanctity of life, the role of technology in God's plan, and the ethics of human genetic manipulation. The ability to manipulate the genetic code of living organisms raises ethical questions about the boundaries of human intervention in the natural world. The ethical dilemma of whether to "play God" and meddle with the sanctity of creation remains a contentious issue (Funk et al., 2016). On the one hand, some argue that since God has bestowed us with the intellectual capabilities to engage in genetic editing, it is our moral obligation to utilize this gift to enhance the world we inhabit. On the other hand, others question whether we should even attempt to manipulate what God has so intricately crafted. One of the primary ethical dilemmas associated with CRISPR is the risk of abuse and exploitation of the technology. CRISPR-Cas9 has the potential to commercialized and used for financial gain rather than furthering God's kingdom. As previously mentioned, CRISPR can potentially deepen social and economic inequalities in healthcare because genetic engineering is an elective procedure and only available to those who can afford it (Funk et al., 2016). These concerns are also grounded in biblical principles that urge us to do good in the world and not let greed dictate our actions, but instead to act in the name of Jesus Christ. Others may argue that CRISPR-Cas9 aligns with God's principles by providing opportunities to improve the lives of those around us. According to the Bible, humans were given the responsibility of caring for creation (Genesis 1:28; 2:15-20). However, the effects of sin have

impacted the natural world (Genesis 3:17-19, Romans 8:19-21), and the Bible anticipates redemption from these effects. As caretakers of creation, some argue that humans have an obligation to mitigate the effects of sin and strive for improvement, using any available means (Funk et al., 2016). Therefore, proponents of this view argue that any scientific advancement can be utilized to improve the state of creation. All in all, some Christians argue that CRISPR/Cas9 is an example of humans overstepping these boundaries, while others may see it as a tool for improving the health and well-being of humanity. While there is no explicit biblical guidance on the topic of genetic editing due to its novelty, it is worth considering the spiritual implications of our actions.

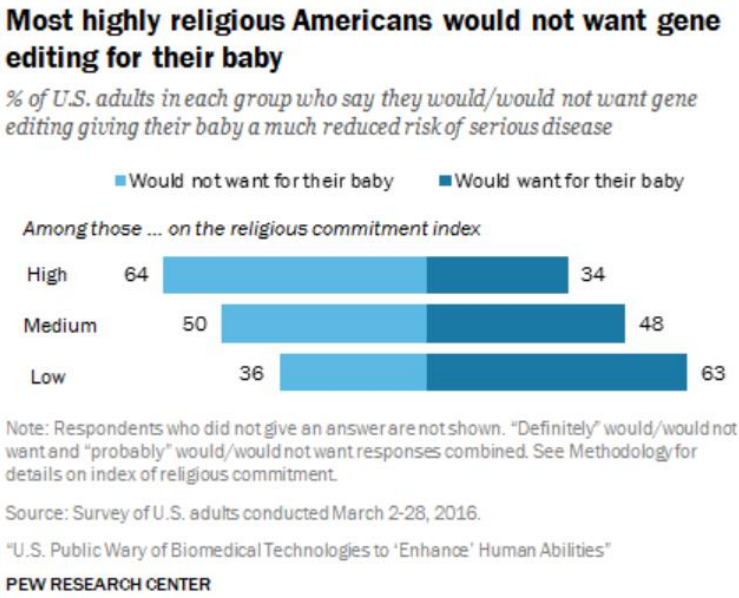

Figure 5. PEW Research Center dated from March 2-28, 2016, detailing the percentage of surveyed highly religious Americans that would genetically edit their baby.

6.2 Public Opinion Statistics: In order to determine what the overall public consensus was in regard to gene editing, the PEW research center surveyed multiple diverse groups of people, including Christians. They found that Americans with strong religious beliefs are more inclined to reject the use of gene-editing technology in their families compared to those

who are less religious. In addition, the majority of adults, especially those with high religious commitment, believe that using human embryos in the development of gene-editing techniques would decrease the acceptability of gene editing (Funk et al., 2016).

It can be concluded that religious affiliation plays a significant role in shaping attitudes towards gene editing, with considerable variation observed amongst different religious groups (Funk et al., 2016). Compared to other groups, White evangelical Protestants, who tend to exhibit greater religious devotion, are less inclined towards using gene editing to reduce the risk of certain serious diseases, with 61% of them indicating their opposition to it. Conversely, a majority of atheists (75%) and agnostics (67%) express their willingness to use gene editing for this purpose (Funk et al., 2016). Overall though, regardless of religious identity, Americans as a whole are confused about the effects about genetic editing in the near future. 68% of adults in the United States express some degree of worry when considering the potential implications of this technology, while around 49% feel strongly positive about it (Funk et al., 2016). Lastly, 30% of adults feel both enthusiastic and concerned, which demonstrates how complex of an issues gene editing truly is.

VII. CONCLUSION

In conclusion, CRISPR-Cas9 is a groundbreaking genetic editing technology that has revolutionized the approach towards treating genetic diseases. Its discovery in bacteria and subsequent adaptation for use in mammalian cells has opened up a world of opportunities to cure diseases caused by genetic mutations. The mechanism of CRISPR-Cas9, which utilizes a guide RNA to direct the Cas9 nuclease to specific genomic locations, provides a highly precise and efficient method of gene editing. Despite some concerns over its potential to

cause off-target effects and raise ethical debates, CRISPR-Cas9 remains a highly promising technology with a vast array of applications. From improving agriculture to treating genetic diseases, directly and epigenetically, CRISPR-Cas9 has the potential to make a huge impact on society. In the coming years, it is anticipated that CRISPR-Cas9 will continue to play a central role in the field of genetic research, as scientists work to fully unlock its capabilities and address any limitations.

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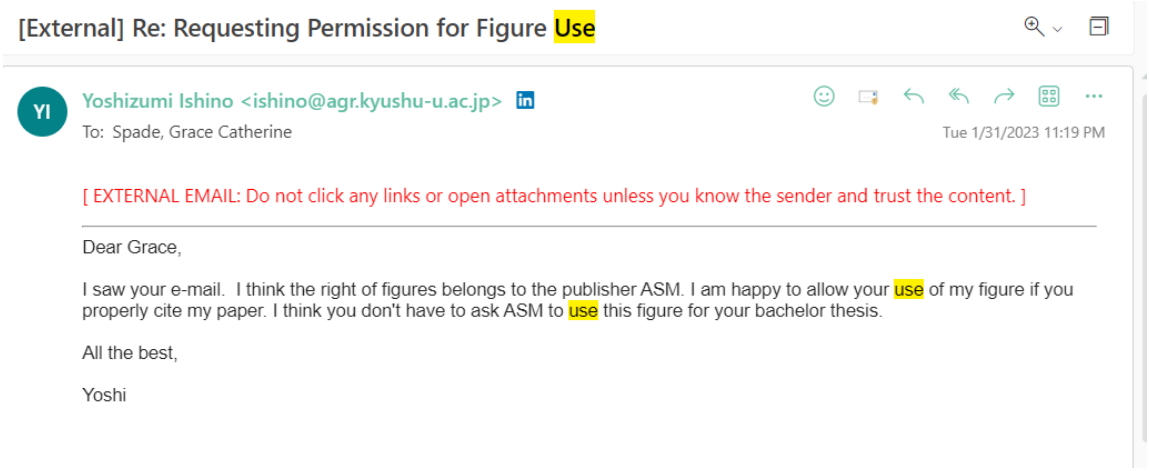

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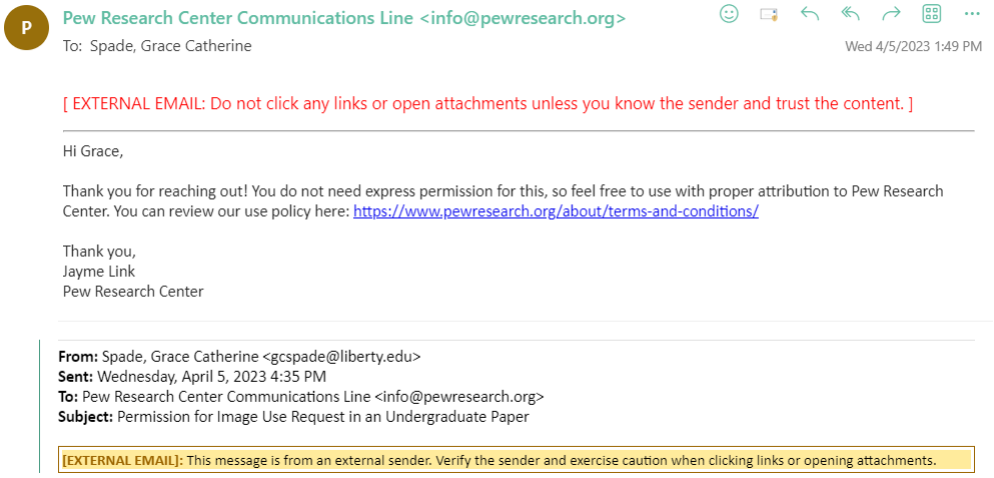

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