CRISPR, OMEGA, and Fanzor:
Mixed Blessings of Genome Editing Technology

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Abstract

A little more than a decade ago, CRISPR-Cas system was identified as a potential gene editing tool. This RNA-guided DNA cleavage system, which naturally provides immunity to the prokaryotic host cell, has been engineered since for its application in agriculture, genomic screening, and hereditary genetic disorder treatment and cures. Recently, a eukaryotic CRISPR-Cas-like system, Fanzor, was reported, under a new class of the RNA-guided system (termed OMEGA). The discovery has increased the potentials of genetic modification more than ever, while simultaneously increasing the need for ethical considerations and guidelines. The history, structure, and functions of the two RNA-guided systems, as well as their current and prospective applications, and the ethical and social issues surrounding genomic modification through specific case studies are discussed in this thesis.

CRISPR, OMEGA, and Fanzor:

Mixed Blessings of Genome Editing Technology

Since the early 1970's when the first DNA splicing and recombination was performed, there has been an ongoing research to engineer endonucleases for genome editing.[1] The main goal of developing gene editing tools is to design enzymes that can target any genomic site with high efficiency, high specificity, and little to no negative side effects.[2] Specifically, editing tools that are effective in eukaryotic cells and animal models of human diseases are desired.[2]

The genome editing tools derived from natural systems that the researchers knew of did not quite fulfill these goals.[2] However, with the discovery of the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR associated proteins (Cas), this changed. Specific engineering of the CRISPR-Cas system has significantly increased the potential of genome editing in the past decade.[1-3] The CRISPR-Cas system has been applied to agriculture, biotechnology, and medicine since then as a genome editing tool.[4,5] In 2021, another system of RNA-guided DNA endonucleases (termed OMEGA) was found in bacteria,[6] and in 2023, Fanzor (Fz), a family of eukaryotic CRISPR-Cas-like endonucleases was discovered.[7]

The effort for genome editing was initiated with the discovery of endogenous spontaneous homologous repair and the realization that this cellular DNA repair mechanism can be used to insert a sequence of desire into the host genome.[2] However, the insertion of DNA sequence using exogenous donor DNA template and spontaneous homologous repair showed low efficiency, as undesirable byproduct events occurred and the template sequences were often incorporated at off-target sites.[2] The introduction of meganucleases helped to reduce this issue.[2] Meganucleases were used to recognize, bind to, and induce double stranded breaks (DSBs) to specific DNA sequence and stimulate homology-directed repair (HDR).[1,2] Once DSBs were

created in the genome by the meganucleases, the cells would repair the break using a template strand.[1] Compared to spontaneous homologous repair, meganuclease-induced HDR had greater efficiency of inserting desired sequence at a target loci.[2] However, two issues remained. Known meganucleases did not target desired sequences most of the times, and non-homologous DNA end joining (NHEJ) occurred at cleavage sites more often than HDR.[1,2] NHEJ repairs the broken DNA strands without using a donor/template strand and creates random insertion or deletion (indel) mutations, making it less desirable for genome editing than HDR.[1,2]

By the 1980's and late 2000's, two new discoveries were made to help scientists overcome the first limitation of meganucleases.[1] Zinc finger proteins (ZFPs), which are proteins with repeating motifs in the zinc-binding domain, were found.[1] The motifs induce proteins to fold in finger-like shapes, which are then used to grip the DNA strand.[1] Because each ZFP recognizes three base pairs on the genome, it was found that multiple zinc fingers linked in a linear manner can be used to detect a target gene.[1] In 1990's, researchers tethered ZFPs to DNA endonucleases to create zinc finger nucleases (ZFNs), which would be used to recognize specific DNA sequences and induce cuts.[1] In 2009, a different DNA-binding motif in proteins from *Xanthomonas* was identified.[1] A protein called transcription activator-like effector (TALE) had these motifs that allowed them to bind and interact with certain locations on the genome.[1] Like ZFPs, TALEs were fused with nucleases to produce TALE nucleases (TALENs) to cut DNA.[1]

While ZFNs targeted DNA with greater specificity than meganucleases, and TALENs showed higher specificity, wider target range, and better cost-efficiency than ZFPs, limitations to these two models existed.[1] ZFN designing process was complicated, making it difficult to design new proteins for every new target site.[1,2] TALENs' relatively large sizes made it difficult to package them in vectors and thus limited their application, despite their specificity.[1]

Additionally, developing ZFNs and TALENs was time-consuming and expensive, which drove the search for a genomic editing tool that could be produced cost-effectively with similar specificity.[3]

CRISPR-CAS System

The History and Natural Function

The search for a better gene editing tool landed the researchers on CRISPR-Cas in 2012, when it was found that a single guide RNA (sgRNA) can be designed and hitched to Cas protein to target a sequence of desire.[1,2] The CRISPR array was initially discovered in 1987, when Ishino et al. noticed regularly interspaced, 32-nucleotides long palindromic repeat sequences in *Escherichia coli*.[1,5] The palindromic repeats were identical from one another while 35-41 base pairs long spacers in between them were the same as plasmid and phage genomes, suggesting that CRISPR-Cas served as an innate, adaptive immune system to hold a memory of past infection and pass it to the future generation.[4,5] In 2007, the researchers confirmed that the system was indeed involved in prokaryotic defense mechanism.[1] By 2012, researchers realized that CRISPR RNA (crRNA) and trans-active crRNA (tracrRNA) can be linked together as a sgRNA and that they could build a specific sgRNA to direct Cas proteins to bind to specific loci of the genome.[2]

As mentioned above, CRISPR-Cas system is an adaptive immune system naturally found in prokaryotes. It prevents reinfection from viral strains using the spacers integrated in the array, as represented by Figure 1.[8] When a prokaryote is infected for the first time, it will cut a short segment of DNA called a protospacer from the invading phage DNA and integrate the DNA to its genome.[1] The protospacer then becomes a spacer sequence in the CRISPR array, functioning as a memory of the invading genome.[1,8] When reinfected by the same phage, the host undergoes

biogenesis of crRNA, where a precursor crRNA is transcribed from the CRISPR array then further processed into mature crRNAs by RNase III.[1,4,8] At the same time, tracrRNA sequence located upstream of the CRISPR array is transcribed.[1,8] Together with the tracrRNA, crRNA forms a guide RNA (gRNA), which then forms a ribonucleoprotein complex with an associated Cas protein and lead the Cas protein to the target DNA containing sequence complementary to crRNA.[1,2,8] During the interference stage, the Cas protein detects the complementarity of the invading DNA via base pairing with crRNA and search for the protospacer adjacent motif (PAM), which is a short sequence following the protospacer, unique to the invading genome.[1,2] PAM recognition is required for Cas protein to identify the target DNA as non-self and induce DSB and thus functions as a checkpoint mechanism of CRISPR-Cas to prevent accidental cleavage of self-DNA.[1,2,8]

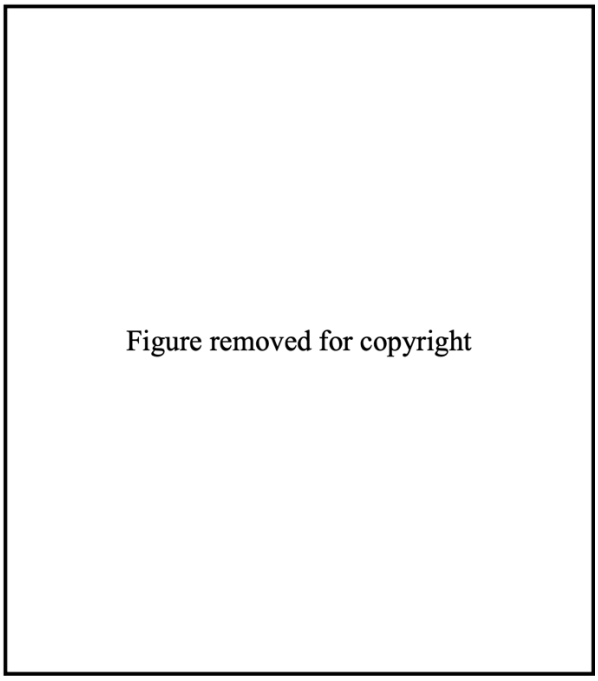

Figure 1. CRISPR-Cas is an adaptive defensive system naturally found in prokaryotes. Segments of invading genome is integrated into the CRISPR array during the initial infection. In subsequent viral infections, the host undergo biogenesis and interference stages to target specific genome. The figure was taken from an original work by Hillary et al.[5]

Cas12 Proteins

Different species of prokaryotes contain diverse Cas proteins, differing slightly in structure and mechanism of action. While both Cas9 and Cas12 will be mentioned throughout the thesis, focus will mainly be on Cas12 for discussion of structure and mechanism as OMEGA and Fanzor are believed to be relatives of Cas12. Cas9 proteins will be highlighted for discussion of application of CRISPR-Cas system as it is the most studied and applied Cas protein.

Cas proteins are largely divided into two classes and six subtypes, with class 1 Cas proteins containing types I, III, and IV and class 2 containing types II, V, and VI.[4,9] Cas9 is a type II Cas protein derived from *Streptococcus pyogenes*, which utilizes both crRNA and tracrRNA (or a specifically designed sgRNA if produced *in vivo*).[1,2] It is commonly used due to its advantage of good balance between PAM complexity and protein size compared to other orthologs.[2] Cas12a is a type V Cas protein and uses only the crRNA as its gRNA.[4] Cas12 protein is derived from *Prevotella* and *Francisella* and is mainly used as an alternative to Cas9 to target sites that Cas9 cannot act on.[5]

Cas12 is a bilobed protein which consists of a recognition (REC) lobe and a nuclease (NUC) lobe, with an arginine-rich bridge between the lobes.[4,5] The REC lobe contains a WED domain that interacts with PAM via H bonding and van der Waals interaction to stabilize the crRNA-DNA interaction.[9] The NUC lobe contains a single RuvC domain that targets both non-target and target DNA strands.[4] This differs from Cas9, which has an HNH domain and two RuvC domains for its nuclease activity.[4,5] Phylogenetic comparison of amino acid sequences between Cas12 and Cas9 subtypes suggests that Cas9 and Cas12 evolved independently from transposon-associated nuclease families.[4]

Cas12 mechanism of action is largely similar to that of Cas9. The gRNA binds to Cas12, inducing a conformational change to align the PAM-interacting amino acid residues and gRNA seed sequence in position.[5] Following this, Cas12 binds to target DNA by identifying T-rich PAM sequence on both target and non-target strands.[4,5] This differs from Cas9, which recognize G-rich PAM only on the non-target strand.[5,9] Once PAM sequence is identified, Cas12 stabilizes the hybridization of 17 base pairs between crRNA and target DNA, inducing a formation of an R loop.[9] The formation of the R loop then facilitates the cleavage of the target DNA, producing staggered DSB approximately 5 nucleotides distal to PAM sequence.[4,5,8] DNA cleavage by Cas12 occurs less frequently if there is a crRNA-target DNA mismatch during base pairing, which makes Cas12 advantageous over Cas9 as it binds to target DNA with greater specificity.[4,5]

CRISPR-Cas and Genome Modification

As mentioned earlier, the CRISPR-Cas system was discovered as researchers were looking for genome editing tools with greater efficacy and specificity. CRISPR-Cas system overcomes the limitations of earlier technologies, mainly due to size and simplicity of Cas proteins compared to ZFNs and TALENs. Cas proteins require a gRNA or a sgRNA, which are easier and cheaper to produce than complex molecules for ZFNs and TALENs.[1,2] Additionally, new sequences can be targeted simply by changing the sgRNA/gRNA sequences, rather than re-designing the entire protein complex.[8] Although requirement of PAM recognition for cleavage does limit the extent of applicability of CRISPR-Cas system, more nucleases with different PAM requirements are being discovered and specifically designed to overcome this challenge.[2] For example, researchers found that small mutations of 3-4 base changes can change PAM specificity of the nucleases.[2] Additionally, many researchers are studying to improve DNA specificity of gRNA and to decrease Cas activity and lifespan in cells to reduce a chance of off-

target activity after target site modification is completed.[2] For example, researchers engineered Cas9 to be activated specifically by light or exogenous small molecules, which not only improved its DNA specificity but also reduced off-target effects.[2]

With further experiments with CRISPR-Cas system, researchers discovered that Cas proteins can be deactivated and/or tethered to other effector proteins and produce epigenomic editing tools, base editors, and prime editors. Attachment of catalytically deficient/dead Cas9 (dCas9)-sgRNA complex, which lacks the endonuclease activity, to a transcriptional activator is used for epigenomic editing.[2,8] Here, specific sgRNA is used to direct the dCas9-transcriptional activator to the target gene and induce expression of the gene, which allows for gene activation.[2,8] On the other hand, by attaching transcriptional inhibitors to dCas9-sgRNA complex, researchers inhibit the expression of specific genes.[8,10] Base editing techniques also utilize CRISPR-Cas system. By tethering Cas9 proteins to deaminase proteins, Cas9 nickases, which induce a single strand nicks on DNA, are produced.[8,9] Single-stranded breaks on DNA greatly increases the efficacy of base substitution and reduces the adverse effects of DSBs (i.e., non-specific indel mutations at cleavage site).[8,9] However, the issue of narrow target window and nonspecific deamination on nontarget RNAs persists.[8] Lastly, prime editors are created by fusing Cas9 with inactivated HNH domain (nCas9) with a reverse transcriptase and modified prime editing guide RNA (pegRNA).[8] The prime editor can bind to a specific DNA strand, induce DSB, and synthesize new DNA strand at the cleavage site using pegRNA.[8] This technology can theoretically correct most genetic mutations with high target specificity, no restriction to editing window, and little to no off-target effects.[8] However, more *in vivo* studies and refinement of the technology are needed before clinical applications as *in vivo* tests and experiments on animal models showed varying efficiency.[8]

As fine-tuning the CRISPR-Cas-based genome editing tools is important, effective delivery of the tools to target cells is also crucial. Typically, the tools are delivered via viral vectors or nonviral vectors. Nonviral vectors include golden- and lipid-nanoparticles, which have limited efficiency but offer low immunogenicity and reduced off-target effects in hosts.[8] Viral vectors are used more commonly and involve transfecting plasmid DNA with coding regions for Cas proteins and gRNA *ex vivo* then injecting the plasmid to target cells using viral vectors.[2,8] Lentivirus (LV), adenovirus, and adeno-associated virus (AAV) are most commonly used for delivery, with specific vector chosen for different circumstances as each have unique advantages and disadvantages regarding cargo size, delivery efficiency, and elicitation of host's response.[8]

Although CRISPR-Cas system is not a perfect tool for genome editing, due to its advantages over other existing tools, it is used in various fields as discussed further later in the thesis. CRISPR-Cas system is used to produce crops that are infection-resistant and yield more high-quality fruits in order to battle famine and food shortages.[4,11] The technology is also applied in human genome screening. It helps to identify specific functions of genes in disease progression and discover genes that are synergetic or antagonistic with therapeutic drugs.[2,8] Furthermore, the system is used to produce new cell lines and animal models for diseases and develop curative treatment to hereditary diseases.[2,11] The field of application of genome editing has potentially been expanded even more with the discovery of OMEGA and Fanzor.

OMEGA System

OMEGA System and TnpB

The different structures and mechanism of action of Cas9 and Cas12 suggest independent evolutionary ancestors, as previously mentioned. More specifically, Cas9 is believed to have descended from IscB protein, a subset of IS200/IS605 transposon superfamily.[12] In-depth study

of the transposon superfamily in 2021 revealed another subset that encode for programmable transposon-encoded RNA-guided nucleases – the TnpB families.[12] The transposon-encoded RNA-guided nucleases are collectively termed obligate mobile element-guided activity (OMEGA), and study of their distribution suggest that RNA-guided mechanisms are more common than previously reported in prokaryotes.[12] Through additional phylogenic assessments, researchers suggested that the nuclease families evolved independently from each other and that TnpB is potential ancestor of Cas12 and Fanzor, a eukaryotic RNA-guided DNA nuclease.[12]

Researchers found several structural similarities between the OMEGA system and CRISPR-Cas system, implying that OMEGA system may have similar application to CRISPR-Cas in research. The nucleases of OMEGA are composed of the effector nuclease and ωRNA guide, just as CRISPR-Cas is composed of Cas effector nuclease and gRNA.[6,9] The effector proteins also contain an RuvC nuclease domain, which cleaves DNA once the proteins identify target adjacent motif (TAM) on the target genome.[6] ωRNA is a highly structured, noncoding RNA with hairpin structures and variable regions that assist the OMEGA effector nucleases with target recognition, like crRNA do for Cas proteins.[6,13]

As one of the IS200/IS605 transposable element families, TnpB is found in diverse groups of archaea and bacteria, with greater abundance than Cas12 in some prokaryotes.[6] Likely due to their genealogic association, TnpB shares structural similarities with Cas12 (Figure 2).[12] TnpB is a bilobed protein with REC and NUC lobes.[6] However, it only contains the functional units of Cas12, making the catalytic domains more compact.[6,9] TnpB gene is also found upstream of ωRNA, just as Cas12 gene is found upstream of the CRISPR array.[6] This contrasts with IscB and Cas9, which have ωRNA and CRISPR array upstream of IscB gene and Cas9 gene, respectively.[6]

Along with the structural resemblances, scientists found similar mechanism of action between the TnpB and Cas12. TnpB interacts with the nucleotides of TAM on target DNA, similar to how Cas12 recognizes PAM on invading DNA.[9] Additionally, the interaction between ωRNA and complementary target DNA sequence induces the formation of R-loop, resulting in DNA cleavage.[9] However, TnpB lacks an interaction with distal TAM region, making the interaction less stable and highly disordered compared to Cas12, which interacts with distal PAM region.[9] This then suggested to researchers that Cas12 acquiring additional domains over time allowed it to have more stable interaction with the target PAM region.[9]

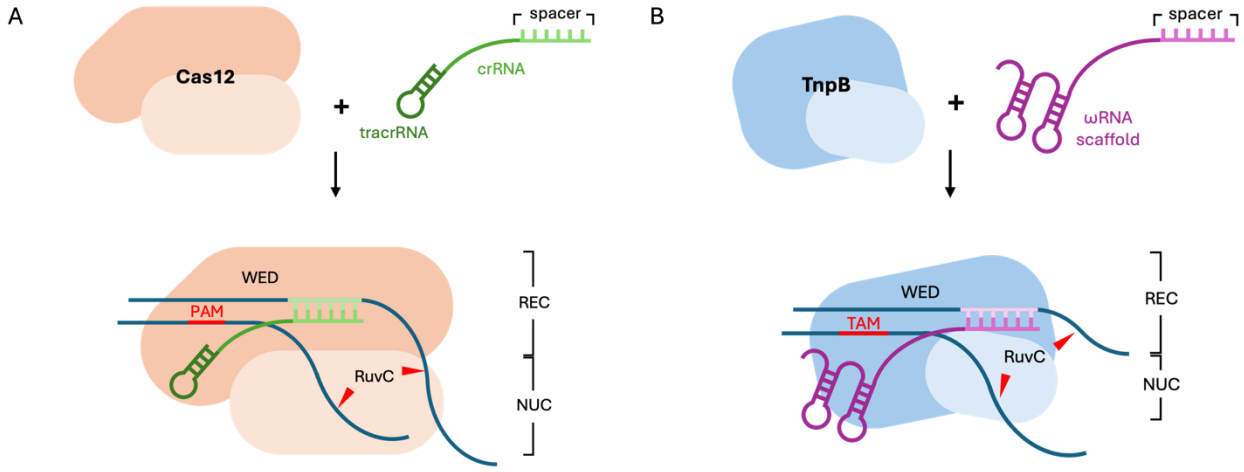

Figure 2. Cas12 and TnpB are similar in structure and mechanism of action. (**A**) Cas12 forms a ribonuclease complex with gRNA composed of tracrRNA and crRNA. The WED domain interacts with G-rich PAM and stabilizes the nuclease's interaction with the gRNA. A single RuvC domain produces staggered DSB. (**B**) TnpB forms a ribonuclease complex with the ωRNA scaffold. The WED domain interacts with T-rich TAM and ωRNA. Like Cas12, RuvC domain cleaves DNA to produce staggered DSB. TnpB contains functional units of Cas12, allowing it to be more compact in size. The figure was adapted from an original work by Yang and Patel with permission.[15]

Based on the mechanical analysis and study of associated molecules, researchers suggest that TnpB is associated with various families of transposases for its potential role in transposition

activity in prokaryotes.[6] For example, after a transposon was removed from the genome, TnpB that targets the site may initiate HDR and restore the site with transposon sequence, resulting in propagation of transposon.[6] However, despite this observation, the exact function of TnpB in prokaryotes is yet to be confirmed.[6]

Fanzor – the Eukaryotic CRISPR-Cas?

Discovery and Diversity

Related to TnpB of the OMEGA system is Fanzor, which was initially reported as a eukaryotic TnpB-IS200/IS605-like protein that regulated transposable element activity via methyltransferase activity.[7] However, further analysis of Fanzor indicated that it is a eukaryotic transposon-encoded protein that can be programmed to be used as RNA-guided DNA endonuclease for genome editing purposes.[7] Two different types of Fanzor proteins, Fz1 and Fz2, are found across the eukaryotic kingdom. Fz1 proteins exist in fungi, arthropods, protists, plants, and eukaryotic viruses, while Fz2 proteins are found more commonly in mollusks, choanoflagellates, viruses, and fungi.[9,13,14]

The phylogenic analysis of Fanzor show its similarities with TnpB, suggesting that Fanzor may be a eukaryotic version of the OMEGA system.[7] The two types of Fanzor proteins appear to have evolved from two different subtypes of TnpB.[7] The researchers proposed that symbiotic relationship between prokaryotes and eukaryotes allowed the OMEGA system to transfer horizontally from bacteria to eukaryotes.[7] Then, the eukaryotic virus spread the CRISPR-Cas-like OMEGA system amongst eukaryotes, and over time, TnpB proteins evolved to become Fanzor in eukaryotes (Figure 3A).[7]

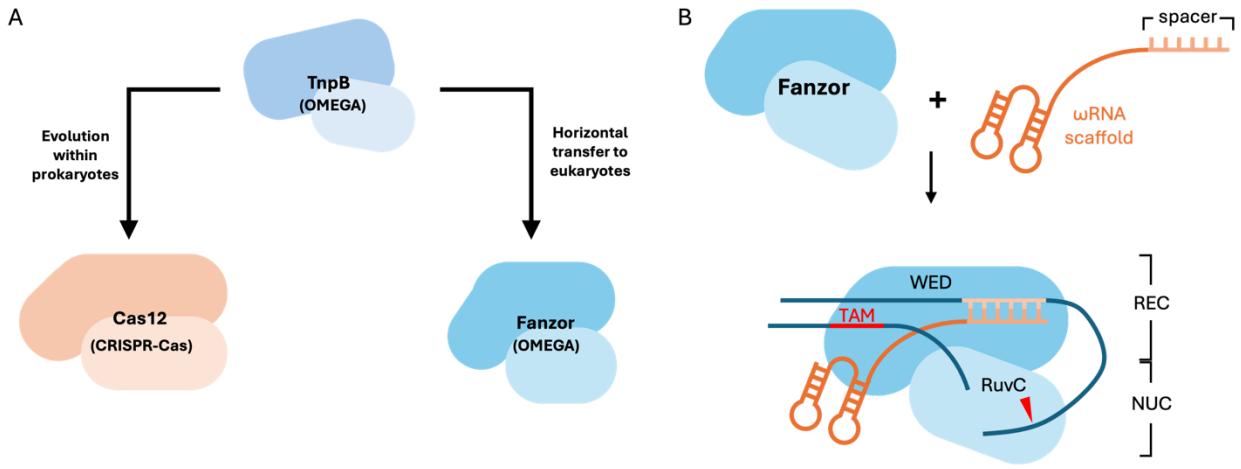

Figure 3. Fanzor is evolutionarily and structurally similar to TnpB and Cas12. (**A**) Symbiotic relationship allowed for horizontal transfer of TnpB from prokaryotes to eukaryotes, which evolved into Fanzor. Within prokaryotes, Cas12 likely evolved from TnpB over time. (**B**) Fanzor forms a ribonuclease complex with ωRNA. The WED domain identifies a diverse sequence of TAM. The conserved RuvC domain cleaves target DNA to produce staggered DSB. Panel B was adapted from an original work by Yang and Patel with permission.

Structure

For structural and functional characterizing of Fanzor and comparison with TnpB and Cas12, Saito et al. derived Fanzor orthologs from soil fungus, Percolozoa, and marine mollusk.[7] Like TnpB and Cas12, Fanzor is a bilobed protein with REC and NUC domains (Figure 3B).[7,9] At a molecular level, Fanzor further express similarity with TnpB, as its core domain is composed of a conserved endonuclease RuvC domain and a ωRNA-recognizing WED domain.[7,9,14] Conserved alternate glutamate, which plays a critical role in nuclease activity, is found in the RuvC domain.[14] It should also be noted however, insertions that were unique to each Fanzor orthologs were found within the core domain.[7]

Fanzor has other differences that sets it apart from TnpB and Cas12. For example, it lacks certain domains and features that are found in Cas12 system, though it is hypothesized that ωRNA scaffolds can functionally substitute for the missing regions.[7] Additionally, a pseudoknot structure on ωRNA, which is commonly found in TnpB and Cas12 were not identified in

Fanzor.[7] Another structural difference is the presence of three α helixes in the WED domain of Fanzor, which are thought to have an important role in recognizing target DNA.[7] Lastly, due to compact core domain and lack of certain structures, Fanzor is smaller in size when compared to TnpB and Cas12.[13]

Mechanism of Action and Function

Biochemical analysis of Fanzor show that Fanzor can perform DNA cleavage with a guide from ωRNA, making it an RNA-guided DNA endonuclease like TnpB and Cas12.[7] First, ωRNA is transcribed and interact with the RuvC domain of Fanzor to stabilize the effector for optimal activity.[9] Then, Fanzor identifies the target DNA that is complementary to its ωRNA and utilizes the WED domain to interact with TAM.[9] Once TAM on the target DNA is confirmed and ωRNA-target DNA complex is formed, Fanzor cleaves the target DNA, creating a staggered DSB.[7,13] However, it appears that Fanzor does not induce collateral non-specific cleavage on target DNAs as Cas12 does.[15]

Through Sanger sequencing of cleaved products, researchers revealed unique cleavage patterns of Fanzor orthologues, and suggested that each Fanzor protein from different species recognize unique TAM sequences.[7] Fanzor orthologs' TAM targets are wider in range and diverse while TnpB and Cas12 prefer T-rich TAMs.[7] Mutating any of the bases in TAM sequence blocked Fz1's ability to cleave the target DNA, indicating that recognition of specific TAM is vital in Fanzor's nuclease activity.[7]

As a newly identified protein family, Fanzor's exact function in nature is yet to be found.[7] However, co-occurrence of Fanzor with transposases suggest that Fanzor may have a role in facilitating transposon activity in eukaryotes, as does TnpB in prokaryotes.[7]

Significance of Fanzor

Although more is yet to be discovered about Fanzor, its discovery is an exciting news to the scientific community. The presence of Fanzor as an RNA-guided endonuclease in eukaryotes implies that a universal RNA-guided mechanism may exist in all kingdoms of life.[7] Additionally, the discovery of RNA-guided endonuclease system with a eukaryotic origin is important as its use in genome editing can help overcome some of the current limitations of CRISPR-Cas (i.e., it may reduce immunogenic reactions in human bodies).[14]

Experiments that have been conducted so far also highlight the potential of Fanzor as human genome editing tool. Using four different Fz1 orthologues and eight guide sequences, eight different loci on human genome were targeted.[7] Three of the four orthologues showed editing efficiency that was comparable to AsCas12f1, a subtype of Cas12 protein.[7] Of these three orthologues, *Spizellomyces punctatus* derived Fz1 (SpuFz1) was utilized to determine how Fanzor's efficacy could be increased. The experimental data showed that when compared to wildtype SpuFz1 and wildtype ωRNA, extending ωRNA scaffold improved the nuclease activity and mutating amino acid residues on ωRNA increased the binding strength between ωRNA on SpuFz1 and target human DNA.[7] Thus, Fanzor has a potential to be modified and tailored for its specific use in eukaryotic genome editing, especially in conjunction with germline genome therapies and disease treatment like CRISPR.[7,13]

Lastly, other characteristics of Fanzor also provide promising applicability of Fanzor in gene editing. Fanzor is relatively smaller in size than Cas12 or TnpB, which provides for an easier delivery as it can be better loaded into vectors.[9,14] Additionally, Fanzor creates a sticky-end DSB like TnpB and Cas12, which is advantageous over blunt-end DSB when inducing HDR

of DNA.[9,13] Furthermore, depending on the host Fanzor is derived from, there is a wide range of TAMs to select from and a broad temperature range (4-70 °C) at which Fanzor can function in.[13]

Research and Application of CRISPR-Cas

As previously discussed, Cas9 is the most analyzed out of all known Cas types and thus most applied in research. Additionally, as OMEGA and Fanzor are relatively new topics, their *in vivo* and clinical applications are yet to be studied. Thus, the thesis will focus specifically on CRISPR-Cas9 system's application in genome editing.

Ever since the realization that specific gRNAs can be designed to target and edit specific genes, CRISPR-Cas system has been utilized in many agricultural, biochemical, and medical research. It has been heavily utilized in medical research to develop animal models, identify gene function, study effects of loss- or gain-of-function of genes, correct genetic disorders, and to treat infectious diseases.[4,5]

CRISPR-Cas system come in handy with genome wide screening. By genetically modifying isolated pluripotent stem cells then growing them with unmodified cells, researchers are able to study the impact of modification on phenotype expression and gene function.[2] The system is also applied in study of cancer related gene functions, as genes that are involved in progression of cancer, drug resistance, and evasion of immune cell-mediated killing can be studied with the technology.[2] CRISPR-Cas provided great advancement to cancer screening as it can help identify genome-specific vulnerabilities so that certain genes can be knocked out in hopes of decreasing the viability of cancer cells.[2] Study of tumor cells' mechanism of evasion of immune system using CRISPR-Cas also help locate genetic deviations in tumor cells that allow for resistance to immune cell killing, while screening genes that are synergetic or antagonistic with certain therapeutic drugs aid with analyzing efficacy of drugs during clinical trials.[2,8]

Lastly, a significant yet perhaps most controversial application of CRISPR-Cas is genome editing for treating and/or curing diseases. Starting with the first application of CRISPR-Cas in clinical trials in 2016, there have been ongoing studies to apply the technology in many disease treatments.[8] New cell and animal models for certain diseases were produced using CRISPR-Cas, leading to initiation of *in vivo* and clinical trials with genome editing technology for treating diseases like sickle cell disease (SCD) and Duchenne muscular dystrophy (DMD).[2,8]

Sickle Cell Disease

Disease Background

Sickle cell disease is one of the most common monogenic blood disorder in African and Hispanic descents.[16-18] The sickled red blood cells (RBCs) have shortened lifespan and can cause chronic hemolysis and hemolytic anemia.[17] Additionally, patients are exposed to greater risk of progressive organ damages, including cardiovascular, renal, and eye complications, as well as pain from vaso-occlusive crises (VOCs) and early mortality.[16-18]

Soon after birth, the human body produces adult hemoglobin (HbA) to replace fetal hemoglobin (HbF) in the RBCs.[17] However, when a single point mutation exists in *HBB* gene, which encodes for the β chain of HbA, sickle cell hemoglobin (HbS) is produced.[17,18] The HbS eventually polymerizes to induce sickling of red blood cells and cause various symptoms of SCD.[17] When a different mutation occurs to *HBB,* hereditary persistence of fetal hemoglobin (HPFH), a condition in which HbF is produced at abnormally high concentration in adulthood, occurs.[17] It was observed that SCD patients with HPFH typically experience milder symptoms due to a correlation between an increased level of HbF and reduced morbidity and mortality.[17] Thus, upregulating HbF by silencing *HBG* repressors such as BCL11A or mimicking mutations to induce HPFH in SCD patients became one of the therapeutic strategies.[17,18]

Treating SCD

The current standard cares for SCD aim to either prevent acute and chronic complications or to manage symptoms.[16,18] There are four drugs approved by the U.S. Food and Drug Administration to help alleviate symptoms, and hematopoietic stem cell transplant and insertion of anti-sickling *HVV* via LV are offered as a means for curative therapy.[16-18] However, because the drugs only alleviate symptoms and the treatments have complications and risks, researchers turned their eyes to CRISPR-Cas based genome editing technology.

The first strategy in treatment development was to disrupt the promotor region of the *HBG* gene, which encodes for γ globin.[17,18] In HPFH patients, mutations to *HBG* promotor region – the binding site of BCL11A – are commonly found.[17,18] Inducing the mutations that naturally exist in HPFH patients to SCD patient-derived cell lines led to an increase in HbF production to a level sufficient to reverse sickling.[17,18] However, the procedure was only done *in vivo* and clinical trials of engrafting the treated cells into SCD population is still needed to determine if the strategy will produce desired clinical effects.[17,18]

The second strategy was to disturb BCL11A gene.[17,18] While other non-hematopoietic roles exists, BCL11A specifically functions to represses HbF expression after birth in hematopoietic pathways.[17] In theory, CTX001 therapy would only target the BCL11A enhancer region on *HBG,* cause reduced BCL11A activity in erythroid cells, and lead to an increase in HbF production while maintaining the nonhematopoietic functions of BCL11A.[17,18] Data from phase II/III clinical trials showed that those who were treated with CTX001 therapy were no longer dependent on transfusion, became free of severe VOC, and experienced improved quality of life.[16,18] Additionally, the level of HbF increased from 0.1% to 43.2%, which is considered a threshold for curative effects, while HbS concentration decreased from 74.1% to 52.3%.[16,18]

Based on these promising results from phase II/III clinical trials, U.S. FDA announced the use of Casgevy therapy (also known as exagamglogene autotemcel (exa-cel). Previously called CTX001) by Vertex Pharmaceuticals and CRISPR Therapeutics as a treatment for SCD patients in December of 2023.[19] This is a major advancement in medicine as this is the first FDA-approved gene therapy utilizing CRISPR-Cas technology.[19] The therapy is designed to first harvest patient's bone marrow stem cells then disrupt BCL11A enhancer and induce HbF production for production of RBCs with normal shapes.[19,20] The genetically modified cells would then be engrafted into patient's bone marrow and allowed to repopulate and fill the bone marrow.[19]

The FDA does warn about the side effects of the treatment, including but not limited to, low levels of platelets and white blood cells, musculoskeletal and abdominal pain, fever, and headache.[19] The FDA and other researchers also point out that because the patients from clinical trials have been followed for only a short amount of time, the long-term effects of the CRISPR-Cas treatment are not fully determined.[16-19] Researchers predict that if the CRISPR-Cas system remains active in cells and start targeting other genomic loci, it can lead to disruption of gene function and genomic instability.[17] Additionally, if large indel mutations that were rarely observed in mice models and *in vivo* experiments occur in patients, it can also lead to detrimental effects as mutation to hematopoietic stem cells can lead to malignancies in the blood cell line.[17,18] Regardless, the announcement of the FDA's approval of Casgevy is an exciting news to patients with other hereditary diseases, at it signals for the beginning of permanent curative treatments to free them from their diseases.

Duchenne Muscular Dystrophy

Disease Background

Just like the SCD, Duchenne muscular dystrophy is a hereditary disease for which CRISPR-Cas based gene therapies are being developed. DMD is an X-linked recessive disease that causes muscle weakness, loss of ambulation, cardiac or respiratory complications, and possibly early death.[10,21] The *DMD* gene encodes for dystrophin, a cytoskeletal protein that helps to connect the cytoskeleton of muscle fibers with extracellular matrix.[10,21] With 79 exons, *DMD* is the largest gene in the human genome and thus, exposed to an increased risk of mutations compared to other genes in the cell.[21] For example, 60% of mutations in DMD patients arise from large deletions, mainly of exons 45-55.[10] The deletions cause shift in reading frame, producing truncated proteins, malfunctioning proteins, or no protein at all.[10]

The current therapeutic approaches include administrating corticosteroids and employing physical therapy.[21] Molecular treatments approved by the FDA include Eteplirsen, which restores the expression of partially functional dystrophin and reduces symptoms.[10] Of the three other FDA-approved drugs are golodirsen and viltolarsen, which are intended to increase protein expression and bring transcription levels up from 2.8% to 42.4% of normal dystrophin level.[10,21]

Treating DMD

CRISPR-Cas based treatments of DMD intend to target mutations on *DMD* and produce a permanent curative effect to free the patients from the causative mutation rather than mitigating the symptoms only. As there are various mutations to *DMD*, different clinical trials develop treatments with different strategies. One of the many strategies involves reframing the axons.[10] In a DMD mouse model, an indel mutation was produced via NHEJ after creating a cut on *DMD* upstream of a premature stop codon with CRISPR-Cas.[10] This led to a reading frame shift with

up to 8% frequency, producing dystrophin in up to 39% of the muscle fibers.[10] The increase in dystrophin level translated to improved muscle strength and showed the potential of CRISPR in correcting DMD open reading frame.[10]

The second therapeutic approach involves deleting exons 44 and 45.[10,21] An *in vivo* deletion experiment on human myoblast from DMD patient restored dystrophin expression in cells.[10,21] In another experiment using a mouse model, when stem cells with exon deletions were injected, not only did dystrophin expression increase but also proper colocalization of dystrophin-associated transmembrane proteins and sarcolemma occurred.[10] Through other exon deletion experiments, the researchers found that editing efficiency is size-dependent – the fewer the exons are deleted by CRISPR-Cas, the less efficient the intervention become.[21]

HDR-mediated gene correction of certain exons also showed success in mouse models.[10] Using CRISPR-Cas-induced HDR and exogenous template DNA for exon 53, up to 0.1% of total genome was corrected.[10] This led to the expression of full-length dystrophin in up to 8.4% of wild type mouse muscles.[10] Editing exon 23 with Cas9 showed correction rates of up to 41% while Cas12 showed correction rates of up to 50%.[10] Larger animal models, such as golden retrievers, showed dystrophin expression restoration to 6-16% of normal levels after correction of exon 7, exhibiting the potential of CRISPR-Cas-and-HDR-mediated gene correction as a therapeutic approach for DMD.[10]

The last therapeutic approach for DMD discussed in the thesis is base editing. Using a Cas9 tethered to a deaminase, an attempt to convert a premature stop codon (TAG) in exon 20 to a glutamine (CAG) was made on a mouse model.[10] The conversion was achieved with a frequency of 3.3% in tibialis anterior muscles.[10] As a result, dystrophin expression increased to up to 17% of normal level, exhibiting the potential of base editors in gene therapy.[10] As 25-35%

of DMD patients have point mutations in *DMD*, many DMD patients may benefit if the base editors are engineered to have greater specificity and efficacy.[21]

Despite the potentials, the CRISPR-Cas technology is not perfect, and limitations to applying the technology for DMD treatment remains. For example, unwanted indel mutation or base editing in non-target DNA are always possible.[10] Additionally, AAV is frequently used to deliver the CRISPR-Cas system and template DNA to the target cells due to its advantages.[8] However, its prolonged use can lead to neural toxicity and cellular immune response, especially if the patient was exposed to the virus prior to receiving CRISPR-Cas based treatment.[8,10] Challenges to HDR-mediated gene therapy also exists: it is restricted to certain phases of cell cycle and occurs at a lower frequency than NHEJ in mammals and thus yields low efficiency.[10]

CRISPR-Cas based treatments for DMD are yet to be approved by governmental authorities. However, other non-CRISPR-Cas-based gene therapies are available,[10] and ongoing research with CRISPR-Cas based technologies suggest that there is a potential for their application in clinical settings when more effective mechanisms and additional studies are done.

The Ethics and Perspectives Surrounding Genome Editing

Ethics and Religion Meet Genome Modification

With the advancement of the technology, questions regarding the boundaries of the application of genome editing technology are brought to light. To what extent can gene editing be utilized, and who establishes the guideline? It seemed that the technology was limited to somatic cell lines until a Chinese researcher He Jiankui announced the birth of CRISPR-edited twins in 2018.[1] This was a groundbreaking announcement not only to the scientific and medical population, but also to the general public. The event various concerns over the ethics of genome editing in humans throughout the various communities.

The role of ethics is to determine the human nature and create a basis for justification or discreditation of scientific advancements.[3] Bioethics exists to guarantee human rights are preserved in research and medicine and to give guidelines and principles to ensure human lives are respected in research.[11] Therefore, regarding genomic modification, there are debates over whether disruptions to genome should be morally forbidden as it will change the very essence of the human nature, and whether genome editing will pre-determine human lives, which will then restrict humanity's freedom of choice in one way or another.[3] Can human dignity be violated with genome editing if it is to help humans achieve the best versions of themselves?[3] The answer to such questions depends on factors such as one's philosophical and theological views, as well as socioeconomical and cultural backgrounds.[3,22]

Theological Perspectives

Religion and spirituality influence one's health, as well as his or her decision making regarding therapeutic interventions.[22] Positive influences include inhibition of specific health risk behaviors, promotion of practices associated with improved health, and reduced stress, while negative influences include discouragement of seeking professionals' help and provocation of guilt, anxiety, or shame due to religious doctrines.[22]

Individuals will have varying degrees of opinion, but studies on major religions like Islam, Judaism, and Christianity, report that at large, religious communities approve the use of genome editing tools as long as it is done for a medical purpose and does not alter the "image of God".[3] Muslims are cautious towards the use of genome editing tools, allowing them for medical purposes only when the safety and efficacy issues have been resolved.[3] Jews and Christians, who view humans as images of God (New International Version, Gen 1:26-27; Gen 2:7),[23] believe the technology should be used more carefully.[3] Jews who favor genome editing claim that use of the

technology is acceptable for medical purposes as it can save lives as the Torah instructs.[3] Christians who favor the technology claim that its application should be encouraged because humanity's advancement of technology reflects God's inherent characteristic of creativity, as long as it is done ethically and with respect to God's creation.[3]

In-depth Review of Christian View

What would be considered "playing God" from Christian perspective? Life is a God-given gift, and thus, manipulation of genome to influence life and death, as well as mankind's future evolution can be considered "playing God".[22] Genes do determine phenotypes, but humans are more than just a dictation by genetics, as God breathed life into humans (Gen 2:7)[23] and gave the ability to think, reflect on actions, feel emotions, and love one another.[22]

Christians who are in favor of genome editing argue that using the technology is using talents given to humanity for wellbeing of others, as good stewards of God (1 Cor 16:14; 1 Pet 4:10)[23] should do.[22] Somatic gene therapy is accepted under this view, because with gene editing, symptoms can be mitigated and the pain and suffering of patients can be reduced, making humans compassionate and loving toward each other.[22]

However, Christians who oppose genome editing also have theological reasonings. Genome modification tools are recreation of the original sin (Gen 3)[23] – it is the temptation to the humanity to become God-like or to have God-like power.[22] If life is a gift of God, and if children are gift of God (Ps 127:3),[23] how can the value of human life, especially of children's lives, be respected and dignity protected, if gene editing has a potential to be abused to alter the embryo, create superhumans, and influence the events in lives of unborn lives?[22]

Thus, Christians need to be able to differentiate the line between therapeutic interventions and experimental interventions. Not only that, but one must also discern between using the

technology developed with the knowledge God has granted to take care of the creation and using it to abuse the power that is given in the hands of the humanity.

Varying Views of the U.S. Public

As the U.S. is populated by people from various cultural, religious, and socioeconomic values, two surveys published by Pew Research Center reflect the varying views of Americans regarding the use of gene editing.

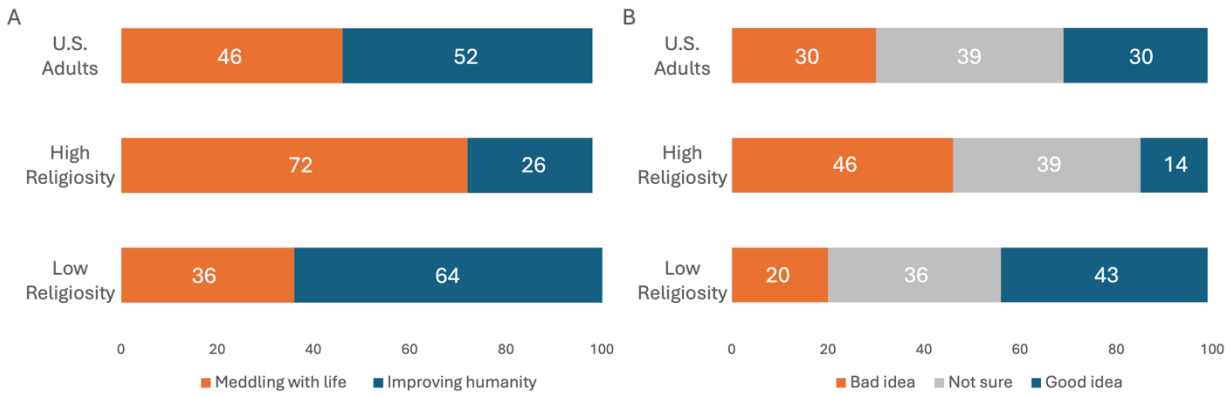

Figure 4. The survey from 2021 Pew Research Center reflects closely divided opinions of the U.S. public regarding germline genome editing. (**A**) Just over a half of the respondents believe that the use of gene editing tools to reduce baby's risk of developing disease can be seen as an equivalence to improving ourselves. However, those who are highly religious are more likely to view this action as meddling with the human nature than those who have low religiosity. (**B**) The responds were near equal in proportion to the question of impact of germline editing to reduce a baby's risk of developing diseases to the society. When religiosity was taken into consideration, those who were highly religious were more likely to believe that germline editing would be a bad idea for the society than those with lower religiosity. The figure was adapted from Pew Research Center with permission. "Americans are closely divided over editing a baby's genes to reduce serious health risk." Pew Research Center, Washington, D.C., (2022) https://www.pewresearch.org/internet/2022/03/17/americans-are-closely-divided-over-editing-a-babys-genes-to-reduce-serious-health-risk/

When asked whether genome editing on babies is simply an alternative way to better ourselves or is meddling with the nature of human life, 46% of responders viewed it the same as improving ourselves while 52% viewed it as crossing the limit (Figure 4A).[24] Those who were more religious were more inclined to respond that the use of technology was meddling with

human nature (72%) than to respond that it was a way to improve humanity (26%).[24] On the other hand, those who reported low religiosity were more likely to respond that it was to aid humanity reach its full potential (64%), rather than crossing the moral boundary (36%).[24] Regarding the use of gene editing tools under a condition that it did not affect the future generations, the percentage of people who though the use of the technology is acceptable increased from 17% in 2016 to 48% in 2021.[24,25] As if to reflect this increase, in the 2021 survey, 71% of responders were in favor of the using the technology to treat a disease in a person who is already born.[24]

Regarding the impact germline editing to reduce a baby's risk of developing disease to the society, the opinion of the U.S. public was almost evenly divided (Figure 4B). Thirty percent of the respondents believed it was a bad idea while an equal amount of individual believed that it was a good idea for the society.[24] The remaining 39% were unsure of whether the edit on germ cells was a good idea or bad idea for the society.[24] When accounting for religion, compared to less religious individuals, those who are highly religious were more likely to view it as a negative action rather than a positive action for the society.[24]

When the participants were asked for their opinion on the outcomes of gene editing, many believed that both positive and negative impacts would happen.[24] However, responders believed that negative impacts, such as abusing the technology in morally unacceptable ways, were more likely to occur than the positive effects.[24] Perhaps due to the belief that the technology can be abused, the majority of the participants responded that they believe gene editing techniques should be tested against higher standard than other medical treatments.[24]

He Jiankui and CRISPR-Babies

The Experiment and The Wave of Ethical Issues

In 2018, He Jiankui announced the birth of genome-edited twins – Nana and Lulu.[1] Using CRISPR-Cas technology in conjunction with the stem cell study, He claimed that he designed the babies to be HIV-resistant by altering the *CCR5* gene.[1] Later, it was quietly announced by the Chinese government that that He was sent to prison for not two, but three genome-edited babies.[26] The information regarding the third baby was not reported – its gender, the altered gene, and any health complications remain hidden.[26]

Despite He's success in creating Nana and Lulu – the designer twins – and the unnamed child, the event threw the world in shock due to many ethical issues tied to this study, few of which will be listed in the thesis. Firstly, He did not test the effects of genetic changes in other cultured cell lines before implanting in the embryos.[1] Thus, He was not fully aware of what the genomic changes would bring to the babies prior to implantation.[1] Secondly, two different modifications were made to the twins,[1] which makes it difficult to be viewed as a "therapeutic treatment" but rather more like an "experimental research". Nana received a frameshift mutation on both alleles, while Lulu received a deletion mutation on one allele.[1] The differences in treatments have a risk of producing different – and potentially unwanted – responses to HIV, which contradicts He's claim of creating "HIV-resistant" babies. Thirdly, the study made it appear that it is ethical to treat one disease by increasing the chance of another.[1] Mutations to *CCR5* may increase resistance to HIV, but they are also associated with greater risk for severe symptoms to West Nile virus, influenza A, and encephalitis.[1,27] Would it be ethical and moral to have individuals experience severe reactions to other diseases in light of treating one disease? If so, who and what differentiates which disease is considered "critical"? Lastly, during the

investigation, the Chinese court discovered that He had kept the experiment a secret from his institution, forged documents regarding ethical reviews while recruiting participants, and led doctors to unknowingly implant the edited embryos.[27,28]

The study not only raised these philosophical ethical issues but also socioeconomic issues. Companies that develop treatments may fight over the patent right or may refuse to reduce the cost of treatment, leading to a situation where the treatment is not readily available for everyone at an affordable cost.[11] In this case, is it morally justifiable for people to be restricted from treatment because of their financial limitations? If insurance can cover the cost of treatment, who decides the extent of the aid? Will all CRISPR-Cas based disease treatments be covered by the insurance, or will the insurance companies decide which diseases will be covered and which will not? Additionally, the selection process of participants for the study, which should prioritize the participants' safety and health, can face ethical challenges. Some may consent to gene editing clinical trials because they see it as their last hope, which puts them at a vulnerable position for possible exploitation, especially for disadvantaged women.[1]

Since the Scandal

Prior to He's announcement, there were some levels of regulations for germline genome editing study established by certain countries and major research organizations.[29] However, with He's study, governments and organizations recognized the limitations of the existing guidelines and began to enhance the regulations. For example, in China, updates between 2019 and 2021 included the following: obligation of researchers to inform the participants of alternative drugs or treatments to gene editing, requirement to provide means for participants to contact the ethics review committee, addition of civil penalties for going beyond the ethical norm of gene editing, and permission of genome modification of human germline cells for clinical purposes only.[29,30]

The guidelines set by the Chinese government still have limits. For example, the new regulations do not apply to the private sector.[29] With how easy genome editing tools are to access and use, it raises concern as it will be difficult to restrict individuals and have their work regulated.[30] In fact, He set up a nonprofit research center to work on hereditary diseases with CRISPR-Cas after being released.[29] Thus, as rules and laws regarding genome are being re-established and enhanced, government authorities and research organizations need to be aware of the possible loopholes and prevent unethical and immoral uses of genome editing technology.

Conclusion

In a timespan of twelve years, significant advancements have been made to biotechnology and medicine due to the discovery of a genome editing tool called CRISPR-Cas. Its appearance has radically changed the direction of therapeutic interventions of various diseases – curative interventions that were previously deemed impossible became a reality. Identification of TnpB and Fanzor of the OMEGA system as novel families of programmable RNA-guided DNA nucleases in 2023 added to the diversity of genome editing technology. Specifically, Fanzor, which has a eukaryotic origin, promises a wide range of applicability in clinical trials of human disease treatment with further research. However, these great advancements come with greater responsibilities. As reminded through the birth of three genetically modified children, the scientific, medical, and religious communities, as well as the general public need to be alert to ethical and social issues surrounding genomic modification and take action to use the technology ethically and morally.

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Appendix – Abbreviations and Terminology

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas	CRISPR-associated protein
Fz	Fanzor
DSB	Double stranded break
HDR	Homology-directed repair
NHEJ	Non-homologous DNA end joining
Indel	Insert or deletion
ZFP	Zinc finger protein
Bp	Base pair
ZFN	Zinc finger nuclease
sgRNA	Single guide RNA
trans-active RNA	TracrRNA
PAM	Protospacer adjacent motif
REC	Recognition (lobe)
NUC	Nuclease (lobe)
WED	PAM/TAM-interacting domain of REC lobe
dCas9	Dead Cas9; catalytically deficient Cas9
nCas9	HNH domain inactivated Cas9
pegRNA	Prime editing guide RNA
LV	Lentivirus
AAV	Adeno-associated virus
OMEGA	Obligate mobile element-guided activity
TAM	Target adjacent motif
SpuFz1	*Spizellomyces punctatus* derived Fanzor
SCD	Sickle cell disease
DMD	Duchenne muscular dystrophy
RBC	Red blood cells
Hb	Hemoglobin
HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HbS	Sickle cell hemoglobin
HPFH	Hereditary persistence of fetal hemoglobin
FDA	(United States) Food and Drug Administration
HSPC	Hematopoietic stem and progenitor cells