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The (Ab)uses of CRISPR-Mediated Human Genome Editing

Brooke Mullen
Assumption College

THE (AB)USES OF CRISPR-MEDIATED HUMAN GENOME EDITING

Brooke Mullen

Faculty Supervisor: David Crowley, Ph.D.

Department of Natural Sciences

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Prologue

If you could change your DNA, would you?

The development of new biotechnology tools renders this feat possible. The future is here: we wield the power of changing the human genome, forever.

DNA carries the genetic information that encodes the sequence of amino acids in proteins, which carry out the essential functions of life. Therefore, genes, in large part, dictate the development and function of organisms.

Changes in the genetic code, called mutations, can occur during DNA replication or as the result of exposure to environmental conditions such as smoking and ultraviolet radiation emitted by the sun. These alterations disrupt the gene's instruction, often resulting in the expression of shortened, or likely nonfunctional, proteins. Of course, not all mutations are 'bad.' Some can have no effect and, on occasion, can be beneficial to an organism.

Mutations are the original source of genetic variation as they introduce new alleles into a population. Natural selection then acts on that variation, increasing the frequency of 'beneficial' alleles and decreasing the frequency of 'detrimental' traits in the population over time. Evolution, however, does not progress toward a 'perfect' population. Natural selection works by selecting the most fit organisms, but harmful alleles persist in a gene pool to contribute to genetic diversity. Therefore, mutations of all sorts can and are passed on from one generation to the next. Hence, "deleterious genes are much more widespread throughout the population than is apparent,

so that every individual carries a … burden of deleterious genes" [1]. In fact, individuals are estimated to carry "on the order of 1,000 deleterious mutations" in their genomes[2].

Many deleterious genes give rise to disease. In fact, a mutation of a single gene can cause over 10,000 human disorders, including, but not limited to, cystic fibrosis, sickle cell disease, muscular dystrophy, red-green color blindness, and Huntington disease[3]. Such mutations, however, can now be *precisely* revised, removed, or replaced using gene-editing technologies. Most recently, CRISPR-Cas9 has been used to accurately repair disease-causing mutations and thus prevent the inheritance of genetic diseases. Many conditions, however, are nonlife-threatening and do not affect an individual's quality of life. Should they be eradicated, too? What about treatable diseases? In addition to modifying existing genes, scientists can insert novel ones. Should scientists be able to introduce enhanced genes (i.e., select for favorable traits) such as a specific eye color, athleticism, intelligence, etc.? Where is the line drawn?

Life-changing scientific discoveries are being made every day but, as with many new discoveries, comes the risk of harmful use. With each scientific breakthrough, scientists and nonscientists should come together to discuss the current state of research, expected future developments and their possible ramifications on society.

CRISPR/Cas9 and Targeted Genome Editing: Discovery, Mechanism, and Applications

In 1987, Yoshizumi Ishino, a Japanese molecular biologist, wanted to learn more about phosphate metabolism in *Escherichia coli*. To do so, he sequenced the DNA fragment containing the *iap* gene, which encodes for a metabolic protein[4]. During genetic analysis, he stumbled upon something unusual, beginning downstream of the *iap* gene: well-organized, repetitive DNA sequences interspaced by short stretches (~20 nucleotides long) of non-repetitive sequences[4]. At the time, scientists did not have sufficient DNA sequencing data at their disposal, so the function of these never-before-seen repetitive sequences remained a mystery.

Unexpectedly, similar repeated sequences were discovered in the archaeon *Haloferax mediterranei* by Francisco Mojica, a Spanish microbiologist, during his doctoral thesis research in 1992[5]. Comparison of its genome sequence with those discovered by Ishino in *E. coli* revealed that they had no similarity[6]. However, the presence of these conserved clusters of repeated sequences in two different domains suggested that they may serve an important function in prokaryotes.

With the function of the clustered DNA repeats still unknown, Mojica shifted his attention to the spacer regions between the repeat sequences. He used BLAST, the basic local alignment search tool, to scan for homologous nucleotide sequences, for similar sequences have the same or similar function[6]. All spacers subjected to analysis matched sequences in bacteriophages, viruses that infect and replicate within bacteria[6]. This finding led Mojica to hypothesize a connection between repeated sequencing content and acquired immunity.

In 2001, Mojica and his colleague Ruud Jansen jointly coined a name for this family of repetitive DNA sequences: "clustered regularly interspaced short palindromic repeats" — CRISPR for short. Jansen then went on to compare the genomic content of CRISPR regions in various archaea and bacteria[7]. In doing so, he observed a set of conserved homologous genes adjacent to the CRISPR regions, indicating that the two have a functional relationship[7]. Furthermore, the conserved gene contexts contained motifs characteristic of helicases and RecB nucleases, suggesting their involvement in DNA repair. Due to their association with the CRISPR regions, Ruud Jansen designated these genes *Cas* (CRISPR-associated) genes. It is important to note that although Mojica and Jansen proposed the acronym CRISPR to describe the repeat-spacer structural pattern in prokaryotic genomes, their function remained enigmatic.

The function of CRISPR as a prokaryotic acquired immune system was experimentally demonstrated in 2007 by Rodolphe Barrangou, Philippe Horvath, and their colleagues. Because these scientists worked for Danisco, a manufacturer of food ingredients, they were interested in controlling phage infection during dairy fermentation by lactic acid bacteria. They observed that after infecting a wild type strain of *Streptococcus thermophilus* bacteria with phage, new spacer sequences were incorporated into the bacterium's CRISPR array[8]. Analysis indicated that the acquired spacers were identical to the phage sequence[8]. Their insertion correlated with increased resistance. These findings suggest that the CRISPR array stores sequences from previous infections and thus functions as a memory bank. Spacer acquisition allows prokaryotes to recognize and fight off future invading organisms.

In addition, Barrangou et al. tested Ruud Jansen's hypothesis that *Cas* genes play a role in DNA repair. To do so, they inactivated two *Cas* genes in the *S. thermophilus* wild type strain:

*Cas*5 (now called *Cas*9) and *Cas*7. They found that inactivating *Cas*7 did not affect bacteriophage resistance[8]. In contrast, phage resistance was lost following the *Cas*5 (now *Cas*9) knockout[8]. From this, Barrangou and his colleagues concluded that *Cas*5/*Cas*9 is the only gene required for phage resistance. Taken together, these findings suggest that CRISPR and its associated Cas9 protein, encoded by the *Cas*9 gene, mediate sequence-specific immunity against viruses in prokaryotes.

Using *Streptococcus pyogenes* bacteria as a model, Jennifer Doudna and Emmanuelle Charpentier discovered the mode of action of the prokaryotic CRISPR-Cas system in 2012. They found that upon plasmid or viral invasion, the bacterium transcribed the acquired spacer sequences into mature RNA called CRISPR RNA (crRNA), which then formed a duplex with a partially complementary RNA molecule[9]. The two-RNA structure directed Cas9 to introduce a double-strand break within the target DNA, the sequence complementary to the crRNA[9]. Doudna and Charpentier concluded that Cas9 could be reprogrammed to site-specifically target any genomic location by changing the target sequence present in the guide RNA. Feng Zhang, a Chinese-American molecular biologist, was the first to adapt the CRISPR-Cas9 system to edit DNA in mammalian (i.e., mouse and human) cells[10].

The Cas9 enzyme can be used to modify any desired genomic target provided that sequence is unique (i.e., after the tenth nucleotide) compared to the rest of the genome and is located just upstream of a Protospacer Adjacent Motif (PAM), a short 2-6 nucleotide conserved sequence that is recognized by Cas9 and serves as a binding signal[11]. Once expressed, the guide RNA (gRNA) binds to Cas9 and forms a stable ribonucleoprotein complex, leaving the gRNA spacer sequence free to interact with its homologous target DNA. If the guide RNA has a region

complementary to the target DNA, and the target is present immediately adjacent to a PAM, it will specifically bind its target sequence through base-pairing. Upon target binding, Cas9 cleaves the target DNA, resulting in a double-strand break (DSB) (Figure 1).

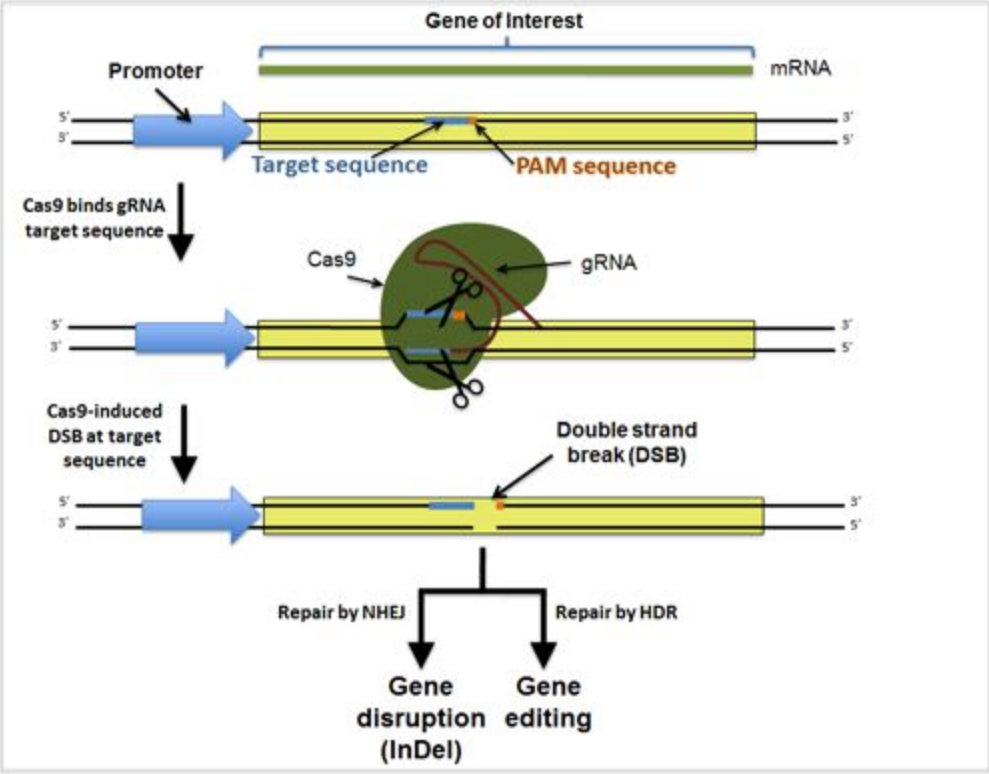

Figure 1. Overview of the CRISPR-Cas9 mechanism of action. A guide RNA (gRNA) recognizes the target DNA sequence, which is located next to a PAM, and binds to the Cas9 nuclease. The resulting complex induces site-specific double-stranded cleavage, which will be repaired by the NHEJ or HDR pathway.

This damage is repaired by cellular DNA repair mechanisms, either via the non-homologous end joining DNA repair pathway (NHEJ) or the homology-directed repair (HDR) pathway (Figure 2). The NHEJ repair pathway is the most active repair mechanism and typically creates small nucleotide insertions or deletions (indels) at the DSB site, resulting in gene disruption[12]. Alternatively, if a repair template is provided by scientists, the double-stranded break can be

repaired with high-precision by the HDR pathway. HDR can be used to insert desirable novel genes in place of the disease-causing genes[13].

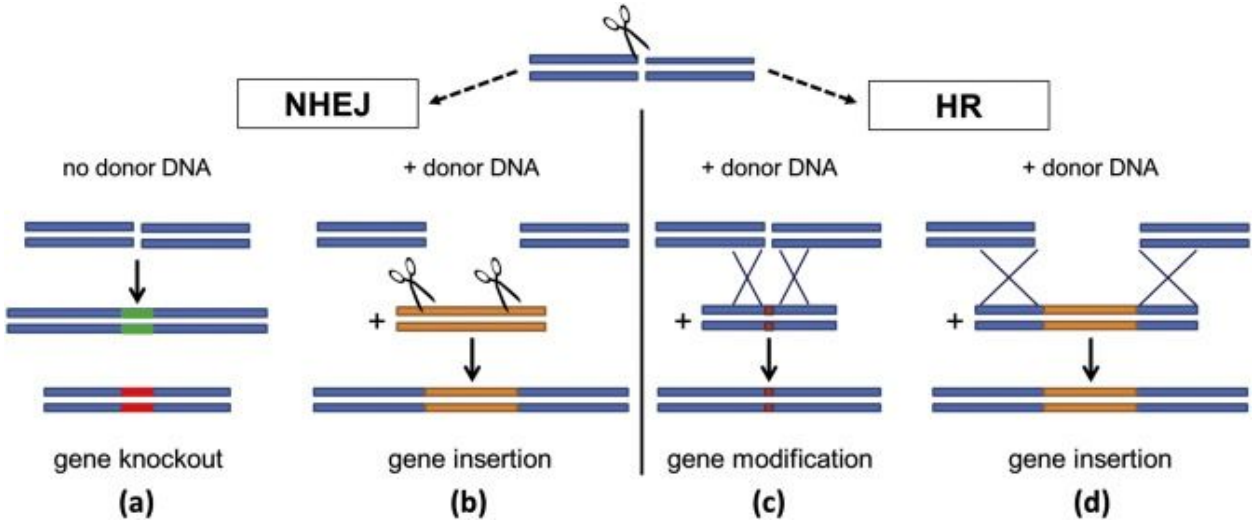

Figure 2. The mechanism of DNA double-strand break repair by the non-homologous end joining (NHEJ) and homologous recombination (HR) pathways. (a) With no donor DNA template, NHEJ simply reattaches the blunt ends of the cleaved DNA, often resulting in insertion (green) or deletion (red) of random nucleotides. (b) If a donor DNA template is provided, it will simultaneously be cut by the same enzyme, and compatible nucleotides will be inserted into the DNA sequence. (c) In HR, a homologous sequence guides repair such that nucleotides can be precisely substituted or (d) inserted at a target locus.

Since some diseases are caused by the mutation of a single gene, CRISPR could be used, in theory, to knock out the disease-causing gene and correct the mutation via the HDR pathway.

Gene knockouts have a profound effect on our understanding of human genetics. Using techniques for manipulating DNA, researchers can inactivate, or 'knock out,' specific genes and observe the effects of removing them. Knockout organisms allow for the assessment of gene function *in vivo*. Models that express the pathophysiology of human disease are a vital resource for providing insight into disease mechanisms, improving diagnostic strategies, and testing

therapeutic interventions. Using CRISPR, scientists can knock out genes more precisely than the conventional knockout vector[14].

Transgenic methods involve the insertion of foreign DNA (a single gene or combination of genes, called transgenes) into the host genome, followed by germline transmission of the gene(s). Because DNA is transferred from one species to another, the phenotype of an organism can potentially change. Transgenic technology has potentially large agricultural and therapeutic value, namely understanding human diseases and developing novel therapeutics, adapting pig organs to be transferred to humans, and improving breeding and food production[15]. Using CRISPR and homology directed repair, scientists can engineer insertions of new DNA at precise locations in the genome of virtually any organism.

The simplicity and efficiency of the CRISPR/Cas9 system allows for the creation of knockout and transgenic organisms. This editing process has a wide variety of applications in crops, animals, and humans. In plants, eukaryotic translation initiation factors are usurped by viruses in order to ensure efficient viral mRNA translation and replication. In 2016, Chandrasekaran et al. used CRISPR/Cas9 to disrupt the function of the eukaryotic translation initiation factor 4E (*eIF4E*) in *Cucumis sativus* L. cucumbers[16]. Cas9/sgRNA constructs were designed to target sequences in the first and third exons of the *eIF4E* gene, creating insertions and deletions (indels) in the process[16]. T3 transgene-free lines homozygous for the *eif4e* mutation demonstrated resistance to several viruses of the family *Potyviridae*: *Cucumber vein yellowing virus*, *Zucchini yellow mosaic virus*, and *Papaya ring spot mosaic virus-W*[16]. Knocking out the *eIF4E* gene can successfully prevent devastating yield losses in *Cucumis sativus* L. cucumbers.

The α-gliadin 33-mer peptide is the most immunodominant gluten peptide in patients with Coeliac disease (CD)[17]. In an effort to produce wheat lines with reduced immunogenic gluten proteins for CD patients, Sáchez-León et al. combined two sgRNA sequences, designed to target DNA sequences coding for 33-mer protein domains, in CRISPR/Cas9 constructs in hexaploid bread wheat and durum wheat cultivars[17]. Most of the resulting transgenic lines showed a significant reduction in α-gliadins containing the immunodominant 33-mer epitope (32-82%), and all of the lines showed a significant reduction in immunoreactivity (85%) compared to the wild type[17]. The CRISPR/Cas9-induced mutations were transmitted to subsequent generations with no detectable off-target effects or transgenes[17]. These reduced-gliadin, nontransgenic wheat lines could provide an alternative to the gluten-free diet for people with gluten intolerance or sensitivity.

Pigs lack the *fat-1* gene, which encodes an omega-3 fatty acid desaturase enzyme that catalyzes conversion of omega-6 fatty acids to omega-3 fatty acids. Pork being the most widely eaten meat in the world suggests that the human diet has a high omega-6/omega-3 ratio, which promotes the pathogenesis of many diseases, including obesity, cardiovascular disease, Alzheimer's disease, rheumatoid arthritis, and inflammatory bowel disease[18, 19]. To improve meat quality, Li and colleagues used the CRISPR-Cas9 system to insert the *fat-1* gene from the roundworm *Caenorhabditis elegans* into the porcine Rosa 26 locus[20]. These *fat-1* knock-in pigs exhibited a significant increase in the level of omega-3 fatty acids and thus a reduction in the ratio of omega-6 fatty acids to omega-3 fatty acids[20]. Transgenic pigs could improve the nutritional value of pork and have potential benefits for the human diet, decreasing the risk of many chronic inflammatory diseases.

In a 2015 study, Long et al. microinjected Cas9, sgRNA, and a HDR template into mouse zygotes to correct the mutation in exon 23 of the *dystrophin* gene, which causes Duchenne muscular dystrophy (DMD)[21]. The zygotes were then implanted into pseudopregnant mice. PCR products corresponding to *dystrophin* exon 23 revealed that the genetically mosaic NHEJ- and HDR-progeny exhibited 2-100% correction of the *dystrophin* gene, with NHEJ being more efficient[21]. Low levels of correction were sufficient to dramatically increase *dystrophin* expression, and high levels of correction (83^{+}%) allowed for the complete restoration of *dystrophin* gene expression in all myofibers of corrected *mdx* progeny[21]. These findings suggest that CRISPR-Cas9 could potentially be used to prevent human inheritance of DMD in the future.

In a 2020 study, Omar Akbari and his colleagues injected an engineered human anti-dengue antibody capable of targeting all four dengue serotypes into the embryos of *Aedes aegypti* mosquitoes[22]. After drinking blood infected with any of the four dengue virus serotypes, the engineered mosquitoes were found to have no detectable virus in their saliva[22]. This suggests that the antibody effectively neutralized the dengue viral particles and prevented infection of the midgut. These newly non-infective *A. aegypti* mosquitoes have no dengue viral particles in their saliva to expel into the bloodstream of a host (i.e., a human), eliminating transmission of the pathogen. In the near future, Akbari's team plans to release these mosquitoes into the wild so that the anti-dengue antibody gene will be passed down through generations, suppressing dengue virus circulation.

Heterozygous *SHANK3* mutations have been found in individuals diagnosed with an autism spectrum disorder[23]. To model ASD, Tu et al. used CRISPR-Cas9 to mutate the *SHANK3* gene in monkeys[24]. Unfortunately, the researchers found that the absence of the *SHANK3* gene in

the primate's prefrontal cortex led to a significant loss of neurons, causing all but one subject monkey to die. A two-year longitudinal study of this monkey revealed that he developed delayed vocalization, frequent repetitive and anxious behavior, enamel dysplasia, and an impaired ability to engage in social interaction, all of which are characteristic of ASD[24]. The *SHANK3*^{M3} monkey also exhibited a significant reduction in body weight, body length, and glucose metabolism compared to control monkeys[24]. These findings are also compatible with an ASD. The researchers then treated the mutant and three control monkeys with 2.5 mg/kg of fluoxetine every day for two weeks. Fluoxetine significantly increased social interaction and eye contact duration, returned glucose metabolic activity to 'normal' levels, and markedly decreased repetitive and anxiety-like behaviors in the *SHANK3*^{M3} monkey. Control monkeys were unaffected[24]. These findings present the reversibility of a subset of ASD-related symptoms in monkeys and show promise to do the same in humans.

Worldwide, there is a chronic shortage of transplantable organs, but xenotransplantation, which involves the transplantation of nonhuman cells, tissues, or organs into human recipients, may be a potential solution[25]. Since porcine (pig) organs are of similar size and physiology to those of humans, pigs are thought to be the optimum donor for the transplants. However, one hurdle to xenotransplantation is that the porcine genome contains porcine endogenous retroviruses (PERVs), which are RNA viruses that can infect human cells *in vitro*[25]. Gene-editing using CRISPR technology could remove PERVs from the porcine genome, paving the way to pig-to-human transplants. For the first time in 2017, Niu et al. used CRISPR-Cas9 to generate genetically modified pigs in which all PERVs were inactivated[26]. In their study, guide RNAs were designed to target the 26 copies of PERVs in the primary porcine fetal fibroblast cell line

(FFF3) genome. Single-cell clones were grown with ~90% efficiency[26]. The addition of an immunosuppressive cocktail allowed FFF3 cells to grow with 100% PERV-inactivation[26]. These modified fibroblasts were then cloned via somatic cell nuclear transfer (SCNT) to produce PERV-inactivated embryos, which were then transferred into surrogate sows. The resulting piglets exhibited 100% PERV inactivation. This study suggests that clinical trials of xenoplantation may occur in the foreseeable future, with organs from genetically modified pigs transplanted into humans.

Clinical trials for treating sickle cell disease (SCD) and β-Thalassemia (β-thal), two genetic blood diseases characterized by a reduction in hemoglobin production, using CRISPR are now underway. In both trials, patients' hematopoietic stem cells were removed, modified, and then returned to the patient. Using the CRISPR-Cas9 system, the *BCL11A* gene, a repressor of fetal hemoglobin (HbF), was deleted, allowing HbF production to increase and the disease to be "cured" [27, 28].

In the future, scientists intend to use CRISPR-Cas9 to correct genetic defects and prevent disease inheritance. In the meantime, researchers are working to improve the technology due to a high frequency of off-target mutations observed. That is, CRISPR has been found to inadvertently alter regions of the genome other than the intended one(s)[29]. Target sequence binding was found to tolerate up to five mismatches depending on their position along the gRNA, meaning there are many possible binding sites within the genome[29]. This may lead to genomic instability and an altered function of otherwise intact genes. Scientists are hoping to reduce or eliminate, if possible, the frequency of off-target effects before CRISPR is employed in

a clinical setting as they present experimental and safety concerns. However, this has not stopped certain researchers from proceeding with human experimentation.

Pushing Scientific Boundaries: How Far is Too Far?

In 2016, a University of Pennsylvania-led team proposed the first-in-human Phase 1 CRISPR gene editing cancer trials (*ex vivo*) in the United States. Investigators described their plans to remove T cells from patients with melanoma, synovial sarcoma, and multiple myeloma and transduce them with a lentiviral vector to express receptors against NY-ESO-1, an antigen expressed on a wide range of tumor types[30]. This transduction would allow their T cells to specifically recognize and kill tumor cells displaying NY-ESO-1. CRISPR/Cas9 would then be used to knock out the *TCR* and *PD-1* genes so that expression of the introduced T-cell receptor (TCR) would be enhanced, leading to improved antitumor activity and prolonged persistence of the T cells[30]. The genetically modified T cells would then be reinfused back into the patient and would, hopefully, target NY-ESO-1^+ cells.

UPenn's proposition was subject to review in June 2016 by the Recombinant DNA Advisory Committee (RAC), who evaluated the pre-clinical evidence used to justify clinical trial initiation. Bioethicists Françoise Baylis and Marcus McLeod performed a second evaluation, wherein they concluded that the "one non-peer reviewed pre-clinical study in mice" with a human lung cancer cell line (not a melanoma, plasma or sarcomatous cell line) "used to justify the first-in-human Phase 1 CRISPR gene editing cancer trial in the United States does not satisfy the ethical requirement of scientific validity"[30]. They also claimed that the study is premature and of no urgent medical need[30]. The investigating team, however, announced their plans to proceed with the trial anyway. On February 7, 2020, the results of the trial were reported: the cancer

progressed in all three individuals (killing one) and off-target effects were detected[31]. The therapy's limitations, voiced by Baylis and McLeod four years prior, have come to light.

Although many clinical trials, such as this one, are being conducted on somatic cells, germline manipulation using CRISPR/Cas9 genome editing has recently attracted much attention. So far, two scientists have attempted to use the CRISPR technology to alter susceptibility to human immunodeficiency virus (HIV). He Jiankui, a Chinese scientist, shocked the world when he announced on November 26, 2018 that he used CRISPR to "create" the world's first genetically edited babies — reportedly HIV-resistance twin girls. Using CRISPR, Jiankui allegedly genetically modified the *CCR5* gene that encodes the CCR5 protein, which enables HIV infection.

Jiankui reportedly had every intention of inducing the *CCR5* delta-32 mutation, a popular target for studying HIV prevention and treatment, into a human embryo created from an HIV-positive father. However, his data suggests that an alternative, never-before-seen mutation was made, of which the effects are unknown[32, 33]. The genetic analysis further revealed that CRISPR modified both *CCR5* genes from one embryo but only one from the other, meaning one of the twins will remain susceptible to HIV infection[32, 34]. It is important to note that Jiankui was aware of the unintended mutation and partial modification introduced by CRISPR-Cas9 prior to implantation[34]. His decision to implant the second, HIV-susceptible embryo anyway contradicted his original "curative" purpose and led scientists to condemn his work as irresponsible, dangerous, and premature. There is no telling what effect this will have on the genetically edited twin babies 'Lulu and Nana' or subsequent generations.

Jiankui arguably would have faced widespread criticism even if the proper mutation was introduced in the embryonic genome. The *CCR5* delta-32 mutation that Jiankui supposedly intended to create would not confer *complete* resistance to HIV infection as there are multiple strains of the virus, all of which exploit different co-receptors to efficiently gain entry into T cells. Furthermore, individuals possessing the *CCR5* delta-32 mutation have been shown to have a reduced lifespan, as well as an increased fatality rate and susceptibility to influenza and West Nile Virus, respectively[35, 36]. There is an unfavorable risk/benefit profile of HIV-prevention in this "clinical trial." The design of the trial was deeply flawed.

Many scientists and ethicists across the world have denounced Jiankui's "clinical trial" as medically unnecessary, for one parent (or even both) being HIV-positive does not necessarily mean that the child itself will be infected with the virus. Further, assisted reproductive technologies can reduce the risk of transmission to essentially zero. He Jiankui, however, argued that it *was* medically necessary as HIV-infected couples are banned from receiving sperm washing and in vitro fertilization (IVF) in China[37]. Jiankui's data revealed that the HIV-positive father's sperm was washed and separated from the semen, where the virus resides. Therefore, the HIV-negative mother was inseminated with virus-free sperm before the gene-editing tool was added. Clearly, He Jiankui not only aimed to eliminate parent-to-child transmission of HIV but to prevent HIV infection for life, and this constitutes genetic enhancement, an alteration that has been long considered off-limits. An expert on Chinese bioethics Jing-Bao Nie, in agreement, said, "The enormous ambition in China, the desire to be the first, collides with the desire to create and enforce standards"[38]. This "clinical trial" was experimentation and not a medical intervention.

Scientists have also objected to Dr. Jiankui's failure to receive adequate informed consent and research ethics review. He Jiankui recruited participants by claiming that he received ethical approval to conduct his research from Shenzhen HarMoniCare Women and Children's Hospital; however, the hospital later denied knowledge of his experiment and asserted that the signatures were forgeries[39]. After deceiving his participants with these forged approval documents, he provided them with a 23-page consent form that was written in technical, English language and did not appropriately explain the relevant risks of knocking out *CCR5*, including off-target effects[40, 41]. It is possible that some participants did not understand what they were consenting to, which begs the question of whether their consent was valid. Furthermore, Jiankui did not inform doctors that they were implanting gene-edited embryos[37, 42]. It is therefore not surprising that Jiankui lost his job and has been sharply criticized by the government for breaching ethical norms.

The parents' "consent" also raises an issue of individual liberties: Should parents be allowed to genetically engineer their children's' genes without their knowledge and consent? Some may say yes, parents should do everything in their power to ensure that their child is in good health. But others may argue that in doing so, they undermine the autonomy of the resulting child. This is an issue that should be explored further before more scientists fiddle with the DNA in germ cells.

Immediately following Jiankui's announcement, the US National Institutes of Health (NIH) director Francis Collins released a statement saying that "the project was largely carried out in secret, the medical necessity for inactivation of CCR5 in these infants is utterly unconvincing, the informed consent process appears highly questionable, and the possibility of

damaging off-target effects has not been satisfactorily explored"[43]. Chinese bioethicist Ren-zong Qiu also spoke about the matter, describing Jiankui's research as "a practice with the least degree of ethical justifiability and acceptability"[41]. Clearly, there is international consensus that the first known application of human germline editing was carried out irresponsibly.

Dr. Jiankui came under investigation for his work, wherein courts found evidence of a third gene-edited baby[44]. Ultimately, the court concluded that Jiankui and his co-workers "had not obtained qualification as doctors to practice medicine, pursued fame and profits, deliberately violated Chinese regulations on scientific research, crossed an ethical line in scientific research and medicine," and "fabricated ethical review documents"[44]. Additionally, Jiankui used "reagents not approved for humans" and "concealed information from those who implanted the embryos"[45]. Jiankui was sentenced to three years in prison for his work, with his assistants receiving shorter sentences. Reaction was fierce. Many scientists and bioethics were up in arms about the ruling, claiming that it is "'ridiculous' that this 'notorious scandal' has been reduced to a case of illegal medical practices" and is "'a minimal sentence for such a brazen action'"[45].

A second incidence of germline genome editing on human embryos for clinical use may soon be underway. Denis Rebrikov, a Russian scientist, has been vocal about his plans to create more HIV-resistant gene-edited babies. Like Jiankui, Rebrikov used CRISPR for *CCR5* deletion, thereby disrupting the HIV co-receptor CCR5 and restricting HIV infection. Rebrikov's experiment is different from that of Jiankui for two reasons: First, Rebrikov modified the *CCR5* gene in embryos created from HIV-positive mothers (who did not respond to other therapies) instead of HIV-positive fathers. The transmission of HIV from father to child is "very rare" while there is great risk in a mother passing it to her child during pregnancy, childbirth, and

breastfeeding[46]. Transmission from father-to-child is so infrequent because the female reproductive tract contains an abundance of dendritic cells, many of which are resistant to HIV-1 infection[47]. This prevents men's infected spermatozoa from efficiently transmitting the virus. In fact, the risk of infection is 1:1000 to 1:10,000[47]. On the other hand, mother-to-child transmission of HIV can occur if the infected cells travel across the placenta and to the fetus, or the baby is exposed to the mother's infected vaginal fluid or blood during labor (water breaking) and delivery, respectively. The transmission of HIV through breast milk is unknown; however, breastfeeding has been found to increase the risk of transmission by 10-15%[48]. For this reason, Rebrikov claims implanting gene-edited embryos into HIV-positive mothers will be more beneficial. Secondly, Rebrikov vowed to not implant the embryo(s) until he was approved to do so by a review board, although he did not mention which one[49]. In this way, Rebrikov would not violate any regulations, making his research more justifiable. It, however, does not change scientists' minds about its unethicalness and medical unnecessariness as there are low-risk methods to prevent HIV infection already in place[50, 51].

Rebrikov has temporarily abandoned his research on HIV prevention not because he submitted to international objections but because he could not find any HIV-positive women who did not respond to antiretroviral therapies and were willing to participate in his study[52]. In the meantime, Rebrikov has begun gene-editing to target inherited deafness. A single-nucleotide guanine deletion at location 35 (*35delG*) in the *GJB2* gene results in the coding of a stop codon prematurely, causing a truncated or nonfunctional protein to be expressed[49]. It is the main genetic cause of autosomal recessive deafness[53]. Rebrikov plans to correct the faulty gene in human embryos created from couples who are homozygous for the *35delG* mutation via CRISPR and

homology directed repair. Many scientists and ethicists have objected to Rebrikov's procedure, saying individuals with two mutated copies of *GJB2* can hear well with cochlear implants or hearing aids and thus there is no compelling medical need to undertake germline therapies[54]. CRISPR co-discoverer Jennifer Doudna calls Rebrikov's project "recklessly opportunistic, clearly unethical and damages the credibility of a technology that is intended to help, not harm"[52]. There is a strong scientific consensus that germline genome editing for clinical purposes is a line that should not be crossed at this time.

In each of the aforementioned cases, researchers disregarded serious and articulated ethical objections and proceeded with their "clinical trials" before questions of safety and efficacy – not to mention ethics – were resolved. To prevent future reckless experimentation, one may look to the history of restriction enzymes as a way of modeling scientific and ethical reflection on new genetic technologies.

Restriction Enzymes: A Case Study on Ethical Reflection in Modern Biotechnology

Like clustered regularly interspaced palindromic repeats, the discovery of restriction enzymes was purely accidental. In the early 1950s, two research teams infected different strains of *Escherichia coli* with (different) bacteriophages. It was observed that the success of bacteriophage infection varied with bacterial strain[55, 56]. In Bertani and Weigle's 1953 experiment, *E. coli* strains S and C were infected with bacteriophage lambda (λ). They observed that phage lambda, when grown and replated on the same strain, produced equal numbers of plaques (a sign of productive infections) on strains S and C. However, when it was propagated first in *E. coli* C and then used to infect *E. coli* S, the rate of infection was significantly suppressed[55]. This was unexpected, for the proportion of productively infected cells should increase together with multiplicity of infection (MOI). When returned to the previous strain, the virus' ability to grow was restored, indicating that the loss was not due to mutation. Werner Arber, a Swiss microbiologist and geneticist, hypothesized that the unsuccessful infection in another strain suggested that natural restriction barriers exist in some bacterial strains, including *E. coli* S. He then set out to test it.

In 1962, Werner Arber and his graduate student, Daisy Dussoix, analyzed the fate of bacteriophage λ DNA upon infection of new host strains. They observed bacteriophage λ DNA degradation in all cases of infection[57]. The bacterial DNA, however, remained intact[57]. Together, they posited that restriction, or suppression of bacteriophage growth, occurs via the cleavage of foreign bacteriophage DNA by restriction enzymes, which eliminate infecting organisms while modification enzymes protect the bacterium's own DNA from degradation[58]. This prediction was

confirmed in 1968 when Werner Arber and his postdoctoral student Stuart Linn isolated two enzymes from *E. coli*. One enzyme (called "methylase") added protective methyl groups to (bacterial) DNA, and the other ("restriction nuclease") cleaved unmethylated (phage) DNA into fragments at specific sites[59]. These enzymatic tools gave researchers a technique to precisely edit DNA for the first time.

Ever since their discovery, researchers have relied on restriction enzymes (REs) to manipulate, analyze, and create new combinations of DNA sequences. When a restriction enzyme finds its recognition site or target sequence, it will wrap around the DNA and introduce a double-strand break (DSB). Some restriction enzymes cut in the middle of a recognition site, producing blunt-end fragments. Most restriction enzymes, however, cut in an offset fashion, yielding sticky end fragments. Any two fragments with complementary sticky ends (regardless of the organism from which they originate) can be isolated and covalently joined together by DNA ligase, forming recombinant DNA. The recombinant DNA (rDNA) could then be introduced into bacteria, such as *E. coli*, via transformation, where it would be cloned once incorporated into a host cell.

Safety concerns about such experiments began to be raised: What risks are associated with applications of rDNA organisms? Because recombinant DNA produces new genetic combinations as a result of combining DNA from disparate sources, the Environmental Protection Agency (EPA) concluded that such techniques "have the greatest potential to pose risks to people or the environment"[60]. This prompted the Asilomar Conference of 1975. One hundred forty-four of the world's most elite molecular biologists, as well as some members of the general public, gathered in Pacific Grove, California to examine the risks and benefits of

recombinant DNA technology[61, 62]. This panel of experts and laypeople decided upon a set of guidelines to be followed by all scientists doing rDNA research and in doing so, set a precedent of scientific self-regulation[63]. The participants of the Asilomar Conference also agreed to reconvene to reassess the biohazards of recombinant DNA experimentation "in the light of new scientific knowledge"[64]. However, as more data accumulated, there was wide agreement among scientists and community members that rDNA experiments were fairly safe. Today, restriction enzymes are routinely used in DNA or RNA manipulation and molecular cloning.

CRISPR Conferences: Moral Considerations for Applications of a Powerful Tool

CRISPR goes well beyond anything the Asilomar Conference discussed. In 1975 no one was talking about heritable genetic modification policies. Technology in the twentieth century did not allow researchers to potentially eradicate the genetic diseases that long seemed out of reach; with CRISPR-Cas9, the unthinkable has become conceivable.

CRISPR/Cas systems for genome editing are poised to revolutionize medicine. The power and promise of this innovation, however, raises many scientific, ethical, and societal questions, all of which were thoughtfully discussed from December 1-3, 2015 at the first International Summit on Human Genome Editing[65]. The members of the Organizing Committee, including Asilomar's Paul Berg and David Baltimore, concluded that it is appropriate to make edits in patients' somatic cells as it allows for the correction of disease-causing gene(s) inherited by an individual without passing the altered genomes onto future generations[65]. They also agreed that research involving the genetic alteration of germline cells can and shall proceed so long as

the modified embryos are not used for impregnation, at least until (1) researchers resolve any and all "safety and efficacy issues," and (2) researchers gain societal approval for their (appropriate) clinical applications[65]. They did not, however, specify how these conditions could be met. Furthermore, germline genome editing should never be used for "enhancement" purposes.

The 2015 International Summit was intended to motivate a much larger conference to think about the governance of human germline editing. Keith Gillis, the Dean of UC Berkeley's College of Natural Resources, had a conversation with Neal Gutterson, a chief technology officer at Corteva Agriscience, about the possibility of hosting a conference that would "invite public discussion around the ethics of genome editing"[66]. This was the birth of CRISPRcon: Conversations on Science, Society and the Future of Gene Editing, first hosted by the University of California, Berkeley on August 16-17, 2017. Individuals in the scientific community, industry, and academia (excluding ethicists) gathered together to voice their hopes and fears for the technology, as well as to discuss potential future applications of CRISPR in health, agriculture, ecosystem conservation, and more.

Beginning the next day and ending on November 29, 2018, the Second International Summit on Human Genome Editing at the University of Hong Kong revisited the clinical use of germline editing. Because He Jiankui's announcement of the world's first genetically edited babies was made days before the Summit, discussion centered around his work. Its members, who for the first time included ethicists, called for a global moratorium on human germline editing, concluding that it was still too soon to permit clinical trials of germline editing due to limited scientific understanding and great risk posed by the newly emerged technology[67]. Furthermore, they stressed that Jiankui's experimental technique, regardless of whether it was

safe or effective, was irresponsible, immature, and should not be repeated until it "conforms with international norms"[67].

In each of the aforementioned CRISPR conferences, safety precautions were outlined but no limit or threshold was set for which experiments could be conducted and under what circumstances, hence how He Jiankui was able to push human experimentation to new limits. There has been no mention of a Third International Summit on Human Genome Editing, though one is necessary to limit further rogue experimentation. Jiankui's study and consequential prison sentence clearly stresses the need to rethink regulations, but more particularly to develop an internationally agreed-upon regulatory framework, and enhance ethical governance of scientific practices. Working together, scientists and nonscientists *must* establish norms and set harmonious regulations for which CRISPR's use is acceptable, prior to future attempts to conduct this research, in order to discourage scientific misconduct and advance human health.

CRISPR technology has the potential to transform human nature at an unprecedented level, making its ethical use everybody's responsibility. The participation of members of the public in scientific discussions has long been an essential condition of scientific research; however, somewhere along the way, scientists became resistant to public 'interference.' So far, scientists have failed to engage the 'ill-informed' public in scientific discourse, though gene-editing research can and will have community-wide impacts. Public participation, however, needs to be fundamentally reimagined so as to create an opportunity for mutual learning and societal decision-making. The public should be involved in scientific discussions, specifically those regarding CRISPR/Cas9, so that they can express their views on how they envision their futures being affected by research and discoveries. People's capacity to express or exercise their

views has the potential to influence what research is culturally and morally acceptable. Clearly, there is a huge need for inclusive deliberation (backed by enforceable norms).

Bill Clinton was the first U.S. president to appoint a National Bioethics Advisory Commission, whereby the U.S. government could endorse ethical standards for biological research[68]. Alternative forms of a bioethics commission have existed for subsequent Presidents, except for President Donald Trump, who has dismissed the panel. Without it, the U.S. government cannot oversee or reflect on experimental science. This is especially harmful when it comes to ethical issues arising from clinical applications. One can only wonder whether the intentional misuses of CRISPR technology could have been prevented had this national bioethics advisory body existed …

There is no question of whether CRISPR can fix mutations that cause heritable diseases. While scientists assess the technology's safety and efficacy, they are overlooking a bigger and more important question which is *should* we be performing these experiments? That question does not belong to science uniquely, but to all of humanity. Should is all about ethics and beliefs and one can not change ethics and beliefs with facts and science alone. The process for deciding answers needs involvement of scientists, ethicists, government officials, and the general public, just to name a few.

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