Introduction to the CRISPR Revolution

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1. CRISPR basic mechanism

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has been the buzzword for genome editing in the past few years. The CRISPR/Cas system evolved as an adaptive immune response in bacteria against foreign DNA, mainly viral and plasmid DNAs (1-4). There are three types of CRISPR systems (I-III), but it is type II that is currently being exploited for our purposes (3, 5-7). This system consists of the Cas9 protein, a mature CRISPR RNA (crRNA), partially complementary trans-activating crRNA (tracrRNA). Together, crRNA and tracrRNA form what is called guide RNA (sgRNA)(8), which with Cas9 protein will cleave complementary DNA if they are adjacent to short sequences known as protospacer-adjacent motifs (PAMs).

The CRISPR/Cas9 system is not the first genome editing technology that has ever been developed. In recent years, we have ZFNs (9-11) and TALENs (12-14) which have been used to mutate specific loci in various plants (15-22). Like CRISPR/Cas9, ZFNs and TALENs induces DNA double strand breaks (DSBs). These targeted DSBs are then repaired either via nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ in general is imprecise, oftentimes causing frame-shift mutations, effectively causing knockouts. On the other hand, HDR is much more favored in that it repairs broken chromosome by homologous DNA template inducing a better repair than NHEJ(23).

Though CRISPR/Cas9 system is very similar to ZFNs and TALENs because they all induce DSB which led to either NHEJ or HDR, there are significant unique advantages that CRISPR/Cas9 has that make it a superior genome editing tool than its predecessors. First, CRISPR/Cas9 has a wider range of sites than ZFNs that are limited because of the absence of zinc finger motifs for all possible DNA triplets(24). For CRISPR/Cas9 system, all that is needed is a 20-bp sequence nearby the PAM. Second, delivery of short sgRNA for CRISPR/Cas9 application is a lot easier than dealing with long vector constructs that are needed for ZFNs and TALENs. Third, CRISPR/Cas9 requires a single cloning step to carry its sgRNA, while two different ZFN and TALEN proteins carrying multiple repetitive ZF or TALE modules are needed. Such constructions are time-consuming and expensive. Last, which is perhaps the most important advantage of CRISPR/Cas9 system is that it is able to target multiple genes given the construction of a library of sgRNA (25). Contemporary array-based oligo production technology (26, 27) can produce enough sgRNAs (millions) to target all genes in the human genome twenty times over (28).

2. CRISPR history

Having discussed the basics of CRISPR, I will give a brief overview of the history of CRISPR. In December 1987, researchers found CRISPR sequences in *Escherichia Coli*, but do not characterize their function (29). In July 1995, CRISPR sequences are found to be common in other microbes, not only *E. Coli* (30). Interestingly enough, this information was first picked up by a food company in March 2007. Scientists at Danisco food company determined that the

repeats are part of a bacterial innate immune system against viruses (31-33). It was not until June 2012 that Jennifer Doudna's group at UC Berkeley reported that CRISPR can be used to perform genome editing (8). In January 2013 CRISPR is used in mouse and human cells, and two months later they started filing patents for their finding on CRISPR (8, 26, 28, 34, 35). In April 2014, MIT and the Broad Institute were granted a patent on CRISPR gene editing, initiating the well-known patent battle. In March 2015, the first concern of CRISPR was reported, namely gene drive that can spread an edited gene rapidly through a population (36, 37). As early as in April 2015, a group in China reported that they have edited human embryos with CRISPR stoking the ethical debate surrounding human genome editing (38). By 2016, applications have spread to livestock animals. Whitelaw's team has altered immune genes in pigs to make them resistant to swine fever(39), while Russell's team made his pigs resistant to a deadly respiratory virus(40). More of these CRISPR applications will be discussed in the next section.

Parallel to the scientific history of CRISPR, the business side of this technology definitely propels this field forward (41). As early as October 2011, biotech companies were founded based on CRISPR technology, such as the Caribou Biosciences from Berkeley which raised $11 million. Following the ability to use CRISPR and human and mouse cells, Editas Medicine was founded in Cambridge, Massachusetts in November 2013, and managed to raise $43 million, while CRISPR Therapeutics in Basel, Switzerland managed to raise $89 million. Following the patent that was granted to the MIT and Broad groups, Intellia Therapeutics was founded in November 2014 raising $15 million.

3. CRISPR current applications
    a. Human applications

Human applications of CRISPR-mediated genome editing falls into three broad categories: disease prevention, therapy, and enhancement.

i. Disease prevention

The best medical application of CRISPR is obviously disease prevention. This can be done when the gene causing the disease is known and can be detected before the disease onset. Adult-onset diseases are best for such prevention mechanisms. Promising work has been done in using CRISPR as a preventive measure in a mouse model of muscular dystrophy, a debilitating neurological disease, where patients progressively lose strength in their muscles. As early as in 2014, scientists have been able to correct the genetic mutation behind muscular dystrophy in their mouse model using CRISPR genome editing (42). Such preventive measure has been done not only in the editing of the host human genome, but also in the editing of the pathogen's genome in the case of HIV. CRISPR allows scientists to cut viral genome to disable them from infecting human cells (43-45).

As great as this sounds, disease prevention comes with a hidden ethical dilemma. Since DNA is present in every single one of our cells, to ensure the most successful CRISPR genome editing possible, it is best to perform such prevention as early as possible in life, namely, early during embryonic development. This then raises very important ethical questions as to whether CRISPR

genome editing should be done in human embryos, which is discussed in a later section of this paper.

ii. Therapy

CRISPR in theory can be used as a genetic therapy option. Khalili's group from Temple University was able to take HIV+ patient cells and use CRISPR to cut out the HIV gene while leaving the patient DNA intact (46-48). This was all in vitro of course, then they proceeded with an in vivo experiment using a mouse model. They created a mouse model that expresses HIV gene in their DNA. They then were able to use CRISPR to cut out the HIV gene in these mice, showing the possibility of using CRISPR in vivo as a therapeutic agent (49-51). Though studies in primates and humans have not been done, this mouse model experiment shows a great leap forward in using CRISPR as a therapeutic agent in the future. There are approximately 3,000 single-gene heritable disease traits (52, 53), which means CRISPR can be used to potentially cure at least these 3,000 diseases (54).

iii. Enhancement

The fact that CRISPR has the potential to be used for prevention and treatment, brings the question regarding enhancement. Can CRISPR be used to create a more intelligent person, with blonde hair and blue eyes? Theoretically speaking, yes that is possible, but only if we have the genetic information behind such traits and whether they are genetically determined. The determination of something as simple as eye color is highly complex genetically. Height is also another of those traits where multiple genes play a role and is still largely unknown. When it comes to traits involving interactions with the environment (intelligence, friendliness, sense of humor, ethical, etc) are far from the reach of genetics alone.

b. Animal applications

Jennifer Doudna has been keeping a list of CRISPR-altered creatures, and so far she has about 40 entries (41). This is only within the three years since she first published her work on CRISPR (8, 10). Scientists engineered a bacteria to produce egg proteins that do not induce egg allergies, bringing about future hypoallergenic eggs once the chicken genome is edited (55). Though many of these animals are altered to create human disease models, CRISPR has been used to alter animals for non-medical purposes.

As previously mentioned, pigs are a big business. Whitelaw's team has altered immune genes in pigs to make them resistant to swine fever (39), while Russell's team made his pigs resistant to a deadly respiratory virus (40). Both of which have saved millions of dollars in the livestock industry. On the topic of pigs, Chinese scientists have used CRISPR to knockout the growth hormone receptor gene in pigs to create micropigs that weigh only half of normal pigs. They were first created as laboratory pig models, but since they are very adorable, they are being sold as pets. They currently cost US $1,600 but that may change as demand rises and as future colorful pigs come into the market in the next few years (56).

c. Plant applications

Just like any GMO applications on plants in the past, CRISPR can be used to modify plants to be pest-resistant, to grow faster, to be more fruitful, etc. There are also decorative applications of CRISPR to create plants with different colors. Regardless, of the application, these plant modifications are not trivial scientifically. CRISPR modifications of rice and wheat have proven to be different than animal modification. CRISPR delivery cannot be done through viral infection or HDR, for example (25). One would think that it would be simpler, but that is not always the case.

d. Other organisms

i. Viral applications

The best DNA delivery system that we currently have is virus, because viruses naturally infect their host with viral DNA. Thus, for better or worse, viruses can be designed by CRISPR to carry whatever DNA we want it to deliver. It has been shown, albeit in mice, that it is possible to design a virus carrying a CRISPR system which can cause human lung cancer when breathed in (57). One can imagine that viruses can be designed to carry various kinds of disease-causing genes which would be detrimental in a biological warfare. Though it would be possible to detect the type of virus and lethal gene that are being used, it might not be easy to design a cure before the damage is done.

ii. Mosquito applications

Mosquitos can be more than just a simple nuisance, when they start carrying deadly diseases such malaria, dengue fever, or recently, the Zika virus. With CRISPR, scientists have been able to edit mosquitos to be resistant to malaria infection, thus disabling them from contracting malaria to humans. Moreover, using CRISPR, the modification done on one chromosome will copy itself to the other chromosome, ensuring all the progenies of these mutant mosquitos to be resistant to malaria as well (58, 59). This specific technology is called ‘gene drive’ which is able to pass genomic modification to 100% of its progenies, altering the population genetics greatly within just a few generations (60). As safeguard against unintended consequences of such gene drives, scientists have developed ‘reverse gene drives’ using CRISPR as well to cut out the original genetic modification, rendering the genome back to wild type (61, 62).

iii. Yeast applications

Yeasts have been known to transform sugar into alcohol, lipids, and other hydrocarbons. With CRISPR technology, scientists at UC Riverside have been successful in engineering yeasts to synthesize lipids and polymers (63). These novel lipids and polymers can be used for the development of various novel substrates: biofuels, specialty polymers, adhesives, and fragrances. The simplicity of yeasts limits what can be produced by their cellular machinery, however, their simplicity allows easy CRISPR manipulations and yeasts are known to yield large quantities of whatever product we engineer them to synthesize.

iv. De-extinction applications

George Church and Vincent Lynch have thought of using CRISPR in a completely different way. They both looked to the past, more specifically, to the woolly mammoths and retrieved their

genetic information. Using CRISPR, Lynch's group was able to show cells with mammoth genes can grow in low temperatures (64, 65). Similarly, mice with those modifications prefer colder areas of their cages (66). Church's group intends to save endangered Indian elephants by transforming them to be cold-resistant so that they can be released and survive with lots of space to roam in Siberia (67).

4. CRISPR dangers

Having discussed the various possible applications of CRISPR ranging from curing 3,000 diseases to bringing back extinct mammoths to life, it is necessary to understand the dangers of CRISPR. I will discuss here three broad categories of problems: scientific, ecological, and social. Though not completely true, a lot of the ecological and social problems will diminish as the scientific problems are solved. Thus I place greater emphasis in discussing these scientific hurdles pertaining to CRISPR.

a. Scientific
    i. Off target

The key to CRISPR targeting system is the sgRNA which is used to determine which gene gets edited. Given the fact that our DNA is long with 3 billion base pairs, it is of course a major concern to get the correct gene edited out of the myriad of base pairs in the genome. To overcome this problem, most scientists have used BLAST bioinformatics tool to ensure that the sgRNA sequence will go to only the desired site in the genome. In addition, scientists have been working to induce HR as the DBS repair mechanism instead of NHEJ which is prone to mutagenesis. Though this is a serious problem in the application of CRISPR technology, it is not impossible to overcome this technical problem, and scientists are overcoming this problem little by little everyday.

    ii. Chimerism

Not all cells in the embryo could be transfected by CRISPR, such that the resulting fetus would turn out to contain a much smaller proportion of edited cells (68). In turn, these edited cells would presumably have different rate of division than non-edited cells. Either way, you will have a chimera baby where some of the cells are edited while others are not. This efficiency problem in transfection of CRISPR can be improved to 100%, but even so, some cells may be able to epigenetically revert CRISPR edits causing the chimera problem again.

    iii. Mutation of sgRNA

Since there is an inherent rate of genetic mutation in our genome, this means that the CRISPR edited genome may mutate as well. More specifically, even the sgRNA may mutate. Thus there has to be a way to track these mutations. This is not always easy to do because once we see a mutation, we do not know if the mutation is due to CRISPR application error, natural inherent mutation error, or an environmentally-induced error. This problem is currently being explored by tagging the gene of interest with Green Fluorescent Protein (GFP) to allow scientists to visualize and track them over time.

iv. Epigenetic factors

CRISPR is predominantly about editing the DNA, however we all know that any biological trait is not 100% determined by the genome. There are other non-genetic factors which could override the genetic information, and they are called epigenetic factors. For example, in our cells, DNA is bound and wound up onto histone proteins. To allow transcription of a specific gene, the histone on that gene must first be opened, unwinding the DNA to allow transcription to take place. Thus it is possible for certain disease-causing genes to be present but not cause any disease because they are 'closed' by histones disabling the transcription machinery to transcribe those disease-causing genes. In the case of CRISPR genes, it is possible that we have inserted a specific edited gene, which in turn is epigenetically repressed in the host, rendering the CRISPR modification powerless. Scientists have now tried to overcome these epigenetic factors by modifying the genes behind those epigenetic machineries themselves.

v. Genetic Background

Any animal model of human disease is constructed under identical genetic background. For example, a genetically modified mice carrying a cancer-causing gene has identical genetic background, to ensure that when the mouse gets cancer, it is caused by the oncogene, and not by other genes in the background of the mouse. To ensure identical genetic background, mouse subspecies are interbred for 10 generations before being used for experimentation.

In the case of CRISPR application, there is always that lurking question whether CRISPR modification will work the same in different genetic backgrounds, because unlike genetic animal models, every person has a different genetic background. Even people of the same race have as big if not bigger genetic variance than people of different race (69-71). Scientists could address this problem by experimenting CRISPR modifications in animal models of various genetic backgrounds, but this is an expensive endeavor, since the experimental costs will be multiplied by the number of genetic background models that are being used. In the end, there is no guarantee that human genetic background variation will pose no problem even if multiple genetic backgrounds in animal models have been tested.

vi. Unintended consequences even if on-target

Similar to unknown epigenetic factors, even if the first off-target problem could be overcome, we do not always know all the possible effects of an on-target CRISPR modification in an organism. There are current examples of this, such as the elimination of sickle-cell disease leads to an increased risk of contracting malaria (72). Such information cannot always be known beforehand, and will accumulate over time as more modifications are done. However, the difficult question is always, how much experimentation is needed before it is ready to be implemented?

vii. Embryonic destruction

CRISPR has resurrected concerns that were discussed in the stem cell debate, which is the use of embryonic stem cells for research or full modification and application. Since CRISPR transfection efficiency is a major problem, one way to bypass this problem is to do the

modification in early in the embryonic zygote, morula or blastocyst stage, where there are still very few cells. However this brings up the problem of not only designer babies, but also embryonic destruction even if the stem cells are used for research purposes only.

Scientists are well aware of this and have proposed various mechanisms to address this issue. First, instead of modifying the zygote itself, scientists have proposed modifying the gametes (sperm and/or egg) which would produce the desired genetic modification when fertilized. Second, if human genetic modification is a concern, we could still use embryonic cells for research purposes for a short period of time, and then not implant them. Third, along similar lines, the source of embryos could be those unused from IVF and ART. Fourth, though not as effective, it is possible to perform CRISPR modifications on iPS cells as needed prior to transplantation. The use of iPS is promising, though it would not work for every scenario due to the lack of totipotency of iPS in general, and also the limited scope of the prevention. However, if such CRISPR-modified iPS is implanted, then we run into the problem of human cloning (72).

b. Ecological

As previously discussed, with the invention of 'gene drives', where CRISPR modified gene on one chromosome will copy itself to the other chromosome, this ensures the 100% propagation of the modified gene to all progenies. This means, one can alter the diversity of phenotypes of that gene in the ecosystem, altering that species survival intended or unintended, for better or worse. A group in UCSD has successfully created yellow fruitflies. If those fruitflies were to escape, it is estimated between half if not all fruitflies will be yellow (73, 74). The ramifications of such actions to the environment is difficult to predict and perhaps better for one to not know.

Understandably, there are times where the use of gene drive would be useful, such as in the elimination of malaria or Zika disease. Thus scientists have developed three safeguards to contain global ecological changes. First, one can create a *split gene drive* system where the components for the gene drive is split between two groups of organisms. For example, one can separate the gene drive sgRNA in a separate mouse from the one carrying the Cas9 gene, such that it is impossible for the modification to take place unless it is mated with a Cas9 mouse in the laboratory. Second, one can create *artificial sequence* in the target organism, and the CRISPR sgRNA in another mouse is designed to target that artificial sequence. Thus, if either mice escaped and mated with wild type mice, no modification would take place due to the absence of such artificial sequence in wild type mice. Third, as one last measure, one can design a *reverse gene drive*, a second gene drive designed to eliminate the initial genetic modification from the previous gene drive (61, 75, 76).

c. Social

With the advent of a new technology, price for its development, access, and application are usually not within the reach of the public, giving leverage only to the wealthy. With the advent of genome editing, this gap would be even more visible if only the wealthy could get preventive medical care using CRISPR while the rest of the population is left behind. Though this is a serious problem, this is not a problem unique to CRISPR.

In fact, CRISPR technology might have exactly the opposite problem. As previously mentioned in the introduction and history of CRISPR sections earlier, scientists have been able to edit genomes for over a decade, however they were more expensive. For example, ZFN genome editing costs US $5,000 or more to order and they are difficult to engineer and expensive. On the other hand, CRISPR only need to order the sgRNA which cost as little as US $30. This is what James Haber of Brandeis calls a democratization and revolution of science(41). In other words, CRISPR might need to be regulated more strictly precisely because anybody will be able to do it for a low cost.

Currently, there is a community of amateur biologists sharing resources to practice CRISPR modification, in a do-it-yourself (DIY) fashion, earning them the nickname "biohackers." Though currently they are limited by lab space and equipment (77), working on yeasts and plants, if they are funded even half of an academic lab, they would be able to do a lot more.

Regardless of what you think could happen with CRISPR, we are only beginning to learn what CRISPR is for, who will use it, and how it should be used. Given the speed of scientific discoveries in the field, it is imperative that scientists engage the public and try their best to explain their discoveries, because something of this magnitude can't be figured out only by a select few scientists.

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