Development, Applications, and Ethical Complications of CRISPR-Cas9 System in Genome Editing

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ABSTRACT

CRISPR-Cas9 technology is a newly developed tool for genome editing with unprecedented simplicity, versatility, and precision. Derived from ancient defense mechanism bacteria used to fight viruses, CRISPR is analogous to adaptive immunity in vertebrates by acting as a word processor in searching and correcting mistakes in DNA strands. Upon its discovery, scientists have harnessed and adopted it to plants, animals, and human embryos in the realm of biology, biotechnology, and medicine. While starting a huge wave of exploring its potential use in various areas, this revolutionary technology also raised fierce debates about human genome engineering since its further development and applications will significantly influence humanity. This review paper focuses on the development, applications, and ethical complications of CRISPR-Cas9 system as an efficient tool in genome editing. It may provide a tutorial base for chemical educators of this exciting field.

INTRODUCTION

CRISPR-Cas9 systems are new weapons in the arsenal of genome editing. Similar to the discovery of restriction enzymes, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) array was found during basic biological research with an entirely different purpose.[1] Later, the mechanism whereby CRISPR served as an adaptive antiviral immunity in bacteria by was understood: Certain endonuclease proteins, called Cas proteins (CRISPR-associated proteins), were guided by RNAs to fight virus. Among all types of Cas proteins, Cas9 exhibits the greatest potential that gives rise to its further extensive use.[2]

DEVELOPMENT

Origin: Bacterial and Archaeal Adaptive Immune System

CRISPR loci were first identified in archaea and bacteria when they systematically drew attention from scientists with their biological function to fight phages and viruses.[1] Structurally, a clustered set of Cas (CRISPR-associated) genes and a unique CRISPR array constitute a CRISPR locus. The CRISPR array is further divided into short repetitive sequences interspaced by distinctive sequences (spacers) in correspondence with exogenous genetic bits (protospacer). The natural CRISPR systems in bacteria and archaea carried out their adaptive antiviral immunity by following a three-step mechanism, namely adaptation, crRNA biogenesis, and interference.[2]

The infection by undocumented DNA starts the acquisition of viral DNA. Upon the detection of the invasion of bacteriophages, bacteria defend themselves in a timely fashion by inserting bits of viral DNA, the protospacer, into their chromosomes at the end of CRISPR loci.[2] To maintain the structure of CRISPR array, bacteria initiate the replication of a repetitive DNA sequence--the repeat.[4]

Next, crRNA biogenesis takes place in two stages. First, the CRISPR locus and the Cas gene are respectively transcribed into a single pre-crRNA and Cas proteins. In this process, different types of CRISPR systems encode distinctive Cas proteins. Specifically, type II system is the only known system that involves a single endonuclease, the Cas9 protein.[1] Afterward, in type II system, Cas9 protein, along with two other endonucleases, aids the finalization of crRNA. Through transcription, a tracrRNA (trans-activating crRNA) is also encoded by a gene proximate to CRISPR locus.[2] It pairs with crRNA via Watson-Crick base pairing rule, a process stabilized by Cas9 protein.[1] The specificity cleavage ensues when an endogenous RNAase cleaves at the repeats, resulting in separate crRNAs. A then undetermined endonuclease then started to truncate the repeats and sections of the spacers, marking the end of biogenesis.[5,6]

The inference, the final step of CRISPR adaptive immunity, entails the formation of DNA-RNA hybrid duplexes and the cutting of target DNA.[2] Protospacer adjacent motif (PAM), directly downstream of CRISPR locus, plays a critical role in crRNA recognition in that it takes the form of "Ny base-Guanosine-Guanosine," or simply NGG, guides the crRNA recognition, and is recognized directly by endonucleases.[2,7] The GG-nucleotides interact with two amino acids in Cas9 proteins and are then, as Farley put it, "pulls away from its complementary CC-nucleotides on the other strand of DNA".[7] The further interaction between N-nucleotide and other amino acids in Cas9 temporarily destabilizes the rock-hard double-helix structure resulting from double and triple hydrogen bonds between two strands

of DNA, setting the stage for crRNA recognition if the crRNA can successfully pair with the direct upstream PAM. Upon the unwinding of target DNA and the subsequent formation of DNA-RNA hybrid duplexes, Cas9 initiates its specific cleavage on target DNA as the completion of CRISPR immunity mechanism.[2] Over time, bacteria can keep a genetic record of encountered infection by bacteriophages and pass on the acquired spacers to their progenies, ensuring a hereditary immunity defense in certain strains of bacteria.[4]

Discovery and Development: From E.coli to Laboratory settings

The origin of CRISPR could be dated back to 1987 in a study conducted by Nakata and colleagues regarding a critical enzyme in the metabolism of E.coli.[8] The scientists noticed a structurally distinctive array of repeats downstream the gene of their interest. The repetitive sequences in that particular array were interspaced by non-repetitive parts, whereas the common tandem repeats are the predominant shape in most repetitive sequences.[1] Later, in light of the advancement in DNA sequencing technology, an increasing number of repeat elements were identified in the bacterial and archaeal genome.[9] Until 2002, Mojica and colleagues coined the term CRISPR to classify said sequences as a unique family of clustered repeats prevalent in bacteria and archaea.[9] After that, the subsequent finding of several intact, different clusters of cas-proteins neighboring the repeats elements enabled scientists to distinguish three different kinds of CRISPR systems.[1] Despite the surging interest sparked by CRISPR loci in the academia, the biological function of the CRISPR remained unrecognized.[1]

In 2005, upon thorough analysis of the spacers sequences, scientists uncovered their "extrachromosomal and phage-associated origin." They noticed genetic similarities in the genome of phage and viruses, marking a milestone in the history of CRISPR.[1] For the first time, scientists raised the hypothesis of CRISPR system being adaptive immunity after they found out the transcript of CRISPR prevented bacteria from getting infected by viruses with corresponding sequences.[10] They failed to determine its underlying mechanism until the first experimental evidence found by Horvath and colleagues in 2007 and a magnitude of ensuing studies shed light on the process that serves as an infection memory.[11] As of 2010, natural CRISPR systems have been harnessed for several biological and biotechnological uses. However, its potential use in genetic engineering remained unexplored to a large extent.[1]

In the next two years, two main studies and several follow-up experiments finally elucidated the potential use of CRISPR technology in genome editing.[1] First, unlike type I and type III CRISPR system, type II system has Cas9 as the sole endonuclease taking charge of target DNA cleavage.[11] Second, a trans-activating crRNA (tracrRNA) that binds with both the Cas9 protein and the crRNA contributes to the formation of crPNA from pro-crRNA.[5,12] The two findings above established the three-component structure of the type II CRISPR system, namely Cas9, crRNA, and tracrRNA.[1] In response, scientists throughout the world have rushed to launch numerous research studies in an attempt to demystify and take control

of such a potentially powerful weapon in genetic engineering.[1] In 2011, type II CRISPR locus was proved to have a transferable nature by a study suggesting the feasibility of the transplantation of CRISPR systems into a different bacterial strain.[1] Also, the relationship between crRNA and Cas9--crRNA guided the Cas9 to the particular locus on the DNA to cleave--was showed to maintain in vitro.[6] Moreover, a single guide RNA (sgRNA) can be fused by hybridizing short sequence of RNA corresponding to the target DNA with a complementary tracrRNA assisting Cas with cleaving the target DNA.[6] In 2013, first success of adopting CRISPR technology in mammalian cells showcased how Cas9 inflicted cleavage with the guidance of multiple programmable sgRNAs or crRNA-tracrRNA hybrids to effectively trigger repair pathways, marking the inception of a huge wave of the adoption of and the research about CRISPR in genome editing in thousands upon thousands of laboratories.[3]

Characteristic: Efficient Tool for Genome Engineering with Unparalelled Potential and Unlimited Use

The CRISPR technology has been employed in numerous experimental trials in laboratory settings. Using short and programmable sgRNA, scientists have simplified the CRISPR system to a two-component system. Also, they have exploited the two repair pathways the cell would take--homology-directed repair (HDR) and non-homologous end joining (NHEJ)--after the target DNA was cleaved.[2] For NHEJ, ordinarily, the sticky ends of resultant sequences attach back to each other in an imprecise fashion.[13] With this repair pathway, researchers can incorporate a new strand of donor DNA into the original one by delivering a specially engineered sequence with homologies on both ends and different hereditary information in between. For HDR, a homologous DNA is used to conduct the repair to insert or modify genes, a mechanism only available in diploid organisms.[14] In this process, theoretically, if we can cleave the double helix at loci of severe genetic diseases, we can adopt CRISPR technology to cause the cell to repair the inherited mutation.[2]

Genome Editing is not a new concept; systems similar to CRISPR-Cas9 have existed for decades.[2] However, CRISPR-Cas9 systems have out-competed past technologies with their practical use in reality. It was well recognized that double-strand breaks in DNA could remarkably facilitate HDR and NHEJ pathways, both of which are conducive to precise genome editing.[1] There are four main categories of nucleases that have been explored and engineered to incise target DNA, yet, except for CRISPR-Cas9 system, the other three have their respective drawbacks.[1] By contrast, CRISPR system significantly refines and streamlines the previous technologies by simplifying the process of engineering DNA-binding proteins to designing merely the short guide RNA sequence of about 20 nucleotides.[1] Furthermore, employing CRISPR technology, scientists can easily realize mass-scale multiplexed targeting by Cas9 protein simply with the introduction of an wide assortment of RNAs instead of various nucleases. As a result, even though CRISPR is not without its peers, its programmable nature, its precise incision on target DNA, and its

potential for multiplex targeting gives rise to its full list of applications in the field of biology, bioengineering, and medicine.[1]

APPLICATIONS

Biological/Biomedical Research: Development of Cellular and Animal Models

In an attempt to find the function of the organization of specific genome genes and regulatory machinery, scientists develop sets of animal and cellular models with CRISPR technology.[1] Simplicity is one of the two principal reasons for the introduction of Cas9 into target cells only require plasmids and corresponding synthesized sgRNA.[1] Moreover, as a tool for multiplex gene editing, the CRISPR-Cas9 technology not only sets a platform for scientists to examine genomes from a larger perspective but also gives rise to potential treatments for polygenic diseases.

Various new cellular models have also been prevalent following the implementation of the CRISPR systems, which show significant advantages in epigenetic control and the analysis of the architecture of genome.[1] Epigenetic modifications directly regulate genome functions, thus pivotal to biological processes. Previous studies have been targeting related enzymes with zinc finger proteins and TAL effectors.[1] As a comparison, Cas9 epigenetic effectors can serve as a more versatile platform to remove or insert genetic information at designated epigenetic loci. Likewise, the spatial architecture of different cellular elements plays a crucial role in determining the functional output of genomes.[1] The spacial organization can be influenced dramatically, yet scientists lack a proper live imaging technology. Compared to previous technologies that are unable to show live processes, Cas9 attached with fluorescent protein realizes live-cell-imaging at multiple loci with multiple colors, a potential that can pave the way for a deeper investigation of genome architecture.

Besides its potential for multiplexed gene engineering, in the cultivation of animal models, CRISPR-Cas systems have two other apparent advantages over other genome editing tools. First, these animal models, in contrast to traditional models that only exhibit non-hereditary changes in phenotype, possess the potential to showcase the causal roles of genetic modification.[1] Second, CRISPR-based engineering makes possible the biological research into more intractable and unconventional animal models.[15,16] As of now, groups of researchers throughout the world have achieved targeted gene mutation in many model organisms, including but not limited to mice and rats, cynomolgus monkeys, and teleost fishes.[15,17]

Laboratory mice are prevalent, basic, and effective animal models for research on human diseases.[17] Nonetheless, production of mice with multiple gene mutations has long been expensive and time-consuming, either by performing single-gene modification multiple times

on one subject or intercrossing single mutants.[16] To streamline the generation of feasible laboratory mice with specific multiple modified genes, Wang and colleagues harnessed CRISPR-Cas technology to simultaneously targeting eight alleles of five different genes in mouse embryonic stem cells. As a result, 10% of cells reported to have all eight alleles modified, with no detectable off-target effects induced.[16] Also, Li and colleagues conducted a similar experiment and reached the same conclusion that CRISPR-Cas is an efficient tool for multiplexed genome engineering in mice.[17-19]

Due to the high similarity between human and non-human primates regarding physiological features and genetic makeup, groups of scientists have endeavored to develop animal models with monkeys to simulate human diseases.[15] They believe transgenic monkeys can replicate human genetic conditions more faithfully than other models, especially in neuroscience where certain diseases, such as Alzheimer's disease, cannot be otherwise modeled in mice.[19] In 2013, Niu and colleagues conducted research to apply CRISPR-Cas system to monkeys, overcoming the difficulty in precisely targeting specific sites in developing monkeys as models. After the co-injection of the Cas9 mRNA and synthesized sgRNA into mouse zygotes, their results indicated no off-target mutations even after close detection. In conclusion, CRISPR-Cas9 systems were thus confirmed as a practical approach to applying on monkey genome.[15]

In addtion to traditional animal models, researchers has long been developing other less tractable animal models.[1] For instance, teleost fishes have always been competent candidates for laboratory animals, exemplified predominantly by two species, killifish and zebrafish.[20] Killifish are common animal models, especially suitable for aging experiment due to their notably short life span averaging 4-6 months.[20] On the other hand, the reason for zebrafish's widespread use in biomedical research stems from their physiological similarities with human.[20] For both animals, scientists, conducting research by co-injecting Cas9 mRNA and sgRNAinto embryos, have already yielded efficient specific gene modifications or even a high-throughput workflow.[20,21] All the studies above reconfirm the unlimited potential in genome engineering CRISPR- Cas9 systems possesses.

Biotechnology: Genetic Optimization of Plant Genome and Development of Biofuel

On the basis of reverse engineering and reconstruction, the application of CRISPR-Cas technology in biotechnology remains predominantly in following directions: optimizing agricultural crops, researching plant genome, and developing sustainable and accessible biofuel which relies on the exploration of new biological pathways in algae and corn.[1,22] We herein present several common features in developing transgenic plants from research reports of studies on crops.

The advancement of technologies in the past decade has given rise to noticeable improvement in agricultural productivity, with the focus on decreasing plant susceptibility to

pests, cultivating their resilience on infertile land, and increasing their nutritional value. In this process, transgenesis, introducing a foreign gene into plant genomes, has earned the most highlight.[23] Nevertheless, its drawbacks--failing to take advantage of natural genomes and potentially undermined long-term effects in food--have severely limited its application. With new technologies like CRISPR, mutagenesis takes place to create new genetic variation in plants. We herein present results in developing transgenic plants from research reports of classic studies on crops.[23]

Generally, all studies certify that CRISPR-Cas9 system efficiently delivers transgene and generates crops with multiple mutations, including rice, wheat, maize, soybean.[22,24-26] Genome-modified rice holds the highest limitation for technology among all four species. As the principle food supply, ideally, genetic modifications performed on rice's genome need to remain transgene-free, generate homozygous strain, and possess the capacity to transmit mutations to further generations.[26] Xu and colleagues reported in their results that approximately half of the samples have biallelic mutations with only rare off-target effects rendered in transgene-positive generations as side effects. Also, despite mutations in first generation being non-inheritable, mutations in the follow generation can be steadily transmitted to offsprings. Similarly, Zhang and colleagues explored CRISPR-Cas system as a one-step, highly efficient system to produce "transgene-free, homozygous mutants".[24] The study set the record as the first successful genome engineering in tetraploid wheat thanks to the simultaneous targeting of all four alleles in the chosen locus. Also, some research groups have been capable of producing by biolistically delivering CRISPR-Cas systems to maize embryo cells with more than 80% of the plants biallelic.[25] Other groups have dedicated to targeted gene editing in soybean, achieving none but two loci with detectable off-target mutations.[22] Worthy of mentioning, various studies have also been done to modify gene of interest in many plants, including but not limited to, Arabidopsis thaliana, liverwort, tobacco, and sorghum.[22] As a by-product, the development of sustainable and accessible biofuel relies on the exploration of new metabolic biological pathways in plants which, in turn, can only be achieved upon the prevalence of CRISPR-Cas9 technology.[1]

Medicine: Potential Treatments and Therapeutics for Various Diseases

Upon its discovery, the CRISPR-Cas9 system has remained a captivating mechanism and generated sensational news coverage, in part due to its unlimited potential as a therapeutic technology to treat genetic disorders. As such, scientists have launched several ongoing trials to test its potential in treating human diseases.

Monogenic recessive disorders are caused by the expression of a recessive trait from a

single gene locus. Cas9 can correct the non-functioning genes that cause diseases such as cystic fibrosis and sickle-cell anemia. Instead of introducing functioning foreign gene copies, CRISPR will enable the edited gene to express in a natural context.[1] In additional to loss-of-function mutation, duplication of genomic sequences causes other monogenic recessive disorders. The ability to simultaneously induce multiple double-stranded breaks (DSBs) in target genes prompts the potential use of Cas9 in treatment for said diseases, especially those with mutations at non-coding zones. Dominant-negative disorders in any gene capable of functioning in spite of one mutant copy can be treated by NHEJ to silence the affected gene.[1,27]

Aside from modifying affected genes to treat genetic disorders, CRISPR-Cas9 also provides a countermeasure against nongenetic disease by causing protective mutation on vegetal cells. For instance, in 2007, Lombardo and colleagues designed a possible protection against HIV by inactivating a receptor in lymphocytes.[28] Although they developed the clinical trial with another gene-editing endonuclease, the same experiment can be conducted with Cas9 with a more desirable result.

Lastly, Cas9 technology holds the capability to engineer therapeutic cells to target cancer, making possible for customized medicine to treat rare genetic disease. In 2013 Couzin-Frankel and colleagues edited CAR receptor on T-cell.[1] Later, studies have been improved upon Couzin-Frankel's study to design a universal source of donor cells by reducing the immune reaction when donor cells were injected into mice.[29] Scientists in London have already applied an experimental method to cure a girl with terminal leukemia by editing the immune cells from the donor's blood to attack cancer.[30]

Limitation in Applications

In the pursuit of efficacious treatment for diseases or editing on the human genome, several general obstacles lying in the path of the development of CRISPR technology. First, it's mainly unclear as to which of the two repair pathways will be use by a particular cell. To target multiple disorders simultaneously, scientists need to strive to "biasing DNA repair toward homology-directed repair".[2] Second, due to the limited knowledge scientists have regarding the expression of particular gene loci, the concept of engineering human genome remains theoretically inchoate. Third, in vivo delivery of the CRISPR system into an organism poses a serious problem that needs a solution before any clinical application of CRISPR. Scientists have been researching blood cells and cells in bone marrow as targets. Fourth, research has shown significant off-target effects as opposed to merely minimal detectable unintended mutations in animal models and plants.[31] Therefore, scientists still need to improve the specificity of Cas9. Last, the long-term implications of introducing a hereditable trait remain unclear. As such, harnessing this technology to clinical trials will be a time-consuming project requiring collective effort for all scientists and researchers throughout the world.[1]

ETHICAL COMPLICATIONS

The CRISPR technology proves to be a cutting-edge, powerful "nature's toolbox for genome engineering".[2] Although CRISPR-Cas9 technology has been commonly used in prokaryotic cells, plant and animal genomes, the experimental trials to edit human genomes remain problematic, inchoate and thus ethically and technically controversial.[22]

In Mar 2015, a group of researchers in China first edited human genes on non-viable embryos with an extra set of chromosomes provided by a fertility clinic.[32] Led by Junjiu Huang, an associate professor at Zhongshan University, the team endeavored to edit a gene responsible for causing beta-thalassemia in thousands of children in southern China. However, out of 54 genetically tested embryos, only 28 of them were accurately mutated, a result too immature for further use in clinical trials. The unpromising experimental results prompt researchers all over the world to seek improvement upon their work and revisit the issue of ethical complications and germline editing.[32] Since then, several groups have conducted similar research to analyze the prospect of harness CRISPR-Cas9 technology to edit human genome.

The studies above have reignited a set of ethical debates that re-examine the legitimacy and justification of the applications of CRISPR. To foster open dialogues and reach a consensus on the path forward, one of the co-inventors of CRISPR technology called for a "global moratorium" for CRISPR technology until scientists thoroughly assess the risks of adopting CRISPR technology on a larger scale.[33] A fierce debate among scientists and researchers all over the world ensued in December, 2015 in Washington D.C..

The primary dichotomy in academia focuses on three issues: off-target mutagenesis, germline editing, and non-therapeutic use. In March 2015, Edward Lamphier, the president and CEO of Sangamo BioSciences in California, and Fyodor Urnov, a senior scientist at that company, collaboratively published an article to warn against germline editing due to off-target effects and potential genetic mosaic.[34] The unwanted results obtained from Huang's study offered ground for his argument.[31,32] According to George Daley, a stem-cell biologist at Harvard Medical School, those preliminary results should deter anyone from hastily using CRISPR to edit human genome with an attempt to eradicate diseases.[32] Second, since germline editing is hereditary, any mutations introduced into a person's genome will be transmitted to their offsprings. Therefore, a wrongful modification will put future generations in peril. Also, potential hazardous effects of germline modification, if any, will not surface until years after birth.[34] Last, concerns have heightened regarding the enhancement, removal, and alteration of genes for non-therapeutic use.[35] Researchers fear that gene-editing research will pave the way for unethical use for CRISPR.[32] For instance, different countries pose regulations on the manipulation of human embryos. Such a discrepancy will result in not only differences in the extent of research on genome engineering, but also unequal access to

CRISPR, giving rise to genetic classism.[35]

Right now, scientists are striving to gain a deeper understanding about this "nature's toolbox." To reduce the risks of off-target mutagenesis, some are trying different technical approaches in experimental trials and refine previous work.[32] Meanwhile, other scientists have endeavored to find endonuclease that pairs with RNA instead of DNA to avoid inheritable modifications to the human genome.[36]

PROSPECT

As a groundbreaking technological breakthrough in biology, the CRISPR-Cas9 has enabled many applications as well as raised many ethical implications. Any further technological advancement and thorough ethical assessment on the way forward require the collective effort of scientists throughout the world. As long as we are constantly achieving a deeper understanding of this technology, we the reason to believe that the CRISPR holds the promise of casting a long-standing influence on humanity.

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