# Genome Editing with CRISPR-Cas9 Systems: Basic Research and Clinical Applications

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## Abstract

**B**ACKGROUND: Recently established genome editing technologies will open new avenues for biological research and development. Human genome editing is a powerful tool which offers great scientific and therapeutic potential.

**CONTENT:** Genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology is revolutionizing the gene function studies and possibly will give rise to an entirely new degree of therapeutics for a large range of diseases. Prompt advances in the CRISPR/Cas9 technology, as well as delivery modalities for gene therapy applications, are dismissing the barriers to the clinical translation of this technology. Many studies conducted showed promising results, but as current available technologies for evaluating off-target gene modification, several elements must be addressed to validate the safety of the CRISPR/Cas9 platform for clinical application, as the ethical implication as well.

**SUMMARY:** The CRISPR/Cas9 system is a powerful genome editing technology with the potential to create a variety of novel therapeutics for a range of diseases, many of which are currently untreatable.

**KEYWORDS:** genome editing, CRISPR-Cas, guideRNA, DSB, ZFNs, TALEN

*Indones Biomed J. 2017; 9(1): 1-16*

## Introduction

In the 1970s, a new horizon in Biology era was started. Through the recombinant DNA development, molecular biologist made it possible to study genes and manipulate DNA molecules for the 1st time, harness them to developed more advanced medicine and biotechnology. Researchers now cannot separate the study of DNA from genome context. DNA sequences can be directly edited to modulate their function in endogenous context virtually, and this can be applied to any organism of choice, allowing them to elucidate the functional organization of the genome at the systems level and also identify causal genetic variations.(1)

Eukaryotic genomes contain billions of DNA bases and are not prone to manipulate. One of the breakthroughs in genome manipulation is the development of gene targeting by homologous recombination (HR) that integrates exogenous repair templates that contain sequence homology to the donor site.(2) HR-mediated targeting has facilitated the generation of knocking and knockout animal models via manipulation of germline-competent stem cells, dramatically advancing numerous areas of biological research.

Genome engineering generally refers to the process, the contexts (*e.g.*, epigenetic marks), or the outputs (*e.g.*, transcripts) of targeted modifications of genomes. Efficient and easy to do in eukaryotic and specific mammalian cells,

this ability holds enormous, not only promise but also challenges to transform basic science, biotechnology and medicine. Therefore, a series of programmable nuclease-based genome editing have been developed to enable this genome engineering, particularly in mammalian species. CRISPR-associated protein 9 (Cas9), an RNA-guided endonuclease from microbial adaptive immune system clustered regularly interspaced short palindromic repeats (CRISPR), is the most rapidly developing among the current generation of genome editing technologies, which can be easily virtually targeted to any genomic location of choice by a short RNA guide.(1)

Studies by Haber and Jasin led to the realization that targeted DNA double-strand break (DSB) could largely stimulate genome editing through HR-mediated recombination events.(3-8) Subsequently, Carroll and Chandrasegaran showed the potential of designer nucleases based on zinc finger proteins for efficient, locus-specific HR.(7,8) Moreover, it was presented in the absence of an exogenous homology repair template which localized DSBs can cause insertions or deletion mutations (indels) via the error-prone nonhomologous end-joining (NHEJ) repair pathway.(9) These early genome editing studies built DSB-induced HR and NHEJ as powerful pathways for the versatile and precise modification of eukaryotic genomes. There are four major classes of customizable DNA-binding proteins that have been engineered so far. To achieve effective genome editing via introduction of site-specific DNA DSBs, such as meganucleases derived from microbial mobile genetic elements (10) zinc-finger nucleases (ZFNs) based on eukaryotic transcription factors (11,12), transcription activator-like effectors (TALEs) from Xanthomonas bacteria (13-16) and very recently the RNA-guided DNA endonuclease Cas9 from the type II bacterial adaptive immune system CRISPR (17,18).

New modes of recognition with simplification of custom nucleases development were needed to answer the challenges associated with the engineering of modular DNA-binding proteins. A short guide RNA, via Watson-Crick base pairing recognizing the target DNA and then targeted the CRISPR nuclease Cas9. Within these CRISPR RNAs, the guide sequence typically corresponds to phage sequences, forming the natural mechanism for CRISPR antiviral defense, yet can be simply replaced by a sequence of interest to retarget the Cas9 nuclease. More achievement gained at an unprecedented scale by introducing a battery of short guide RNAs rather than a library of large, bulky proteins to multiplexed targeting by Cas9. The high efficiency of Cas9 as a site-specific targeting nuclease, plus the possibility of highly multiplexed modifications make a high possibility for a broad range of biological applications across basic research to biotechnology and medicine.

The era of straightforward genome editing boosts ethical questions that will need to be addressed by scientists and society widely. How can we utilize this powerful tool in such a way as to assure maximum benefit while minimizing risks?(19) The identification of the CRISPR-Cas9 technology underscores the way in which many inventions having advanced molecular biology and medicine emerged, through basic research on natural mechanisms of DNA replication, repair, and defense against viruses. In many cases, key methodologies turned up from the study of bacteria. The CRISPR-Cas9 technology originated through a similar process, once the mechanism underlying how CRISPR-Cas9 system works was understood, it could be harnessed for applications in molecular biology and genetics that were not previously envisioned.(19) This review will give us the insight of how far this genome-editing technology which is applied as a new hope in treating currently "impossible" illness.

## CRISPR-Cas Immune System in Procaryotes

It was conceived after a yogurt company in 2007 identified an unexpected defense mechanism that its bacteria use to fight off viruses. Then the birth announced in 2012 followed by rapid and crucial progress within a year. Now, it has matured into a molecular marvel, and much of the world, not just biologists, are taking notice of the genome-editing method CRISPR, Science's 2015 Breakthrough of the Year.(20) The battle for survival between bacteria and the viruses which infect them (phages) has led to the evolution of many bacterial defense systems and phage-encoded antagonists of these systems. CRISPR and the Cas genes comprise an adaptive immune system that is one of the most widespread means by which bacteria defend themselves against phages.(21-24)

CRISPR-Cas systems are classified into three major types, namely type I, type II and type III, and also into 12 subtypes, given their genetic content and structural and functional differences.(25,26) These types and subtypes core defining features based on the Cas genes and the proteins they encode. The immensely varies genetics and functions illustrate how diverse the biochemical functions they can carry throughout CRISPR-mediated immunity in each different steps. The RNA recognition motif is

widely spread in many Cas proteins, and most of the Cas families of proteins carry functional domains which interact with nucleic acids, such as DNA binding, RNA binding, helicase and nuclease motifs.(25-29) Cas 1 and Cas 2 occurs genetically across types and subtypes, while Cas 3, 9 and 10 respectively designated as the signature genes for type I, II and III. Type II system phylogenetically has only been identified in bacteria, yet there is a bias for type I systems in bacteria and type III systems in archaea and hyperthermophiles.(30)

Adaptive immunity occurs in three stages [for recent reviews, (30-32)]. Those stages are: (i) insertion of a short sequence of the invading DNA into the CRISPR array as a spacer sequence; (ii) transcription of precursor CRISPR RNA (pre-crRNA) which goes through maturation to yield individual crRNAs, each composed of a repeat portion and an invader-targeting spacer portion; also (iii) crRNA-directed cleavage of foreign nucleic acid because of Cas proteins at sites complementary to crRNA spacer sequence (Figure 1). In this overall theme, three CRISPR-Cas system types (I, II and III) use distinct molecular mechanisms to achieve nucleic acid recognition and cleavage.(22,25) The protospacer adjacent motif (PAM), or also known as a short sequence motif adjacent to the crRNA-targeted sequence on the invading DNA, has significant role in the stages of adaptation and interference in type I and type II systems.(33-36) The type I and type III systems use a large complex of Cas proteins for crRNA-guided targeting.(37-42) However, the type II system requires merely a single protein for RNA-guided DNA recognition and cleavage, a property that proved to be highly useful for genome engineering applications.(19,43,44)

CRISPR systems are adaptable immune mechanisms used by many bacteria to protect themselves from foreign nucleic acids, such as viruses or plasmids.(30,45-47) Type II CRISPR systems incorporate sequences from invading DNA between CRISPR repeat sequences encoded as arrays in the bacterial host genome. Transcripts of the CRISPR repeat arrays are processed into crRNA, each harboring a variable sequence transcribed from the invading DNA, or known as the "protospacer" sequence, and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA, which is known as the transactivating crRNA (tracrRNA) (48), and these two RNAs complex with the Cas9 nuclease (43). Only when PAMs located adjacent to the protospacer-encoded portion of the crRNA, Cas9 will be directed to cleave complementary target-DNA sequences. The type II CRISPR system from *Streptococcus pyogenes* has been adjusted for inducing sequence-specific DSBs and targeted genome editing.(43) In simple words, genome editing could be performed if the Cas9 nuclease and a guide RNA, amount to a fusion of a crRNA and a fixed tracrRNA can be incorporated into and/or expressed in cells or an organism.(49)

Targeted genome editing using engineered nucleases has rapidly moved from being a niche technology to a mainstream method used by many biological researchers. This widespread adoption has been greatly fueled by the emergence of CRISPR technology, a new approach for generating RNA-guided nucleases, for example Cas9. Genome editing mediated by these nucleases has been used to quickly, easily and efficiently modify endogenous genes in a broad variety of biomedically relevant cell types and in organisms that have traditionally been challenging
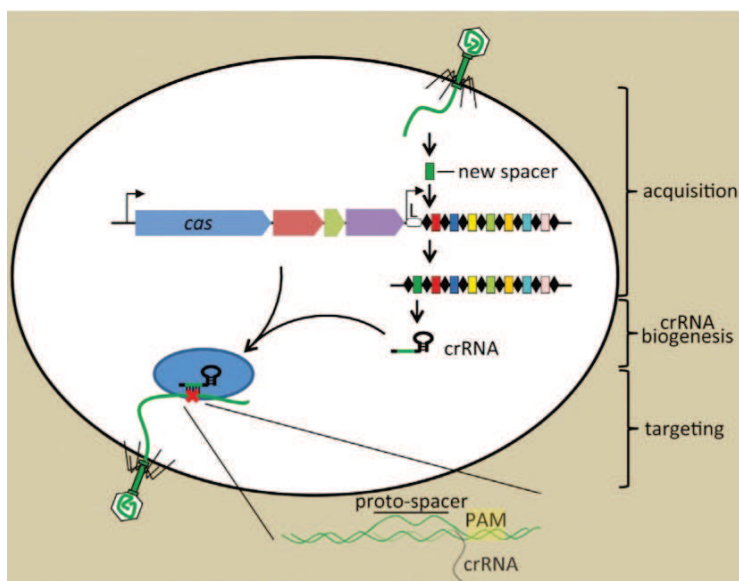


**Figure 1. The Three Stages of CRISPR Immunity.**(30) (Adapted with permission from Cell Press).

to manipulate genetically. The potency of these systems to perform targeted, hugely efficient alterations of genome sequence and gene expression will certainly transform biological research and spur the development of novel molecular therapeutics for human disease.(49)

## Genome-Editing Technologies

We need to understand the complex and dynamic functions of multiple genes network to able precisely manipulate genes, involving genes pathways which drive many essential cellular activities, including genome replication and repair, cell division and differentiation, also disease progression and inheritance.(50) The science of genetics relies heavily on the analysis of mutations and the phenotypes they cause. Many geneticists explore targetable nucleases to particular genes of interest which provide the ability for any direct mutations and precise sequence changes. This will continually create excitation to improve more new technologies.(51-53) The development of tools to generate DNA breaks, activate (54), repress or label genomic loci (55,56) and remodel chromatin (57) in a controlled, targeted manner will hugely aid the studies of a broad range of biological issues, including gene and genomic functions. The ability to specifically modify the genome also holds significant promise for targeted gene therapies.(58)

Gene therapy has historically been defined as insertion of new genes into human cells. Current technologies in this field facilitate a new paradigm to achieve a therapeutic effect by precisely manipulating one or more sequences of the human genome, include correcting gene mutations that cause disease, adding genes to specific sites in the genome for therapeutic purpose, or removing destructive genes or genome sequences.(59) One way to explore the function of genes is to agitate their expression through repression. The dominant tool for programmed knockdown of mRNAs is RNA interference (RNAi).(60) Yet, RNAi has pervasive problems with off-target effects, which can be especially confounding in the context of large-scale screens.(61-63) RNAi is mediated by cytoplasmic argonaute proteins so that gene silencing could be performed by depletion of cytosolic mRNA targets.(64)

The discovery of using targeted DNA DSBs to stimulate the endogenous cellular repair machinery soon become the foundation to gene editing. Breaks in the DNA are typically repaired through one of two major pathways, which are homology-directed repair (HDR) or NHEJ.(65)

HDR depends on strand invasion of the broken end into a homologous sequence and subsequent repair of the break in a template-dependent manner.(66) Maria Jasin lab reported an original work about stimulating the efficiency of gene targeting through HR in mammalian cells via a DSB introducing at the target site.(6,67,68) Alternatively, NHEJ functions to repair DSBs without a template through direct religation of the cleaved ends.(69) This repair pathway is error-prone and often leads to indels at breaking site. Stimulation of NHEJ by site-specific DSBs has been used to disrupt target genes in a huge variation of cell types and organisms by taking some benefit of these indels to alter the reading frame of a gene.(9,70-73) Armed with the capability to harness the cell's endogenous DNA repair machinery, it is now feasible to engineer a broad variety of genomic changes in a site-specific manner.(59,74) The repairing process a DSB is shown by Figure 2.

A concerted effort by various academic and industrial groups to develop programmable DNA recognition and cleavage technologies was inspired by the demonstration of a site-specific DSB could be utilized to attain gene interruption and gene targeting in otherwise refractory cells.

Over the last 15 years, this effort has yielded several designer endonuclease platforms, including meganucleases (75,76), ZFNs (77,78), TALENs (79,80) and CRISPR (19,81) (Figure 3). ZFNs consist of DNA-binding modules derived from natural transcription factors which are linked to the nuclease domain of the Type IIS restriction enzyme, FokI.(18,19) To cut the DNA, two ZFN molecules are required in a single site target, then the nuclease domain will be dimerized. TALENs employ DNA-binding modules from bacterial TALEs linked
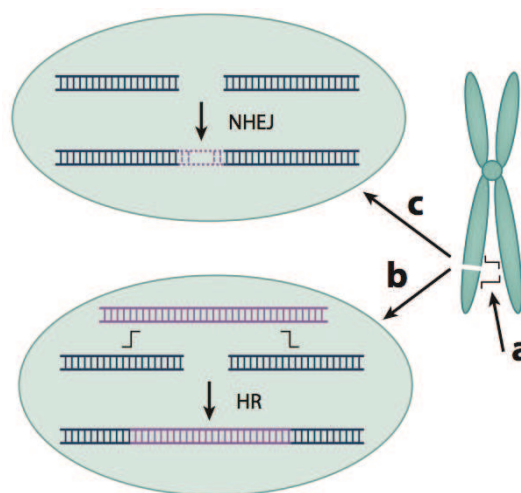


**Figure 2. Repair of a DBS .**(74) (Adapted with permission from Annual Reviews).

to the same FokI cleavage domain.(20) The CRISPR/ Cas RNA-guided nucleases (RGNs) count on base-pairing between a guide RNA and the DNA target for recognition and on a multifunctional Cas9 protein for cleavage.(21, 22) We will discuss all of these types of reagents in detail after this. There are members of another class which have long recognition sites (15–30 bp) but do not have distinct binding and cleavage domains known as the homing endonucleases (HE) or meganucleases.(Figure4).

Once an endonuclease is designed to cut a given target sequence, it must be delivered to the therapeutically relevant cell. While there are few limitations on delivery to cultured cell lines, delivery to primary cells *ex vivo* (such as hematopoietic stem cells and T-cells) and *in vivo* delivery (such as to the liver) have many of the same limitations as other classical "gene therapy" approaches. It is the constraints on the immunogenicity and packaging capacity of the delivery modality.(81) Specificity of genome-editing reagents is paramount in therapeutics, as

off-target mutations could lead to unintended side-effects. The inherent specificity of a given enzyme (independent of the target choice and its relative abundance of near-cognate matches in the genome) is dictated by both the DNA-binding specificity of enzyme and the catalytic mechanism employed to introduce the DNA break.(81)

A number of factors contribute to the absolute efficiency of a given gene-editing procedure, but most paramount is the quality of the nuclease. Considerations and observations for the overall editing rate, the number of edits that can be made simultaneously, and how editing outcome can potentially be influenced by the unique biochemistry of the different platforms. In addition to the quality and attributes of the nuclease, efficiency of editing can depend on several platform independent variables including the cell type (75), cell cycle (82), epigenetics at the target site (83,84) and delivery kinetics.

We still facing some tricky challenges on the technological side, about how to guarantee off-target
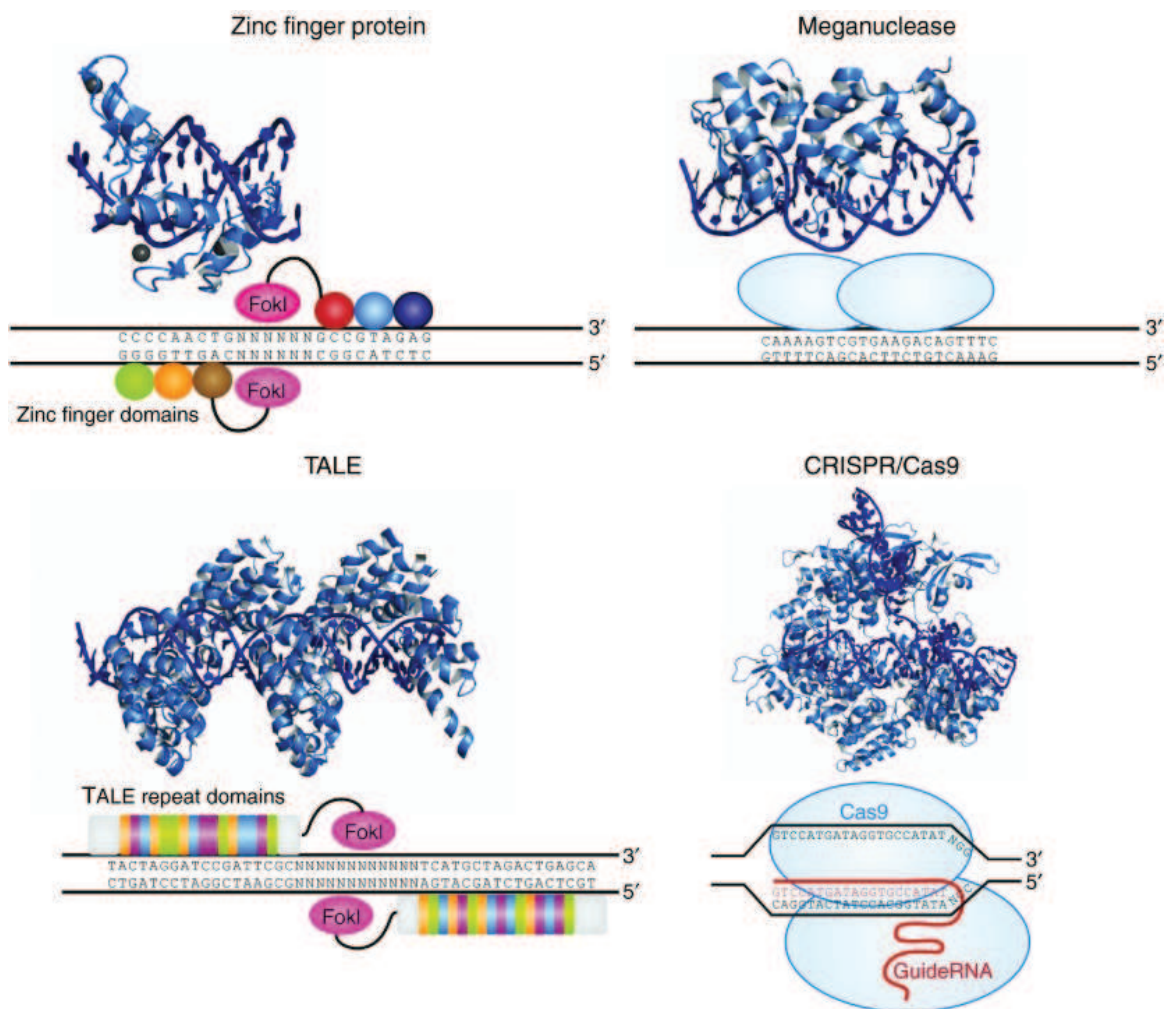


**Figure 3. Common DNA targeting platforms for genome editing.**(59) (Adapted with permission from American Society of Gene & Cell Therapy).
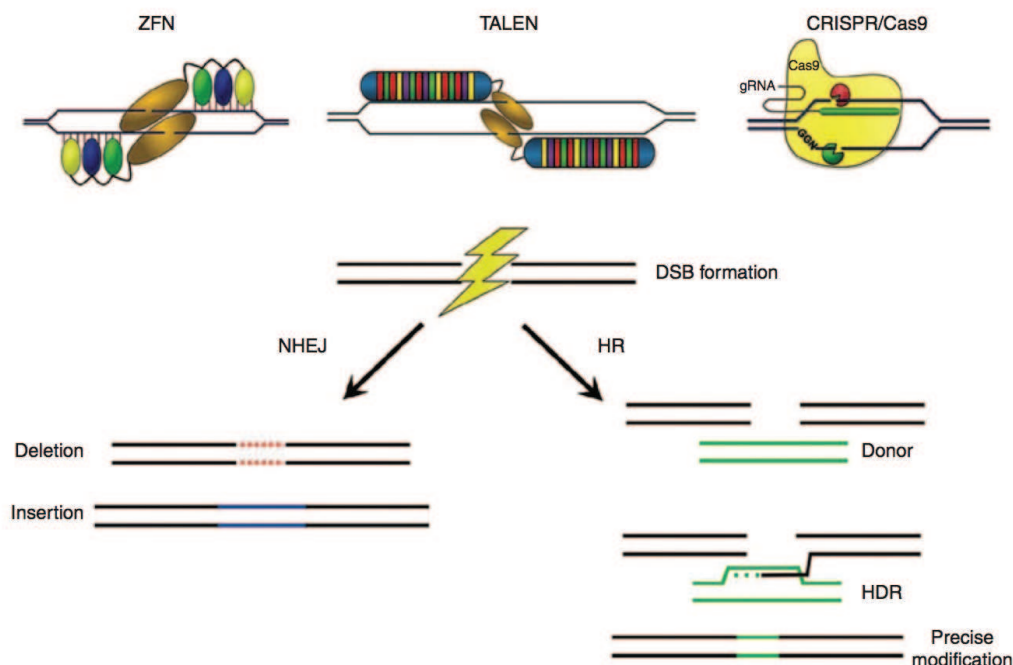
**Figure 4. Classes of designer nucleases and gene-editing outcomes.** Targeted DSB can be induced using ZFNs, TALENs, or CRISPR/Cas9. DNA breaks are repaired via endogenous repair pathways such as NHEJ and HR.(58) (Adapted with permission from American Society of Gene & Cell Therapy).

cleavage will not lead to mutations and compromise the expected benefits. Good progress is being made in both minimizing (85-88) and detecting (89) off-target effects, but the bar should be set very high before heritable modifications are pursued. We don't have decent control over the types of modifications produced at the genomic target. After CRISPR/Cas (or ZFN or TALEN) cleavage, cellular repair continues to introduce local mutations or copy sequences from a homologous donor DNA. The second process was more covetable for most therapeutic applications, but the former typically dominates and must be minimized. After directing most of the current research to solve this issue, no effective approach has yet arrived. Any effective alternatives in current practices, particularly pre-implantation screening should be pointed out, and on the clinical side we also need to identify conditions as reasonable candidates for germline treatment.(90) These alternatives have their own drawbacks, both practical and ethical. It seems possible that germline gene correction may ultimately be suggested as an alternative to such procedures, once technical standards have been met.(91)

Emerging gene-editing technologies are nearing a revolutionary phase in genetic medicine: accurately modifying or repairing causal genetic defects. Nowadays, these can carry out via programmable rare-cutting endonucleases, generate a targeted DNA breaks, involving any manipulation on DNA sequence, such as knocking out a deleterious gene, introducing a particular mutation, or directly repairing a deformed sequence by site-specific recombination, then engage and exploit endogenous DNA repair pathways to deliver site-specific genetic changes.(81)

## CRISPR-Cas 9: A Genome Engineering Tool

Few discoveries transform a discipline overnight, but biologists these days can manipulate cells in ways impossible before, thanks to a peculiar form of prokaryotic adaptive immunity mediated by CRISPR. Many delicate studies performed and make clear how these immune systems function in bacteria and convince researchers about the technological potential of Cas9, an RNA-guided DNA cleaving enzyme, in genome engineering.(92) Precise genome engineering in live cells at any locus promises to facilitate basic research and to enable personalized medicine. In particular, the latest development of the CRISPR-Cas9 system into a versatile and easy-to-use editing tool (43) has been celebrated as a scientific breakthrough in the field. As genome engineering is adapted to clinical applications, a high level of precision, especially the avoidance of editing at sites other than the intended target, will be indispensable.(93)

The CRISPR-Cas system is widely found in bacterial and archaeal genomes as a defense mechanism against invading viruses and plasmids.(30,47,94-97) To target DNA, the type II CRISPR-Cas system from *Streptococcus pyogenes* depend on one protein, which is the nuclease Cas9, and two noncoding RNAs, namely crRNA and tracrRNA, which further can be fused into one single guide RNA (sgRNA). The Cas9/sgRNA complex binds double-stranded DNA sequences which consist of a sequence match to the first 17-20 nucleotides of the sgRNA if the target sequence is followed by a PAM (Figure 5). After the two independent nuclease domains in Cas9 bound, they will each cleave one of the DNA strands three bases upstream of the PAM, and leaving a blunt end DNA DSB. DSBs can be repaired mainly through either NHEJ pathway or HDR. NHEJ typically leads to short indels near the cutting site, whilst HDR can be used to introduce specific sequences into the cutting site, if exogenous template DNA is available. This finding bricked the way of the Cas9 application as a genome-engineering tool in other species. In this review, we only focus on target specificity of the CRISPR-Cas9 system, so for further discussion, we refer readers to other excellent articles as follows: (1,98-100).

Following the 2012 publication of Jinek, *et al.,* three studies in January 2013 illustrated that CRISPR-Cas9 represents an efficient tool to refine the genomes of human cells.(17,18,43,101) The "humanized" versions of *Streptococcus pyogenes* Cas9 (17,18,101) and *Streptococcus thermophilus* Cas9 (17) were coexpressed with custom-designed sgRNAs (17,18,101) or with tracrRNA coexpressed with custom-designed crRNAs in human embryonic kidney,



**Figure 5. The CRISPR-Cas9 system.** The sgRNA (purple) targets the Cas9 protein to genomic sites containing sequences complementary to the 5' end of the sgRNA. The target DNA sequence needs to be followed by a PAM.(100) (Adapted with permission from Higher Education Press and Springer-Verlag Berlin Heidelberg).

chronic myelogenous leukemia, or induced pluripotent stem cells (iPSC) (17,18,101), as well as in mouse cells (17). The expected alterations in the target DNA were detected, indicating that site-specific DSBs by RNA-guided Cas9 had stimulated gene editing by non-homologous end joining repair or gene replacement by HDR. Targeting with multiple sgRNAs, also referred to as multiplexing, was successfully achieved.(17,18) RNA-programmable *Streptococcus pyogenes* Cas9-mediated editing has now been applied to various human cells and embryonic stem cells.(1,49,98,102-105). Although direct comparisons can be inconvenient to assess because of differences in target sites and protein expression levels, some analyses display that CRISPR-Cas9-mediated editing efficiencies can reach 80% or more depending on the target, which is as high as or higher than the levels observed using ZFNs or TALENs.(104,106)

Detailed characterization of the Cas9-guide complex and its interference mechanism in the past (38,107) has led to a revolution of CRISPR-based genetic engineering (108,109). The revolution includes directed recombination in bacteria (110,111), transcriptional activation and repression of synthetic regulons (112,113) and genome editing in eukaryotic cells, ranging from yeast to plant and from zebrafish to human (114). In addition for the directed silencing of the gene expression alternative system, we may also use RNA targeting by type III-B systems, as well as by Cas9. In terms of applications of CRISPR-associated nucleases in general, and Cas9 in particular, the sky seems to be the limit. Many improvements could be endeavor such as lowering the stringency of its PAM dependence and reducing its off-target cleavage. It's crucial to also improve our understanding of the fundamental details of CRISPR-Cas structure and function to optimize further expansion of CRISPR-Cas system applicability.(115)

The significant advantages of the CRISPR/Cas system versus ZFNs and TALENs are as follows: (1) a single protein is required, and it is always the same, no protein engineering is needed; (2) targeting depends on base pairing, so sgRNA design requires only knowledge of the Watson-Crick rules; (3) new sgRNAs are very easily produced, (4) because of advantages 1-3, it is feasible to attack multiple targets simultaneously with mixed sgRNAs.(74)

The epigenome is a layer of regulatory information superimposed on the genome. It's involved in the positions, compositions, and modifications of nucleosomes as well as modifications of DNA bases.(116) The epigenetics
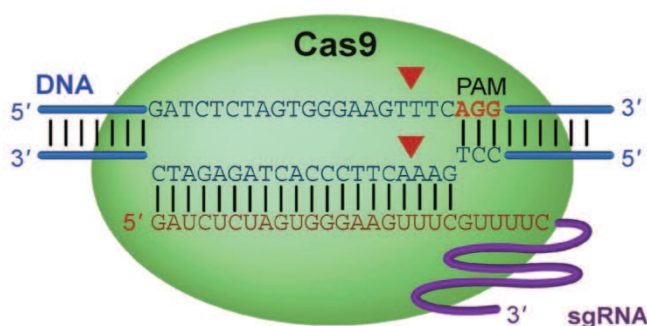
community has done a major ongoing effort that has already yielded millions of putative regulatory elements to map the epigenomic components genome-wide in many cell types. Recent reports in Nature Biotechnology by Hilton, *et al.,* and in Nature Methods by Kearns, *et al.,* have now addressed this need with an epigenome editing technology based on CRISPR-Cas9.(116-118) The studies that combining CRISPR-Cas9 ability in directing effectors to specific genomic sites with well-characterized, histone-modifying domains functionally characterized the chromatin states of specific genomic elements and show the robust, specific transcriptional outcomes of histone modifications modulation.(119) The outcome of those studies suggest that using CRISPR-Cas9 for targeted epigenome editing was very promising to be a simple but powerful method for probing the effects of histone modifications at specific loci.

## CRISPR-Cas 9 Applications

The Human Genome Project has earned a fairly complete catalog of cellular components, and a major goal is moving forward will be to classify all genetic elements involved in normal biological processes and disease.(120) With advances in gene editing enabled by the CRISPR-Cas system (17,18,43,121), it is no longer quixotic to seek a comprehensive picture of cellular circuitry for human cells (122).

Journeyed from dreams about treating inherited disorders, gene therapy now encircled a wide range of strategies, started from the assumption that monogenic diseases would be the easiest to target. Classically, this therapy focused on enhancing endogenous DNA gene expression. These approaches rely on variety of viral vectors to transfer functional genes, adjusted to the virus intrinsic ability to effectively transduce in human cells.

Thus, gene editing grows further on a thought about utilizing targeted disruption, insertion, excision and correction in both *ex vivo* and *in vivo* settings to permanently modify a genomic sequence of interest. Yet we foresee these advances to revolutionize larger without ignoring the safety concerns, modification efficacy and gene-editing tools to target cell types.(123) CRISPR-Cas9 nuclease system raises the hopes for a robust and precise modifying genomic sequences, make it possible to study of gene function at nucleotide resolution. To find a permanent way of correcting genetic mutation will rise an important advance in future therapy.(50)

In the year of 2012, Cas9, programmed initial demonstration to cut various DNA sites *in vitro*, was published in a flurry of papers in the next year, suggested that this platform could efficiently function in various cells and organisms.(43) Initial proof-of-principle studies displayed that Cas9 could be targeted to endogenous genes in bacteria (110), cultured altered human cancer cell lines and human pluripotent stem cells in culture (17,18,101,121), also in a whole organism, the zebrafish (106). Subsequently, Cas9 has been used to refine genes in yeast (124), tobacco (125,126), thale cress (127), rice (127,128), wheat (127), sorghum (129), mice (130,131), rats (132), rabbits (133), frogs (134), fruit flies (135,136), silkworms (137) and roundworms (138).

Here we highlight a few examples that illustrate the power of the technology. The first instance is the precise reproduction of tumor-associated chromosomal translocations that come about during carcinogenesis through an illegitimate non-homologous joining of two chromosomes. The ability of CRISPR-Cas9 to introduce DSBs at defined positions has made it possible to generate human cell lines and primary cells bearing chromosomal translations resembling those described in cancers such as lung cancer (137), acute myeloid leukemia, and Ewing's sarcoma (138,139) (Figure 6). An improved method to generate liver cancer or myeloid malignancy models in mice facilitated by CRISPR-Cas9 was recently reported (140,141). CRISPR-Cas9 thus provides a robust technology for studying genomic rearrangements and the development and progression of cancers or other diseases.(19)

Cas9 has already been widely used as a research tool, but the research expanded excitingly to develop Cas9 as a therapeutic technology for treating genetic disorders. Monogenic recessive disorder due to loss-of-function mutations (such as cystic fibrosis, sickle-cell anemia, or Duchenne muscular dystrophy) will be a prospect case to be corrected causatively by Cas9. This has numbers of advantages over traditional methods of gene augmentation which transfer functional genetic copies trough viral vector-mediated overexpression-particularly that the newly functional gene is expressed in its natural context. For disorders with haplosufficient affected genes (such as transthyretin-related hereditary amyloidosis or dominant forms of retinitis pigmentosum), Therapeutic benefit possibly achieved by using NHEJ to inactivate the mutated allele.

In addition to repairing mutations underlying inherited disorders, Cas9-mediated genome editing could also use to combat non-genetic or complex diseases by acquaint
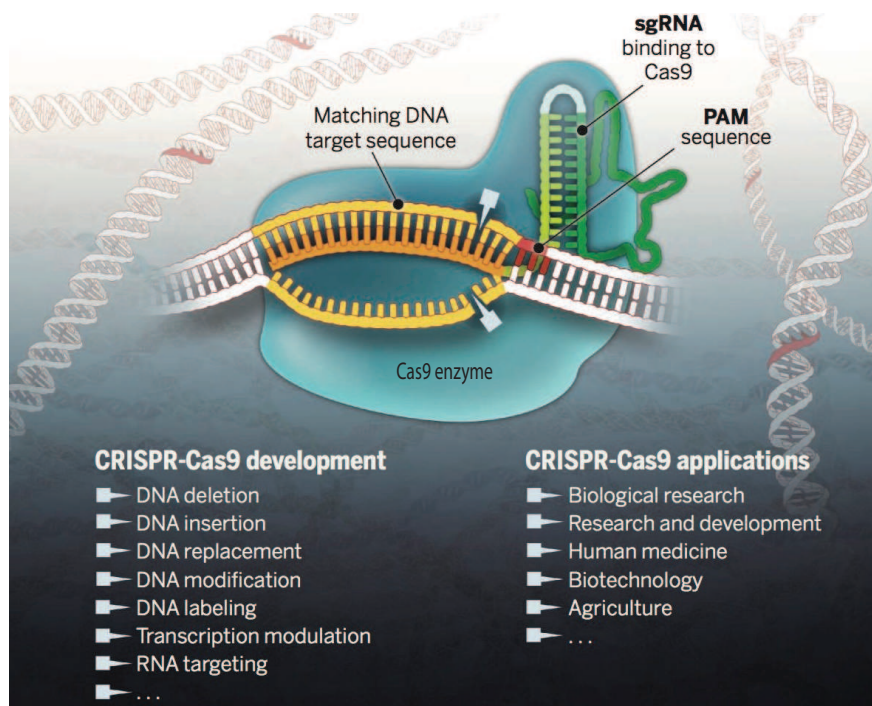
**Figure 6. The Cas9 enzyme generates breaks in double-stranded DNA by using its two catalytic centers (blades) to cleave each strand of a DNA target site (yellow) beside a PAM sequence (red) and matching the 20-nucleotide sequence (orange) of the sgRNA).**(19) (Adapted with permission from American Association for the Advancement of Science).

protective mutations in somatic tissues. For example, NHEJ-mediated inactivation of the C-C chemokine receptor type 5 (CCR5) in lymphocytes (142) may be a feasible strategy for circumventing HIV infection, while deletion of protein convertase subtilisin/kexin type 9 (PCSK9) (143) or angiopoietin (144) may provide therapeutic effects against statin-resistant hypercholesterolemia or hyperlipidemia. The unique advantage of this method rather than using sgRNA-mediated protein knockdown is the ability to achieve permanent therapeutic benefit in one-time treatment. Of course every gene therapies proposed therapeutic use must count the favorable benefit-risk ratio.(1)

The ability to manipulate any genomic sequence by gene editing has created diverse opportunities to treating many different diseases and disorders (Figure 7). We will discuss the major categories of disease indications that have been pursued in preclinical models, as well as highlight the ongoing or planned clinical trials using gene-editing strategies. Gene editing applied most straightforward *ex vivo* in gene knocking out for autologous cell therapy using the relatively efficient NHEJ mechanism, where somatic cells can be isolated, modified and delivered back to the patient. By this method, viral infection or replication can be prevented. Hence, the most advanced gene-editing strategy to date is the *ex vivo* modification of T-cells to knock out the CCR5 co-receptor used for primary HIV infection. The initiative study showed that in mice engrafted with T-cells in which the CCR5 gene had been knocked out by ZFNs, the

viral loads decreased and cluster of differentiation (CD)4+ T-cell counts increased.(145)

Beyond addressing HIV infection, all of the gene-editing platforms have also been applied to various other viral pathogens (146), including hepatitis B virus (147-154), herpes simplex virus (155-157), and human papillomavirus (158). Viral genomes will be removed by degradation following nuclease cleavage and by targeting crucial genes needed for genome stability, maintenance, and replication.

Cancer immunotherapy has been largely recognized as one of the biggest advances in biomedical research in recent years.(159) In particular, adoptive T-cell immunotherapy, in which autologous T-cells are designed to attack cancer antigens *ex vivo* and transferred back to the patient, has been impressively successful at treating some cases of lymphoma, leukemia, and melanoma.(160) Undeterred with those successes and promising ongoing clinical trial, gene editing could even more improve the potency of T-cell immunotherapy, as showed by many studies focused on knocking out the endogenous T-cell receptors with engineered nucleases.(161-164)

Similarly, the successful of CD34+ hematopoietic stem cell (HSC) gene editing and human pluripotent cells capable of differentiating into erythroid progenitors has opened new options for treating other hematologic disorders, including treating specific E6V point mutation in the β-globin gene account for sickle cell disease, and other types of mutations to β-globin as the cause of β-thalassemia.
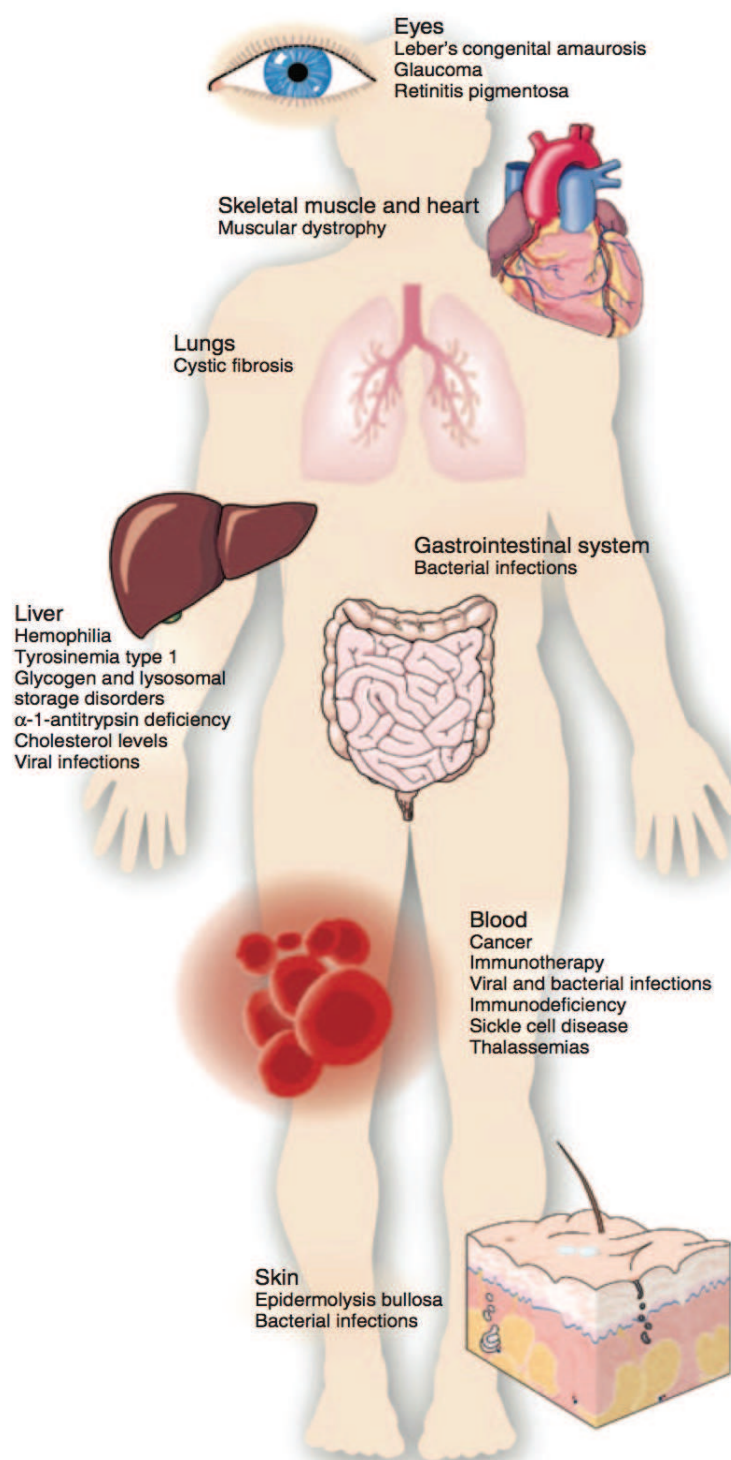
**Eyes**
Leber's congenital amaurosis
Glaucoma
Retinitis pigmentosa

**Skeletal muscle and heart**
Muscular dystrophy

**Lungs**
Cystic fibrosis

**Liver**
Hemophilia
Tyrosinemia type 1
Glycogen and lysosomal
storage disorders
α-1-antitrypsin deficiency
Cholesterol levels
Viral infections

**Gastrointestinal system**
Bacterial infections

**Blood**
Cancer
Immunotherapy
Viral and bacterial infections
Immunodeficiency
Sickle cell disease
Thalassemias

**Skin**
Epidermolysis bullosa
Bacterial infections

**Figure 7. Diversity of targets for therapeutic genome editing.**(59) (Adapted with permission from American Society of Gene & Cell Therapy).

These globin mutations have been corrected by gene editing both in human iPSC that can be differentiated into functional erythrocytes (164-167) and directly in CD34+ HSCs.(168) Similar approaches have been developed for targeted integration  of therapeutic transgenes into safe harbor sites in human iPSCs for α-thalassemia (169) and Fanconi anemia (170).

In applications to the human therapy, we need to make sure that the treatment will cure the one intentionally addressed without causing another unexpected condition. Auspiciously that we are able to direct subtle changes to the endogenous target, so dangers inherent in earlier methods  for delivery of therapeutic genes could be avoided.(171)

## Ethical Implications of Human Germline Genome Editing

In human and non-human modifying, genome engineering technology didn't offer lateral potencies. While in humans this technology preferred directed for curing a genetic disease, in other organisms, it present methods to reshape the biosphere for the advantage of the environment and human societies. However, we still do not know the unknown risks to human health and well-being yet.(172)

The CRIPSR-Cas9 system is quite simple that allows any researcher with knowledge of biology molecular to modify genome, and conduct a previously difficult or impossible study as feasible. For example, a study on CRISPR-Cas9 to correct genetic defects in whole animals by an introduction of DNA sequence, such as replacing a mutated gene underlying liver-based metabolic disease in a mouse model.(173) The technique also enables DNA sequence changes in pluripotent embryonic stem cells (18) which can then be cultured to produce specific tissues, such as cardiomyocytes or neurons (174). Those basic researches will lay a frame of the foundation for real strategies to cure human disease. The ability of CRISPR-Cas9 to precisely replicate the genetic basis for human diseases in model organisms can be utilized to lead an unprecedented insight into previously enigmatic disorders.

CRISPR-Cas9 technology, as well as other genome engineering methods, can be used to alter the DNA in the nuclei of reproductive cells which transmit information from one generation to the next (an organism's "germline"). Thus, it is now possible to carry out genome modification in fertilized animal eggs or embryos, thereby shifting the genetic makeup of every differentiated cell in an organism and so ensuring that the alterations will be passed on to the organism's progeny. It was also not impossible to apply this simple and widely available to set changes in human germline.(172)

Early this year, about a month after the National Academy of Sciences (NAS) International Summit on Human Gene Editing, Stafford Academy for Technology (STAT) and the Harvard School of Public Health conducted a poll of adults in the US on gene editing. Their main finding was that an overwhelming majority of Americans (83%) believe it should be illegal to use gene editing to "improve the intelligence or physical characteristics" of unborn babies. The summit stated a consensus, not a new law but did conclude that it would be "irresponsible to

proceed (with germline genetic manipulations) without resolution of safety issues and broad social consensus on application."(175)

Although it was very possible to bring CRISPR-Cas-9 to modify human germline, we have to consider that patient safety is paramount among the arguments against modifying the human germline (egg and sperm cells), because we can never be assured if the mosaic embryo created, its germline may carry the genetic alteration or not. Philosophically or ethically justifiable applications for this technology are moot until it becomes possible to prove safe outcomes and gain reproducible data over multiple generations. Due to safety and serious ethical reasons, around 40 countries until today, discourage or even ban germline modifications in human. Some countries indeed prohibited it a decade before the technical feasibility was confirmed in rats in the year 2009.(71,90)

## Conclusion

The story of how a mysterious prokaryotic viral defense system turn into one of the most powerful and versatile platforms for engineering biology irradiate the importance of basic science research. Just as recombinant DNA technology benefited from the basic investigation of the restriction enzymes which are central to warfare between phage and bacteria, the latest generation of Cas9-based genome engineering tools are also based on components from the microbial antiphage defense system. It is likely that the future solutions for efficient and precise gene modification will be found in as of yet unexplored corners of the rich biological diversity of nature.

At last, by understanding the current studies of CRISPR application in creating tissue-based treatments for cancer and other currently untreatable diseases, every scientists' dream about genetic manipulation is getting closer. Charpentier described its capabilities as "mind-blowing." For better or worse, the simple truth is, we all now live in CRISPR's world, and what next this method will grow, the sky is the limit.

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