Vol:2, Pg:91-114, Yr: 2021-AJABTR

**A Mini Review on CRISPR -Cas: The Cutting Edge Of DNA Editing**

Chitta Ranjan Santra

Netaji Nagar Day College, Netaji Nagar, Kolkata, West Bengal 700092 , India

Corresponding: crsantra@gmail.com

**Abstract**

The ability to control and modify DNA—the code of life, has long been cherished by the scientists. They want to use genome editing to investigate different diseases that affect humans. With this easier access to DNA sequences, scientists are on the verge of a third revolution that will deeply impact our lives, to the extent that computers have changed society: we are entering the era of “gene editing”, following the era of “gene reading”. Gene editing is the rational and precise modification of DNA sequences program in living cells and organisms. Nuclease-based gene editing is already widely used in research as a cost-effective, fast, and easy way to conduct genetic experiments. Other recent approaches to targeted genome modification – zinc-finger nucleases [ZFNs] and transcription-activator like effector nucleases [TALENs]– enable researchers to generate permanent mutations by introducing double-stranded breaks to activate repair pathways. These approaches are costly and time-consuming to engineer, limiting their widespread use, particularly for large scale, high-throughput studies. A new gene editing technology known as CRISPR-Cas9 offers the potential for substantial improvement over previous technologies in that it is simple to use and inexpensive and has a relatively high degree of precision and efficiency. These characteristics have led many in the scientific and business communities to assert that CRISPR-Cas9 will lead to groundbreaking advances in many fields, including agriculture, energy, ecosystem conservation, and in the investigation, prevention, and treatment of diseases. The advent of facile genome engineering using the bacterial RNA-guided CRISPR-Cas9 system in animals and plants is transforming biology. This article is an aim to understand CRISPR (CLUSTERED REGULARLY INTERSPACED PALINDROMIC REPEAT) biology from its initial discovery through the elucidation of the CRISPR-Cas9 enzyme mechanism, which has set the stage for remarkable developments using this technology to modify, regulate, or mark genomic loci in a wide variety of cells and organisms from all three domains of life. CRISPR-Cas9 has triggered a revolution in which laboratories around the world are using the technology for innovative applications in biology. The power of this technology to systematically analyze gene functions in mammalian cells, study genomic rearrangements and the progression of cancers or other diseases, and potentially correct genetic mutations responsible for inherited disorders. CRISPR-Cas9 is having a major impact on functional genomics conducted in experimental systems. Its application in genome-wide studies will enable large-scale screening for drug targets and other phenotypes and will facilitate the generation of engineered animal models that will benefit pharmacological studies and the understanding of human diseases. CRISPR-Cas9 applications in plants and fungi also promise to change the pace and course of agricultural research. Future research directions to improve the technology will include engineering or identifying smaller Cas9 variants with distinct specificity that may be more amenable to delivery in human cells. Understanding the homology- directed repair mechanisms that follow Cas9-mediated DNA cleavage will enhance.

**2. Introduction**

****  Humans have an estimated 100 trillion (1012) cells. If the DNA contained in each cell's nucleus was completely unfolded, it would measure nearly 2 meters in length. In other words, if the all the DNA from every cell in a person's body were patched up together they would form a strand of 200 billion kilometers, or more than 1,000 times the distance between Earth and the Sun. We can imagine that the genome is a book. There are twenty three chapters, called CHROMOSOMES. Each chapter containsseveral thousand stories, called GENES. Each story is made up of paragraphs, called EXONS, which are interrupted by advertisements called INTORNS. Each paragraph is made up of words, called CODONS. Each word is written in letters called BASES. [1 ]

The four letters A, T, G, and C represent the molecules that make up our DNA, which is subject to potentially deadly or disabling mutations from the moment of conception. A single letter can change our fate.The four letters are four bases -Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). A always pairs with T, while G always pairs C. Such pairs are called base pairs. In RNA, thymine is replaced by uracil. In 1953, Watson and Crick discovered the double helical structure of DNA. The Human Genome Project (HGP) was initiated in 1990 and completed in 2003 and which aimed to sequence the whole human genome. Human beings have 23 pairs of chromosomes in every cell, which makes 46 chromosomes in total. These chromosomes contain 3164.7 million (~3 x109) nucleotide bases (A, T, G, and C). Genes are part of DNA, which codes for protein. The average gene consists of 3000 bases, but sizes vary greatly. The total number of genes is estimated at 20,000 -25,000. Throughout the DNA there are gene-rich areas and gene-poor areas. The 99.9% of DNA sequence of all people are exactly same. The functions of 50 % of gene discovered to date are unknown. There are coding (introns) and noncoding regions (exons), which are sometimes called "junk DNA". Only approximately 2% of our genome encodes proteins. It was also determined that a large part of the non-protein coding regions of human genome are functional. This so-called junk DNA is composed either of deactivated genes that were once useful for our non-human ancestors (like a tail), or parasitic DNA from virus that have entered our genome and replicated themselves hundreds or thousands of times over the generations, but generally serve no purpose in the host organism. One famous retrovirus that copies itself into the human genome is HIV. Genome size is not related to the complexity of life. For example, the genome of *Polychaosdubium*, a microscopic unicellular being, has been reported to contain more than 200 times the amount of DNA found in the human genome [ 2 ]. The information generated by the human genome project has been started to act as the source book for biomedical fields - genetic diseases, ageing, cancer, developmental biology and neurobiology, where scientists are just beginning to understand the underlying molecular mechanisms. The human genome project is expected to immensely benefit medical science. The number of identified disease genes had risen to more than 6,000. HGP focused on the DNA sequence of an individual. The next step was to analyze DNA sequences from different populations. This catalog of human genetic variation – Hap Map will help us to understand and eventually treat the genetic diseases that afflict mankind, as well as the many multifunctional diseases in which genetic predisposition plays an important role. New technologies emanating from the genome project will also find application in other fields such as agriculture and the environmental sciences. But despite discovering genetic changes associated with many cancers, Alzheimer's disease, and thousands of other diseases with deleterious genetic mutations, we have only just discovered how to directly edit DNA. Coupling this fundamental discovery with further clinical exploration has the potential to transform human health, vastly increasing our scientific knowledge and leading to new therapies for previously incurable illnesses.

Human chromosomes contain all different sorts of genes, some bad,some good. The “bad” genes, or ones that cause deformities and disease, can be altered using genetic editing. The techniques are often used in manipulating genes and making life healthier and scientists have endeavored to develop new technologies to modify or manipulate the genome. Precise gene editing and regulation of genomic information is essential to understanding the function of a given gene.

**3. Gene Editing**

For decades, scientists have altered genes using radiation or chemicals. These methods produce unpredictable results. The invention of recombinant DNA technology in the 1970s allowed scientists to insert new DNA into genes in a directed way, but inserting a specific gene or sequence within the genome remained technically challenging and imprecise. Gene editing is a newer technique that is used to make specific and intentional changes to DNA. Gene editing can be used to insert, remove, or modify DNA in a genome. All gene editing technologies involve an enzyme known as a nuclease for cutting the DNA, in addition to a targeting mechanism that guides the enzyme to a specific location on the DNA strand (i.e., a gene within the genome). Gene editing has traditionally involved the insertion, removal, or modification of a single gene, but with CRISPR-Cas9 technique multiple genes can be targeted simultaneously. Such multi-gene editing is generally referred to as genome editing.

Genome editing, or genome editing with engineered nucleases (GEEN) is a type of genetic engineering in which DNA is inserted, deleted or replaced in the genome of a living organism using engineered nucleases, or "molecular scissors. In 2007, Capecchi, Evans and Smithies were awarded a Nobel Prize in Physiology & Medicine for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells [2 ]. However, to truly understand a gene’s role in human biology and disease, human cells and tissue must be studied. This approach will enable researchers to observe the effects of a mutation, SNP [Single Nucleotide Polymorphism]or deletion in combination with the added layers of regulation present within the cell, including post-translational modification, epigenetic changes associated with chromatin structure and transcriptional mechanisms [3].

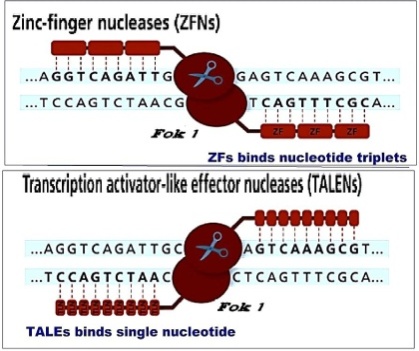
Humans were genetic engineers long before anyone knew what a gene was. They could give living things new traits—sweeter kernels of corn, flatter bulldog faces- through selective breeding. But it took time, and it didn't always pan out. Scientists bombarded seeds and insect eggs with x-rays, causing mutations to scatter through genomes like shrapnel. If one of hundreds of irradiated plants or insects grew up with the traits scientists desired, they bred it and tossed the rest. That's where red grapefruits came from, and most barley for modern beer. Genome modification has become less of a crapshoot. Recombinant DNA is the general name for taking a piece of one DNA, and combining it with another strand of DNA. Recombinant DNA is also sometimes referred to as "chimera." By combining two or more different strands of DNA, with restriction endonucleases (cut at specific sequences) and ligases (join) scientists are able to create a new strand of DNA. The most common recombinant process involves combining the DNA of two different organisms. Different vaccines, bacterial insulin etc prepared by this technique.

**4. The Basics of Gene Editing**

The gene editing toolbox encompasses several types of molecular scissors. Zinc finger nucleases (ZFN) were one of the first editing tools to show specific targeting of the genome as exemplified nearly 25 years ago in Drosophila [4]. More recently, transcription activator-like effector nucleases (TALENs) derived from plant pathogens were discovered and applied to gene-editing approaches [5]. These were shown to be simpler to design and less expensive than ZFNs but still required significant expertise and resources. CRISPR/Cas9 is derived originally from an endogenous bacterial defense mechanism. In its most simplified form, it functions with two modified components: a short chimeric single guide RNA (sgRNA) and a Cas9 nuclease. The sgRNA acts as homing guide for Cas9, thus making it easy to target different genomic loci by simply replacing the sgRNA sequence in the presence of the same nuclease. It is this flexibility and ease of use which has allowed CRISPR/Cas9 to be adopted widely, where it has been used to target DNA sequences from a number of different organisms. CRISPR/Cas9 has been described by one of the pioneers of gene editing, Harvard University chemist George Church, as ‘a real gift from biology’. We will now discuss below the gene editing technologies briefly.

Homologues Recombination - HR: Historically, targeted gene inactivation, replacement or addition via homologous recombination has been achieved and it was a powerful method capable of providing conclusive information for evaluating gene function [6]. However, the use of this technique has been hampered by several factors, including the low efficiency at which engineered constructs are correctly inserted into the chromosomal target site, the need for time-consuming and labor-insensitive selection/screening strategies, and the potential for adverse mutagenic effects. RNA interference - RNAi : Targeted gene knockdown by RNA interference (RNAi) has provided researchers with a rapid, inexpensive and high-throughput alternative to homologous recombination [7]. However, knockdown by RNAi is incomplete, varies between experiments and laboratories, has unpredictable off-target effects, and provides only temporary inhibition of gene function.

**5. Zinc Finger Nucleases - ZFN**

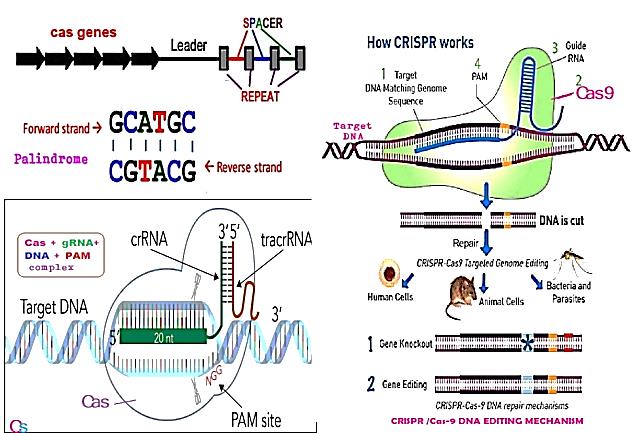
 In the past decade, a new approach has emerged that enables investigators to directly manipulate virtually any gene in a diverse range of cell types and organisms. This core technology – commonly referred to as “genome editing” – is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a non-specific DNA cleavage module [8,9]. These chimeric nucleases enable efficient and precise genetic modifications by inducing targeted DNA double-strand breaks (DSBs) that stimulate the cellular DNA repair mechanisms, including error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR) [10]. Zinc finger is an ideal platform for the design of novel DNA binding domain. The ZFP region provides a ZFN with the ability to bind a specific base sequence. This region contains a tandem array of Cys-His2 fingers, each recognizing approximately 3 base pairs of DNA, previous studies of individual ZFNs used three fingers to construct a 9-bp target, which enabled ZFN dimmers to specify 18bp of DNA per cleavage site. More recent studies added up to six fingers per ZFN for increasing the speciality. The Fok1 DNA cleavage domain is fused to DNA binding domain. It can cleave both DNA strands when two nuclease domains unite to form a functional endonuclease.

**6. Transcription Activator-Like Effectors Nucleases' -TALEN**

The DNA-binding domain of TALENs is made of transcription activator-like effector (TALE) domains. There are four different TALE domains, one for each DNA base, so they can be engineered to bind to specific DNA sequences much more easily than ZFNs. Like ZFNs, the nuclease part of TALENs is normally a Fok1 nuclease [11]. Two Fok1 molecules must come together to make a cut in the DNA, so two TALENs are made, one for each strand.TALEs are naturally occurring proteins from the plant pathogenic bacteria genus Xanthomonas, and contain DNA-binding domains composed of a series of 33–35-amino-acid repeat domains that each recognizes a single base pair. TALE specificity is determined by two hyper variable amino acids that are known as the repeat-variable di-residues (RVDs). Like zinc fingers, modular TALE repeats are linked together to recognize contiguous DNA sequences. However, in contrast to zinc-finger proteins, there was no re-engineering of the linkage between repeats necessary to construct long arrays of TALEs with the ability of targeting single sites in a genome.

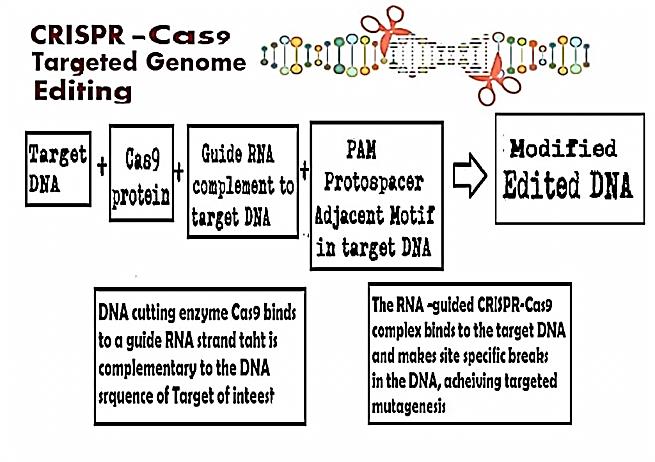
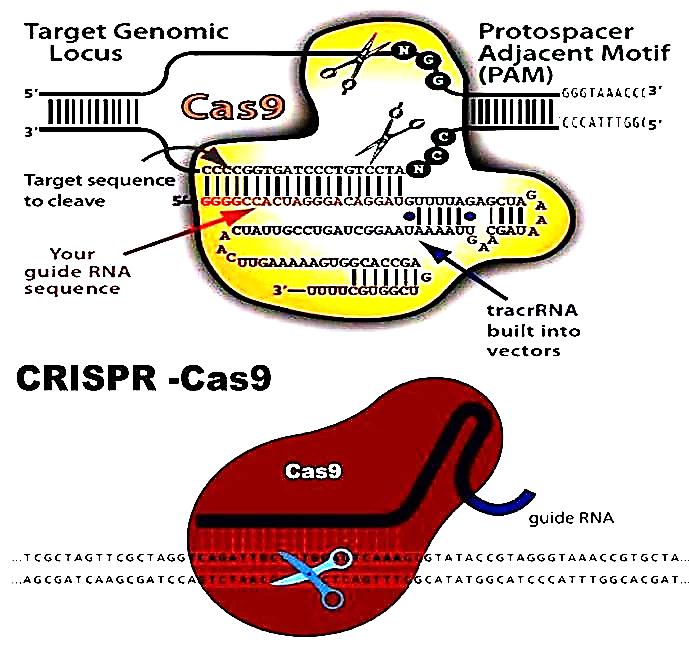
Molecular biologists learned to delete or replace specific genes using enzymes called zinc-finger nucleases, ZFN,; the next-generation technique used enzymes named TALENs. ZFNs and TALENs comprise a powerful class of tools that are redefining the boundaries of biological research. These chimeric nucleases are composed of programmable, sequence-specific DNA-binding modules linked to a non-specific DNA cleavage domain. ZFNs and TALENs enable a broad range of genetic modifications by inducing DNA double-strand breaks that stimulate error-prone nonhomologous end joining or homology-directed repair at specific genomic locations. However, difficulties of protein design, synthesis, and validation remained a barrier to widespread adoption of these engineered nucleases for routine use.

**7. [CRISPR] : The new frontier of genome engineering with CRISPR-Cas9**

CRISPR-Cas9 is the most common, cheap and efficient system used for genome editing. CRISPR stands for ‘CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS’. CRISPR is the DNA-targeting part of the system which consists of RNA molecule, or ‘guide’, designed to bind to specific DNA bases through complementary base-pairing?. Cas9 stands for CRISPR-associated protein 9, and is the nuclease part that cuts the DNA. The CRISPR-Cas9 system was originally discovered in bacteria that use this system to destroy invading viruses?

**8. CRISPR**

The field of biology is now experiencing a transformative phase with the advent of facile genome engineering in animals and plants using RNA-programmable CRISPR-Cas9 [12]. The CRISPR-Cas9 technology originates from type II CRISPR-Cas systems, which provide bacteria with adaptive immunity to viruses and plasmids. The CRISPR associated protein Cas9 is an endonuclease that uses a guide sequence within an RNA duplex, tracrRNA: crRNA, to form base pairs with DNA target sequences, enabling Cas9 to introduce a site-specific double-strand break in the DNA. The dual tracrRNA: crRNA was engineered as a single guide RNA (sgRNA) that retains two critical features: a sequence at the 5’ side that determines the DNA target site by Watson-Crick base-pairing and a duplex RNA structure at the 3’ side that binds to Cas9. This finding created a simple two-component system in which changes in the guide sequence of the sgRNA program Cas9 to target any DNA sequence of interest. The simplicity of CRISPR-Cas9 programming, together with a unique DNA cleaving mechanism, the capacity for multiplexed target recognition, and the existence of many natural type II CRISPR-Cas system variants, has enabled remarkable developments using this cost-effective and easy-to-use technology to precisely and efficiently target, edit, modify, regulate, and mark genomic loci of a wide array of cells and organisms.

CRISPR /CAS 9: A gift fom mother nature. The CRISPR system is an adaptive immune mechanism present in many bacteria and the majority of characterized Archaea. CRISPR-containing organisms acquire DNA fragments from invading bacteriophages and plasmids before transcribing them into CRISPR RNAs (crRNAs) to guide cleavage of invading RNA or DNA[13]. This CRISPR immune system works through the cooperation of many diverse Cas-proteins. Based on differences in their components and mechanisms of action, CRISPR systems have been divided into two major classes. RNA guided target cleavage in class 1 systems (types I, III, and IV) requires a large complex of several effector proteins, but in the class 2 systems [type II, putative types V and VI], only one RNA-guided endonuclease [e.g., Cas9 in type II and Cpf1 (CRISPR from Prevotella and Francisella-1) in type V] is required to mediate cleavage of invading genetic material.

In general, a CRISPR system works in three stages to carry out a full immune response to invading foreign DNA. In the first stage, or acquisition stage, DNA fragments of invading plasmids or phages (termed protospacers) are incorporated into the host CRISPR locus as spacers between crRNA repeats. In the second stage, Cas proteins are expressed, the CRISPR array containing acquired spacers is transcribed into pre-crRNA, and the pre-crRNA is cleaved and processed into mature crRNAs by Cas proteins and host factors. The fully processed crRNA is a guide that contains a spacer sequence responsible for targeting it to the invading genome, as well as all or part of the crRNA repeat sequence, which allows for recognition of the crRNA by Cas proteins and other RNA components. In type II CRISPR systems, the presence of a noncoding trans-activating CRISPR RNA (tracrRNA) that hybridizes with the crRNA repeat sequence is critical for crRNA processing, Cas9 binding, and Cas9-mediated target cleavage. In the third stage, Cas proteins recognize the appropriate target with the guidance of the crRNA and mediate the cleavage of the invading genome, thus protecting the host cells from infection. The action of many CRISPR systems depends on the presence of a sequence-specific PAM that is adjacent to the crRNA target site in the invading genome. The absence of this PAM sequence at the CRISPR locus in the host genome protects it from self-cleavage in type I and type II CRISPR systems.

**9. History of CRISPR**

The CRISPR story began in 1987. While studying the iap enzyme involved in isozyme conversion of alkaline phosphatase in E. coli, Nakata and colleagues reported a curious set of 29 nt repeats downstream of the iap gene [14]. Unlike most repetitive elements, which typically take the form of tandem repeats like TALE repeat [ Transcription activator-like (TAL) effectors specifically bind to double stranded (ds) DNA through a central domain of tandem repeats. Each TAL effector (TALE) repeat comprises 33-35 amino acids and recognizes one specific DNA base through a highly variable residue at a fixed position in the repeat. Structural studies have revealed the molecular basis of DNA recognition by TALE repeats. Unlike most repetitive elements, which typically take the form of tandem repeats like TALE repeat monomers, these 29 nucleotide repeats were interspaced by five intervening 32 nucleotide nonrepetitive sequences.

|  |  |  |
| --- | --- | --- |
| 1987 | Ishino et al | First report of CRISPR clustered repeats |
| 2000 | Mojica et al | Recognition that CRISPR families are present throughout prokaryotes |
| 2002 | Jensen et al | Coined CRISPR name, defined signature of Cas |
| 2005 | Mojica et al &  Pourcel at al | Identified foreign origin of spacers, proposed adaptive immunity function |
| 2005 | Bolotin et al | Identified PAM |
| 2008 | Marraffini et al | CRISPR acts upon DNA targets |
| 2008 | Brouns et al | Spacers are converted into mature crRNAs that acts as small guide RNA. |
| 2009 | Hale et al | Type II-B Cmr CRISPR complexes cleave RNA |
| 2010 | Gameau et al | Cas9 is guided by spacer sequences and cleave target DNA via double Stranded Breaks (DSBs) |
| 2011 | Deltcheva et al | TracrRNA forms a duplex structure with cr RNA in association with Cas9 |
| 2011 | Sapranauskas et al | Type II CRISPR systems are modular and can be heterologuesly in other organisms |
| 2012 | Jinek et al &  Gasiunas et al | In vitro characterization of DNA targeting by Cas9 |
| 2013 | Cong et al &  Mali et al | First demonstration of Cas9 genome engineering in Eukaryotic cells |
| 2014 | Wang et al &  Shalem et al | Genome-wise functional screening with Cas9 |
| 2014 | Jinek et al | Crystal structure of apo-Cas9 |
| 2014 | Nishimasu et al | Crystal structure of Cas9 in complex with guide RNA and target DNA |

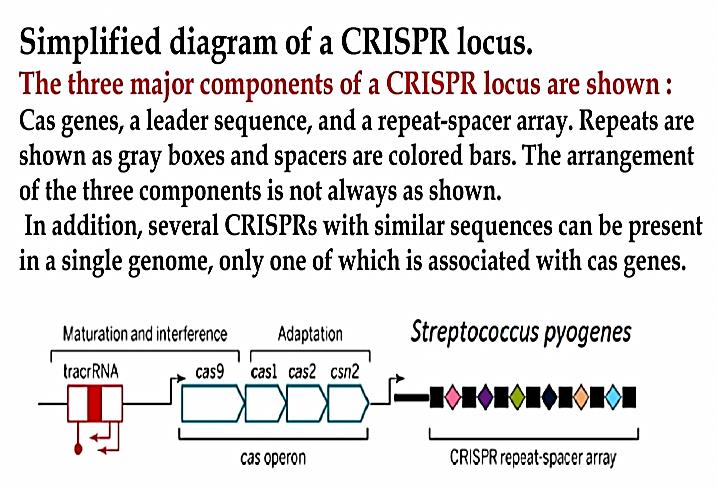
**10. CRISPR: Bacteria Immune System**

It does not happen very often in a science history when a single discovery becomes a technological keystone, especially in biology. CRISPRs (clustered regularly interspaced short palindromic repeats) discovery and a path to its application in genetic engineering is a perfect example of such breakthrough, which is comparable to the discovery of the polymerase chain reaction or development of the next-generation sequencing technology. To date, there are few known defensive strategies in archaea and bacteria that can be referred to as the multilayer prokaryotic immune system: the restriction-modification system, the adsorption inhibition, abortive infection, blocking DNA injection, and the CRISPRs discovered during 1980s. CRISPR-Cas9 has taken the world by storm with the promise of making gene editing much easier and faster than ever before [15]. Since it was developed in 2012, this gene editing tool has revolutionized biology research, making it easier to study disease and faster to discover drugs. It has the potential for treating diseases ranging from cancer to type2 diabetes. The technology has been moving full-steam ahead, with a trial in humans already started, even as the repercussions of gene editing remain largely unknown.

In 2012, scientists turned CRISPR from a bacterial shield into a gene-editing tool. They replaced the bacterial CRISPR RNA system with a modified guide RNA. This RNA acts as a kind of 'wanted poster' — it tells a bounty hunter enzyme called CAS 9 where to look. So far scientists have used it to reduce the severity of genetic deafness in mice, suggesting it could one day be used to treat the same type of hearing loss in people. They’ve created mushrooms that don’t brown easily and edited bone marrow cells in mice to treat sickle-cell anemia. Down the road, CRISPR might help us develop drought-tolerant crops and create powerful new antibiotics. CRISPR could one day even allow us to wipe out entire populations of malaria-spreading mosquitoes or resurrect once –extinct species like the passenger pigeon [16].

A CRISPR clinical trial in humans is already underway in China, in which cancer patients' T-cells are edited to remove a protein that halts immune responses. The cells are then reinserted into the patients. The first CRISPR clinical trial in the U.S. has also been approved, which will involve three edits to T cells. The researchers will remove T cells from 18 patients with several types of cancers and perform three CRISPR edits on them. One edit will insert a gene for a protein engineered to detect cancer cells and instruct the T cells to target them, and a second edit removes a natural T-cell protein that could interfere with this process [17]x19].

The enzyme scans the cell's genome to find a DNA match then slices for the DNA in the cell's enzymes. To repair damage at that point, scientists can change or add DNA within the cell. By feeding CAS9 the right sequence or guide RNA, scientists can cut and paste parts of the DNA sequence, up to 20 bases long, into the genome at any point." CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeat, is at the most basic level a very precise way of tinkering with genes. Whereas gene editing was once a very imprecise and expensive process, scientists can now go into your DNA and essentially cut and paste it at specified places. The technology can be traced back to bacteria, which protect themselves by cutting out invading viruses' DNA and inserting it into their own, then replicating the new sequences to prevent future viral invasions. In 2012, researchers refined the system and revealed that any DNA (not just bacteria) has this ability — and the process works in humans.

The development of efficient and reliable ways to make precise, targeted changes to the genome of living cells is a long-standing goal for biomedical researchers.Gene targeting (also, replacement strategy based on homologous recombination) is a genetic technique that uses homologous recombination to change an endogenous gene. The method can be used to delete a gene, remove exons, add a gene, and introduce point mutations. Gene targeting can be permanent or conditional. Conditions can be a specific time during development / life of the organism or limitation to a specific tissue, for example. Gene targeting requires the creation of a specific vector for each gene of interest. However, it can be used for any gene, regardless of transcriptional activity or gene size.Gene targeting has been widely used to study human genetic diseases by removing ("knocking out"), or adding ("knocking in"), specific mutations of interest to a variety of models. Previously used to engineer rat cell models, advances in gene targeting technologies are enabling the creation of a new wave of isogenic human disease models. These models are the most accurate in-vitro models available to researchers to date, and are facilitating the development of new personalized drugs and diagnostics, particularly in the field of cancer. Recently, a new tool based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes*  has generated considerable excitement.

**11. The Biology of Cas9**

The functions of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) genes are essential in adaptive immunity in select bacteria and archaea, enabling the organisms to respond to and eliminate invading genetic material. These repeats were initially discovered in the 1980s in *E. coli*[9], but their function wasn’t confirmed until 2007 by Barrangou and colleagues, who demonstrated that *S. thermophilus*  can acquire resistance against a bacteriophage by integrating a genome fragment of an infectious virus into its CRISPR locus [16].

Three types of CRISPR mechanisms have been identified, of which type II is the most studied. In this case, invading DNA from viruses or plasmids is cut into small fragments and incorporated into a CRISPR locus amidst a series of short repeats (around 20 bps). The loci are transcribed, and transcripts are then processed to generate small RNAs (crRNA – CRISPR RNA), which are used to guide effector endonucleases that target invading DNA based on sequence complementarity [17]. In the acquisition phase, foreign DNA is incorporated into the bacterial genome at the CRISPR loci. CRISPR loci is then transcribed and processed into crRNA during crRNA biogenesis. During interference, Cas9 endonuclease complexed with a crRNA and separate tracrRNA cleaves foreign DNA containing a 20-nucleotide crRNA complementary sequence adjacent to the PAM sequence.

One Cas protein, Cas9, has been shown, through knockdown and rescue experiments to be a key player in certain CRISPR mechanisms (specifically type II CRISPR systems). The type II CRISPR mechanism is unique compared to other CRISPR systems, as only one Cas protein (Cas9) is required for gene silencing [12]. In type II systems, Cas9 participates in the processing of crRNAs, and is responsible for the destruction of the target DNA. Cas9’s function in both of these steps relies on the presence of two nuclease domains, a RuvC-like nuclease domain located at the amino terminus and a HNH-like nuclease domain that resides in the mid-region of the protein [13]. To achieve site-specific DNA recognition and cleavage, Cas9 must be complexed with both a crRNA and a separate trans-activating crRNA (tracrRNA or trRNA), that is partially complementary to the crRNA. The tracrRNA is required for crRNA maturation from a primary transcript encoding multiple pre-crRNAs. This occurs in the presence of RNase III and Cas9.

During the destruction of target DNA, the HNH and RuvC-like nuclease domains cut both DNA strands, generating double-stranded breaks (DSBs) at sites defined by a 20-nucleotide target sequence within an associated crRNA transcript. The HNH domain cleaves the complementary strand, while the RuvC domain cleaves the noncomplementary strand. The double-stranded endonuclease activity of Cas9 also requires that a short conserved sequence, (2–5 nts) known as protospacer-associated motif (PAM), follows immediately 3´- of the crRNA complementary sequence. In fact, even fully complementary sequences are ignored by Cas9-RNA in the absence of a PAM sequence [18].

## 12. Cas9 and CRISPR as a New Tool in Molecular Biology

## The simplicity of the type II CRISPR nuclease, with only three required components (Cas9 along with the crRNA and trRNA) makes this system amenable to adaptation for genome editing. This potential was realized in 2012 by the Doudna and Charpentier labs [19]. Based on the type II CRISPR system described previously, the authors developed a simplified two-component system by combining trRNA and crRNA into a single synthetic single guide RNA (sgRNA). sgRNA programmed Cas9 was shown to be as effective as Cas9 programmed with separate trRNA and crRNA in guiding targeted gene alterations.

To date, three different variants of the Cas9 nuclease have been adopted in genome-editing protocols. The first is wild-type Cas9, which can site-specifically cleave double-stranded DNA, resulting in the activation of the double strand break (DSB) repair machinery. DSBs can be repaired by the cellular Non-Homologous End Joining (NHEJ) pathway [20], resulting in insertions and/or deletions (indels) which disrupt the targeted locus. Alternatively, if a donor template with homology to the targeted locus is supplied, the DSB may be repaired by the homology-directed repair (HDR) pathway allowing for precise replacement mutations to be made [21,22]. Cong and colleagues [23] took the Cas9 system a step further towards increased precision by developing a mutant form, known as Cas9D10A, with only nickase activity. This means it cleaves only one DNA strand, and does not activate NHEJ. Instead, when provided with a homologous repair template, DNA repairs are conducted via the high-fidelity HDR pathway only, resulting in reduced indel mutations. Cas9D10A is even more appealing in terms of target specificity when loci are targeted by paired Cas9 complexes designed to generate adjacent DNA nicks [24].

The third variant is a nuclease-deficient Cas9 (dCas9) [25]. Mutations H840A in the HNH domain and D10A in the RuvC domain inactivate cleavage activity, but do not prevent DNA binding. Therefore, this variant can be used to sequence-specifically target any region of the genome without cleavage. Instead, by fusing with various effector domains, dCas9 can be used either as a gene silencing or activation tool. Furthermore, it can be used as a visualization tool. For instance, Chen and colleagues used dCas9 fused to Enhanced Green Fluorescent Protein (EGFP) to visualize repetitive DNA sequences with a single sgRNA or nonrepetitive loci using multiple sgRNAs [26].

Applications as a Genome-editing and Genome Targeting Tool : The CRISPR/Cas9 system has been widely adopted and already been successfully used to target important genes in many cell lines and organisms, including human , bacteria [27], zebrafish, C. elegans , plants , Xenopustropicalis , yeast , Drosophila , monkeys , rabbits , pigs , rats and mice . Several groups have now taken advantage of this method to introduce single point mutations (deletions or insertions) in a particular target gene, via a single gRNA [7, 27,28]. Using a pair of gRNA-directed Cas9 nucleases instead, it is also possible to induce large deletions or genomic rearrangements, such as inversions or translocations [29]. A recent exciting development is the use of the dCas9 version of the CRISPR/Cas9 system to target protein domains for transcriptional regulation [30,31]. The CRISPR/Cas9 system requires only the redesign of the crRNA to change target specificity. This contrasts with other genome editing tools, including zinc finger and TALENs, where redesign of the protein-DNA interface is required. Furthermore, CRISPR/Cas9 enables rapid genome-wide interrogation of gene function by generating large gRNA libraries [32,33] for genomic screening.

**13. CRISPR used in COVID-19 diagnostic**

- US FDA has granted Sherlock Biosciences an emergency use authorization (EUA) for its COVID-19 diagnostic assay for Covid-19, beating out other companies and academic groups trying to use the powerful gene-editing technology to figure out who is infected with the novel coronavirus. Sherlock’s test the first use of CRISPR technology for anything, allows the company to scale up production of its assay for use by laboratories that do complex diagnostics. Like those standard tests, Sherlock’s assay detects the presence of the viral RNA. It starts with a respiratory specimen from, for example, the mouth, nose, or lungs. To make the viral genome easier to identify, scientists convert it into DNA, which can be copied over and over. The method they use—isothermal amplification—is done at a constant temperature, unlike the method used by most conventional diagnostics, polymerase chain reaction. Then, the sample goes through Sherlock’s CRISPR gauntlet. CRISPR-Cas chops up invasive viral RNA; scientists have turned it into a technique that makes precise cuts in genetic code through various Cas enzymes. Sherlock’s system uses Cas13, which is a little more flexible in what genetic regions it can target than other Cas enzymes. The CRISPR part of the assay involves converting the amplified DNA back into RNA, which is the type of genetic information the Cas13 enzyme recognizes. The enzyme is led to any viral RNA in the sample based on “guides,” short bits of RNA scientists add to the reaction that match the actual code of the virus. Once it’s there, the enzyme cuts the viral RNA. The assay targets two distinct parts of the SARS-CoV-2 genome: the recipe for the nucleocapsid (N-protein), which helps the virus assemble itself, and *ORF 1ab*, a stretch of the genome that leads to the precursor of an enzyme that helps the virus copy itself. These targets were chosen over better known ones like the SARS-CoV-2 spike protein and the protease because viruses are a bit sloppy when they copy their genetic information. Sometimes they make mistakes, and while those mistakes may not affect the virus’ ability to copy itself and infect, it might affect the precision of a diagnostic based on CRISPR. Once it’s activated, the Cas13 enzyme cuts other nucleic acids as well as the viral RNA. Within the assay are strands of genetic material that have a fluorescent molecule at one end, and a molecule that quenches, or blocks, the fluorescence, on the other. As activated Cas13 chomps its way around the genetic material in the sample, it cuts those strands, freeing the fluorescents bits from the quenching bits. That most fluorescent plate readers can read the test.

The SHERLOCK COVID-19detection protocol works in three steps and can be completed in 1 hour, starting from nucleic acid extraction as used for qRT-PCR [Quantitative reverse transcription PCR] tests. Step (1) – 25 min incubation – isothermal amplification of the extracted nucleic acid sample using a commercially available recombinase polymerase amplification (RPA) kit; Step (2) – 30 min incubation – detection of pre-amplified viral RNA sequence using Cas13;& Step (3) – 2 min incubation – visual read out of the detection result by eye using a commercially-available paper dipstick.

**14. The Future of CRISPR/Cas9**

The rapid progress in developing Cas9 into a set of tools for cell and molecular biology research has been remarkable, likely due to the simplicity, high efficiency and versatility of the system. Of the designer nuclease systems currently available for precision genome engineering, the CRISPR/Cas system is by far the most user friendly. It is now also clear that Cas9’s potential reaches beyond DNA cleavage, and its usefulness for genome locus-specific recruitment of proteins will likely only be limited by our imagination.

Launching a new chapter in the fast-moving cancer immunotherapy field, scientists have blended two cutting-edge approaches: CRISPR, which edits DNA, and T-cell therapy, in which sentries of the immune system are exploited to destroy tumors. Two women and one man, all in their 60s—one with sarcoma and two with the blood cancer multiple myeloma—received CRISPR-altered versions of their own cells [34]. The uses of CRISPR–Cas9 to manipulate cells and organisms continued to mount, it seemed inevitable that researchers somewhere would test the technique in human eggs, sperm or embryos, with a view to creating heritable alterations in people.

**15. Ethical Issues**

The fact that CRISPR-Cas9 is among the important discoveries of the 21st century is widely accepted in the scientific community and related industries. However, the rapid rise of CRISPR-Cas9 has led to new bioethical, social, and legal issues in medicine, agriculture, livestock, and the environment.

The most notable concern is that of the generation of ‘designer babies’. However, there remain viable technical reasons why this technology is not ready for embryo modification, and most Centre on eliminating or selecting-out deleterious off targets effects. Another factor that negates the use of edited embryos lies in the fact that few known genetic conditions exist for which pre implantation genetic diagnosis (PGD) cannot be used to screen-out embryos with pathogenic mutations. Moreover, three parent babies (in the case of mitochondrial disease) cannot be used to exclude inheritance of disease associated mutations. Nevertheless, as recently commented by Paul Knoepfler, patients carrying incurable mutations are likely to have a higher degree of risk tolerance than scientists [35].Researchers in China announced that they had used the nascent gene editing tool CRISPR–Cas9 to modify the genomes of human embryos, triggering a major ethics debate [36].

## **CRISPR Prospects -** CRISPR/Cas9 gene editing technology is being touted as one of the biggest biotechnology breakthroughs of the century with Jennifer Doudna of the University of California, Berkeley, and Emmanuelle Charpentier of the Helmholtz Centre for Infection Research in Braunschweig, Germany, receiving Nobel Prize in Chemistry,2020.

Regardless, CRISPR/Cas9 has been revolutionary facilitating a wealth of research unmatched in over three decades since the inception of genome editing. It has opened up gene editing to the broad scientific community and allowed what used to take years to do to be achieved in a matter of weeks. Recent research has also propelled CRISPR/Cas9 system beyond just gene editing tool allowing study of DNA epigenetics and understanding what non-protein coding segments of our genome encodes. Its potential for research, human medicine and agriculture will likely only be limited by our imagination.

CRISPR/Cas9 may well be considered one of the most important biological tools identified in recent years. There is no end to the number of ways this system can be tweaked for a vast array of molecular exploits. This is especially the case in stem cells, where the application of CRISPR/Cas9 technologies will likely be profound. Although hurdles remain, especially the warranted concern of off-target effects, continued improvements in Cas9 and guide RNA engineering will allay the fears of even the most ardent critics of CRISPR-based therapy. Already, there is unprecedented investment by large and small biotechnology and pharmaceutical industry players alike, which shows the palpable excitement generated by the vast array of possibilities brought by this technology. Eventually, the early hype will subside, but there is little doubt that CRISPR/Cas9, and its application in stem cell engineering, will have an extraordinary impact in the progress towards curing many previously intractable diseases.

**16. Conclusions**

Biologists are using CRISPR–Cas9 to better understand genomes — not just by editing DNA, but by devising variations on the technique to precisely manipulate the activity of genes. And, armed for the first time with a method that can easily introduce genetic changes to many animals, researchers have edited a veritable menagerie of beasts — from ferrets to elephants to koi carp — in an attempt to combat disease, improve agriculture and even make designer pets. The repurposing of bacterial CRISPR–Cas immune systems as genome engineering tools has heralded an era in which RNA-programmed genome editing is a democratized and broadly accessible technology. In the clinic, therapeutic success is likely to be attained in localized tissues (liver, blood, eye), with longer-term goals of targeting systemic diseases dependent on future delivery options. Screen-based drug discovery approaches, together with the ability to use RNA-programmed genome editing technology to produce disease-recapitulating cell line models and animals, will continue to identify potential therapeutic targets. The application of genome-wide, Cas9-based screens to complex diseases, such as leukemia, provides intriguing opportunities for the selection of therapeutic targets and the design of anti-cancer drugs . Over the long term, the potential for CRISPR-enabled production of synthetic tissues or animals and immune-compatible donor organisms for xenotransplantation is vast. In the short term, with the proof of concept already provided for the correction of genetic diseases, such as Duchenne muscular dystrophy and beta-thalassemia, there is potential for gene and antiviral CRISPR-based therapies. Investigations of toxicity and safety will need to accompany advances in our understanding of CRISPR-system efficacy to ensure an appropriate risk-benefit profile for therapeutic interventions. Notwithstanding the promise of RNA-programmed genome editing in somatic cell therapy, a key outstanding issue is whether applications in zygotes and human germ line cells should be considered in the light of the associated ethical issues. The pace of the science is faster than our grasp of the regulatory ramifications, an issue that is being addressed by the scientific community, together with key stakeholders. For better or for worse, CRISPR–Cas9 is transforming biology. We are now at the dawn of the gene-editing age.

**Acknowledgement:** Author is grateful to Professor B K Chaudhuri ,Indian Association for the Cultvatin of Science , Kolkata, for his suggestion and interest in the work.

**References**

# [1] [Jared Diamond](https://www.amazon.in/Jared-Diamond/e/B000AQ01ZS/ref=dp_byline_cont_book_1)  The Rise And Fall Of The Third Chimpanzee: how our animal heritage affects the way we live Paperback – 28 May 1992

[2]. The Nobel Prize in Physiology or Medicine 2007, Press release, 8 October 2007

[3]. Francis Robert and Jerry Pelletie: Exploring the Impact of Single-Nucleotide Polymorphisms on Translation Front Genet. 2018; 9: 507.

[4]. [Marina Bibikova](https://pubmed.ncbi.nlm.nih.gov/?term=Bibikova+M&cauthor_id=12136019)[1](https://pubmed.ncbi.nlm.nih.gov/12136019/#affiliation-1), [Mary Golic](https://pubmed.ncbi.nlm.nih.gov/?term=Golic+M&cauthor_id=12136019), [Kent G Golic](https://pubmed.ncbi.nlm.nih.gov/?term=Golic+KG&cauthor_id=12136019), [Dana Carroll](https://pubmed.ncbi.nlm.nih.gov/?term=Carroll+D&cauthor_id=12136019). Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases.Bibikova Genetics, 2002;161 (3):1169–75.

[5]. [Jeffrey C Miller](https://pubmed.ncbi.nlm.nih.gov/?term=Miller+JC&cauthor_id=21179091)[1](https://pubmed.ncbi.nlm.nih.gov/21179091/#affiliation-1), [Siyuan Tan](https://pubmed.ncbi.nlm.nih.gov/?term=Tan+S&cauthor_id=21179091), [Guijuan Qiao](https://pubmed.ncbi.nlm.nih.gov/?term=Qiao+G&cauthor_id=21179091), [Kyle A Barlow](https://pubmed.ncbi.nlm.nih.gov/?term=Barlow+KA&cauthor_id=21179091), [Jianbin Wang](https://pubmed.ncbi.nlm.nih.gov/?term=Wang+J&cauthor_id=21179091), [Danny F Xia](https://pubmed.ncbi.nlm.nih.gov/?term=Xia+DF&cauthor_id=21179091), [Xiangdong Meng](https://pubmed.ncbi.nlm.nih.gov/?term=Meng+X&cauthor_id=21179091), [David E Paschon](https://pubmed.ncbi.nlm.nih.gov/?term=Paschon+DE&cauthor_id=21179091), [Elo Leung](https://pubmed.ncbi.nlm.nih.gov/?term=Leung+E&cauthor_id=21179091), [Sarah J Hinkley](https://pubmed.ncbi.nlm.nih.gov/?term=Hinkley+SJ&cauthor_id=21179091), [Gladys P Dulay](https://pubmed.ncbi.nlm.nih.gov/?term=Dulay+GP&cauthor_id=21179091) et al.: A TALE nuclease architecture for efficient genome editing. Nat Biotechnol. 2011; 29 (2):143–8.

[6]. Capecchi M R : Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. 2005; 6: 507–512.

[7]. [Michael T McManus](https://pubmed.ncbi.nlm.nih.gov/?term=McManus+MT&cauthor_id=12360232)[1](https://pubmed.ncbi.nlm.nih.gov/12360232/#affiliation-1), [Phillip A Sharp](https://pubmed.ncbi.nlm.nih.gov/?term=Sharp+PA&cauthor_id=12360232). Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. 2002; 3: 737–747.

[8]. F[yodor D Urnov](https://pubmed.ncbi.nlm.nih.gov/?term=Urnov+FD&cauthor_id=20717154)[1](https://pubmed.ncbi.nlm.nih.gov/20717154/#affiliation-1), [Edward J Rebar](https://pubmed.ncbi.nlm.nih.gov/?term=Rebar+EJ&cauthor_id=20717154), [Michael C Holmes](https://pubmed.ncbi.nlm.nih.gov/?term=Holmes+MC&cauthor_id=20717154), [H Steve Zhang](https://pubmed.ncbi.nlm.nih.gov/?term=Zhang+HS&cauthor_id=20717154), [Philip D Gregory](https://pubmed.ncbi.nlm.nih.gov/?term=Gregory+PD&cauthor_id=20717154)Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 2010; 11:636–646;

[9]. Carroll D : Genome engineering with zinc-finger nucleases Genetics.2011; 188: 773–782.

[10.] Wyman C. KanaarR : DNA double-strand break repair: all's well that ends well. Annu. Rev. Genet.2006; 40:363–383.

[11]. J. Keith Joung, Jeffry D. Sander: TALENs: a widely applicable technology for targeted genome editingNat Rev Mol Cell Biol. 2013 Jan; 14(1): 49–55.

[12]. J.A Doudna and E. Charpentier: Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science 2014, 346, 1077.

[13] [Haifeng Wang](https://pubmed.ncbi.nlm.nih.gov/?term=Wang+H&cauthor_id=27145843)[1](https://pubmed.ncbi.nlm.nih.gov/27145843/#affiliation-1), [Marie La Russa](https://pubmed.ncbi.nlm.nih.gov/?term=La+Russa+M&cauthor_id=27145843): CRISPR/Cas9 in Genome Editing and Beyond Annu. Rev. Biochem. 2016, 85; 227-64. .

[14]. [Y Ishino](https://pubmed.ncbi.nlm.nih.gov/?term=Ishino+Y&cauthor_id=3316184)[1](https://pubmed.ncbi.nlm.nih.gov/3316184/#affiliation-1), [H Shinagawa](https://pubmed.ncbi.nlm.nih.gov/?term=Shinagawa+H&cauthor_id=3316184), [K Makino](https://pubmed.ncbi.nlm.nih.gov/?term=Makino+K&cauthor_id=3316184), [M Amemura](https://pubmed.ncbi.nlm.nih.gov/?term=Amemura+M&cauthor_id=3316184), [A Nakata](https://pubmed.ncbi.nlm.nih.gov/?term=Nakata+A&cauthor_id=3316184): Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene productJ. Bacteriol. 1987,169, 5429–5433.

# [15]. Rama Devi Mittal: Gene Editing in Clinical Practice: Where are We?

Indian J. ClinBiochem. 2019 Jan; 34(1): 19–25.

# [16]. [Nathan Singh](https://www.ncbi.nlm.nih.gov/pubmed/?term=Singh%20N%5BAuthor%5D&cauthor=true&cauthor_uid=29039115), [Junwei Shi](https://www.ncbi.nlm.nih.gov/pubmed/?term=Shi%20J%5BAuthor%5D&cauthor=true&cauthor_uid=29039115), [Carl H. June](https://www.ncbi.nlm.nih.gov/pubmed/?term=June%20CH%5BAuthor%5D&cauthor=true&cauthor_uid=29039115), [Marco Ruella](https://www.ncbi.nlm.nih.gov/pubmed/?term=Ruella%20M%5BAuthor%5D&cauthor=true&cauthor_uid=29039115). ,Genome-editing technologies in adoptive T cell immunotherapy for cancer: CurrHematolMalig Rep. 2017 Dec; 12(6): 522–529.

[17]. [Rodolphe Barrangou](https://pubmed.ncbi.nlm.nih.gov/?term=Barrangou+R&cauthor_id=24766887)[1](https://pubmed.ncbi.nlm.nih.gov/24766887/#affiliation-1), [Luciano A Marraffini](https://pubmed.ncbi.nlm.nih.gov/?term=Marraffini+LA&cauthor_id=24766887)[2](https://pubmed.ncbi.nlm.nih.gov/24766887/#affiliation-2)CRISPR-Cas systems: Prokaryotes upgrade to adaptive immunity Mol Cell. 2014 Apr 24; 54(2): 234–244.

[18]. [Thomas Gaj](https://pubmed.ncbi.nlm.nih.gov/?term=Gaj+T&cauthor_id=23664777)[1](https://pubmed.ncbi.nlm.nih.gov/23664777/#affiliation-1), [Charles A Gersbach](https://pubmed.ncbi.nlm.nih.gov/?term=Gersbach+CA&cauthor_id=23664777), [Carlos F Barbas 3rd](https://pubmed.ncbi.nlm.nih.gov/?term=Barbas+CF+3rd&cauthor_id=23664777): ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013 Jul; 31(7): 397–405.

[19]. D C. Swarts, Cas Mosterd,Mark W. J. van Passel, Stan J. J. Brouns.

CRISPR interference directs strand specific spacer acquisition (2012), PLoS One, 7:e35888.

[20]. Martin Jinek1,2,\*, Krzysztof Chylinski3,4,\*, Ines Fonfara4, Michael Hauer2,†, Jennifer A. Doudna1,2,5,6,‡, Emmanuelle CharpentierA Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity (2012), *Science,*337, 816–821.

[21]. F Ann Ran 1, Patrick D Hsu, Chie-Yu Lin, Jonathan S Gootenberg, Silvana Konermann, Alexandro E Trevino, David A Scott, Azusa Inoue, Shogo Matoba, Yi Zhang, Feng Zhang. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity :Cell. 2013 Sep 12; 154(6): 1380–1389.

22]. Søren Overballe-Petersen, Klaus Harms, Ludovic A. A. Orlando, J. Victor Moreno Mayar, Simon . Bacterial natural transformation by highly fragmented and damaged DNA (2013) *Proc. Natl. Acad. Sci. U.S.A.*110,19860–19865.

[23]. [Chunling Gong](https://pubmed.ncbi.nlm.nih.gov/?term=Gong+C&cauthor_id=15778718)[1](https://pubmed.ncbi.nlm.nih.gov/15778718/#affiliation-1), [Paola Bongiorno](https://pubmed.ncbi.nlm.nih.gov/?term=Bongiorno+P&cauthor_id=15778718), [Alexandra Martins](https://pubmed.ncbi.nlm.nih.gov/?term=Martins+A&cauthor_id=15778718), [Nicolas C Stephanou](https://pubmed.ncbi.nlm.nih.gov/?term=Stephanou+NC&cauthor_id=15778718), [Hui Zhu](https://pubmed.ncbi.nlm.nih.gov/?term=Zhu+H&cauthor_id=15778718), [Stewart Shuman](https://pubmed.ncbi.nlm.nih.gov/?term=Shuman+S&cauthor_id=15778718), [Michael S Glickman](https://pubmed.ncbi.nlm.nih.gov/?term=Glickman+MS&cauthor_id=15778718)Mechanism of nonhomologous end-joining in mycobacteria: a low-fidelity repair system driven by Ku, ligase D and ligase C (2005), *Nat. Struct. Mol. Biol.* 12, 304–312.

[24]. Le Cong1,2,\*, F. Ann Ran1,4,\*, David Cox1,3, Shuailiang Lin1,5, Robert Barretto6, Naomi Habib1, Patrick D. Hsu1,4, Xuebing Wu7,. Multiplex Genome Engineering Using CRISPR/Cas Systems (2013), *Science*, 339, 819–823.

[25]. [F Ann Ran](https://pubmed.ncbi.nlm.nih.gov/?term=Ran+FA&cauthor_id=23992846)[1](https://pubmed.ncbi.nlm.nih.gov/23992846/#affiliation-1), [Patrick D Hsu](https://pubmed.ncbi.nlm.nih.gov/?term=Hsu+PD&cauthor_id=23992846), [Chie-Yu Lin](https://pubmed.ncbi.nlm.nih.gov/?term=Lin+CY&cauthor_id=23992846), [Jonathan S Gootenberg](https://pubmed.ncbi.nlm.nih.gov/?term=Gootenberg+JS&cauthor_id=23992846), [Silvana Konermann](https://pubmed.ncbi.nlm.nih.gov/?term=Konermann+S&cauthor_id=23992846), . Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity (2013), *Cell,* 154, 1380–1389.

[26]. [Lei S. Qi](https://www.ncbi.nlm.nih.gov/pubmed/?term=Qi%20LS%5BAuthor%5D&cauthor=true&cauthor_uid=23452860),1,2,8,\* [Matthew H. Larson](https://www.ncbi.nlm.nih.gov/pubmed/?term=Larson%20MH%5BAuthor%5D&cauthor=true&cauthor_uid=23452860),2,3,8 [Luke A. Gilbert](https://www.ncbi.nlm.nih.gov/pubmed/?term=Gilbert%20LA%5BAuthor%5D&cauthor=true&cauthor_uid=23452860),2,3,8 [Jennifer A. Doudna](https://www.ncbi.nlm.nih.gov/pubmed/?term=Doudna%20JA%5BAuthor%5D&cauthor=true&cauthor_uid=23452860),4,5. Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression (2013) ,*Cell,* 152, 1173–1183.

[27]. [Baohui Chen](https://pubmed.ncbi.nlm.nih.gov/?term=Chen+B&cauthor_id=24360272)[1](https://pubmed.ncbi.nlm.nih.gov/24360272/#affiliation-1), [Luke A Gilbert](https://pubmed.ncbi.nlm.nih.gov/?term=Gilbert+LA&cauthor_id=24360272)[2](https://pubmed.ncbi.nlm.nih.gov/24360272/#affiliation-2), [Beth A Cimini](https://pubmed.ncbi.nlm.nih.gov/?term=Cimini+BA&cauthor_id=24360272)[3](https://pubmed.ncbi.nlm.nih.gov/24360272/#affiliation-3), [Joerg Schnitzbauer](https://pubmed.ncbi.nlm.nih.gov/?term=Schnitzbauer+J&cauthor_id=24360272)[1](https://pubmed.ncbi.nlm.nih.gov/24360272/#affiliation-1), [Wei Zhang](https://pubmed.ncbi.nlm.nih.gov/?term=Zhang+W&cauthor_id=24360272)[1](https://pubmed.ncbi.nlm.nih.gov/24360272/#affiliation-1), . Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system (2013) *Cell,* 155, 1479–1491.

[28] Pyne ME, Moo-Young M, Chung DA, Chou CP. Coupling the CRISPR/Cas9 System with Lambda Red Recombineering Enables Simplified Chromosomal Gene Replacement in Escherichia coli (2015)*Appl Environ Microbiol* 81:5103–5114.

[29]. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O. Crystal structure of Cas9 in complex with guide RNA and target DNA (2014) *Cell,* doi:10.1016/j.cell.2014.02.001

[30]. Yuanwu Ma, Xu Zhang, Bin Shen, Yingdong Lu, Wei Chen, Jing Ma, Lin Bai, Xingxu Huang & Lianfeng ZhangGenerating rats with conditional alleles using CRISPR/Cas9 (2014) *Cell Res.* 24, 122–125.

[31]. Daisuke Mashiko 1, Samantha A M Young, Masanaga Muto, Hirotaka Kato, Kaori Nozawa,. Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes (2014) *Dev. Growth Differ.* 56, 122–129.

[32]. Scott J Gratz 1, Jill Wildonger 2, Melissa M Harrison 3, Kate M O'Connor-Giles. CRISPR/Cas9-mediated genome engineering and the promise of designer flies on demand

(2013) *Fly,* 249.

[33]]. Prashant Mali 1, John Aach, P Benjamin Stranges, Kevin M Esvelt, Mark Moosburner, Sriram Kosuri, Luhan Yang, George M Church. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering (2013) *Nat. Biotechnol.*31, 833–838.

[34] . Jennifer R. Hamilton, Jennifer A. Doudna*.* Knocking out barriers to engineered cell activity. Science: *06 Feb 2020, DOI: 10.1126/science.aba9844*

[35]. [Paul Knoepfler](https://ipscell.com/author/pknoepfl/). ‘Just freakin do it: patients voice impatience on CRISPR for genetic diseases’, The Niche, 15-Dec-2015. .

[36]. [Jing-ru Li](https://www.ncbi.nlm.nih.gov/pubmed/?term=Li%20Jr%5BAuthor%5D&cauthor=true&cauthor_uid=30614228), [Simon Walker](https://www.ncbi.nlm.nih.gov/pubmed/?term=Walker%20S%5BAuthor%5D&cauthor=true&cauthor_uid=30614228), [Jing-bao Nie](https://www.ncbi.nlm.nih.gov/pubmed/?term=Nie%20Jb%5BAuthor%5D&cauthor=true&cauthor_uid=30614228), [Xin-qing Zhang](https://www.ncbi.nlm.nih.gov/pubmed/?term=Zhang%20Xq%5BAuthor%5D&cauthor=true&cauthor_uid=30614228). Experiments that led to the first gene-edited babies: the ethical failings and the urgent need for better governance2019 Jan; 20(1): 32–38; doi: 10.1631/jzus.B1800624

--------------------------------