IJPSR (2016), Vol. 7, Issue 8 **(Review Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 28 March, 2016; received in revised form, 11 May, 2016; accepted, 30 June, 2016; published 01 August, 2016

CRISPR/Cas9 TECHNOLOGY- A NEW BOON IN GENOME EDITING

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Keywords:

Genome editing, CRISPR-Cas9 technology, CRISPR-Cas9 mechanism, Genetic engineering, CRISPR/Cas9 applications

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ABSTRACT: Targeted genome engineering provides the ability to precisely modify genetic information in order to study gene function, biological mechanisms, and disease pathology. Historically, random mutagenesis or low-efficiency homologous recombination were used to modify the genomes of cell lines or animal models. However, new advances in the design of sequence-specific endonucleases have enabled more effective, targeted editing of the genome. The most recent and fastest growing method for genome editing is based on the Clustered Regions of Interspersed Palindromic Repeats (CRISPR) viral defense system found in bacteria and archaea. The CRISPR/Cas9 system is much easier to customize and optimize because the site selection for DNA cleavage is guided by a short sequence of RNA rather than an engineered protein as in the systems of zinc finger nucleases (ZFN), transcription activator–like effect or nucleases (TALEN), and meganucleases. Derived from a remarkable microbial defense system, Cas9 is driving innovative applications from basic biology to biotechnology and medicine. The simplicity of the CRISPR-Cas9 system has enabled its widespread applications in generating germline animal models, somatic genome engineering, and functional genomics screening and in treating genetic and infectious diseases. This technology will likely be used in all fields of biomedicine, ranging from basic research to human gene therapy.

INTRODUCTION: Ever since the discovery of restriction enzymes in 1970, the holy grail of molecular biologists has been site specific manipulation of mammalian genomes, including the human genome [1]. The triumphal sequencing of the Human Genome Project offered new insights into the fundamental inner workings of humans, promising a big step toward curing humankind of most diseases. Sequencing the genome was an incredible challenge but, in broader perspective, was only the first small step.

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DOI: 10.13040/IJPSR.0975-8232.7(8).3336-47

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (8).3336-47

The most difficult challenge lies ahead; deciphering the cryptic meaning of the 3.3 billion base pairs of DNA, by assigning functions to the tens of thousands of genes, and determining how they work together to make us human. This is the grand biological promise yet to be fulfilled, and with the recent development of new biotechnological tools, the biggest discoveries are yet to come [2]. A new generation of genome engineering technologies based on the class of RNA-guided endonucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)-associated Cas9, and their rapid applications are now bringing a further revolution in biology and medicine [3].

Genome Editing:

In the strictest sense, genome editing means making stable, permanent, and heritable changes to

the genetic code, to accomplish many potential goals.

The process begins with stimulation of a DSB at the target site. DSBs are lethal if left unrepaired, so eukaryotic cells have several mechanisms in response (**Fig. 1**). The first is homologous recombination (HR), whereby cells use a homologous copy of the broken chromosome as a repair template. HR is a relatively error-free process. The template is normally the sister chromatid during G2 in mitosis, but can also come from a DNA fragment introduced exogenously, which can mediate a "knock in" of desired DNA sequences to the target site. The second major mechanism for DSB repair is non-homologous end joining (NHEJ), which occurs when no homologous template is available. NHEJ is simply the re-connection of the broken chromosome ends. However, NHEJ is error-prone, and frequently leads to small insertions or deletions ("indels") at the break site. Indels can disrupt a gene by causing frame shifts, and, therefore, gene knockouts [4].

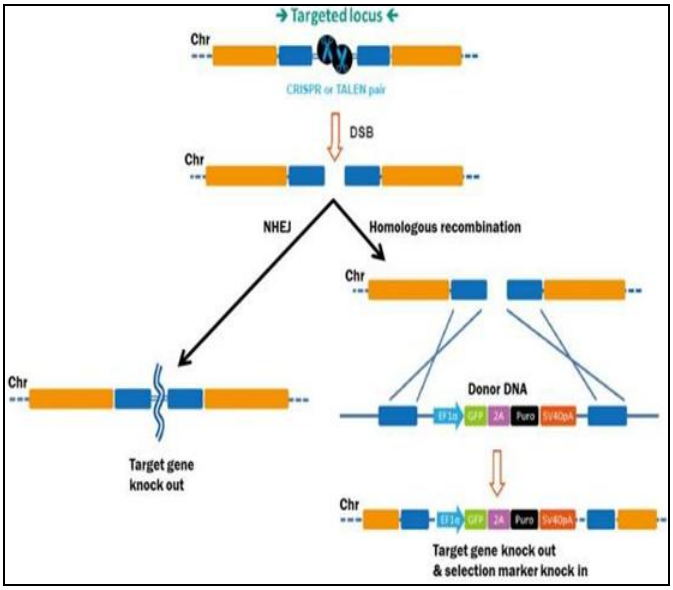

FIG. 1: PATHWAYS FOR THE REPAIR OF DSBS INDUCED BY GENOME EDITING TOOLS.
Left: Non-homologous end joining. Right: HR in the presence of a donor template.

History of Genome Editing Timeline:

Late 1860s- The discovery of DNA by Friedrich Miescher [5].

Late 1880s- Phoebus Levene did extensive research about the DNA molecules [5].

1920s- Erwin Chargaff discovered the primary chemical components of DNA and the way that they attach one another [5].

1953- James Watson and Francis Crick found the three-dimensional double helix structure of DNA [6].

1970s- Frederick Sanger, as well as the contributions from many other scientists and organizations, was able to independently invent a method of genome sequencing [6].

Around 1975- The Sanger Method, which is also known as the Chain Termination Method, evolved into the method of "shotgun" sequencing (described in "Present Technology"). Shotgun sequencing caused genome sequencing to become much quicker and to be the most widely used method [5].

1983- Kary Mullis invented the Polymerase Chain Reaction (PCR). The PCR is able to make many copies of DNA segments in a simple and inexpensive way, such as diagnosing diseases, identifying bacteria and viruses, and recognizing criminals for crime scenes [5].

1990- The Human Genome Project began.

1984- The U.S. Department of Energy (DOE), National Institutes of Health (NIH), and international groups held conferences to discuss the human genome [6].

1988- The idea of mapping the human genome was presented in order to find genetic maps, physical maps, and the complete nucleotide sequence map of the human chromosomes [6].

2003- Scientist were able to accurately map the human genome [5].

September, 2012- Genome editing began with the discovery of epigenetic editing [7].

April, 2013- The CRISPR/Cas system was used on zebrafish[7].

June, 2013- The CRISPR method is used as a user-friendly transcriptional repressor [7].

November, 2013- Epigenetic editing targets DNA demethylation, the process that is able to remove a methyl group from DNA nucleotides, which induces gene expression[7].

February 5, 2014- Chinese researchers conducted experiments on monkeys using the CRISPR/Cas9 method of genome editing [7].

April, 2014- The CRISPR/Cas9 method was able cure its first human related genes found in mice. This was able to happen by correcting a mutation to create a healthy phenotype.

May, 2014- Genome editing and induced pluripotent stem cells (iPSC) develop a "heart-on-chip" technology to reveals specific mutations of a heart abnormality. A synthetic heart is then created based on that information. Researchers use whole genome editing in human pluripotent stem cell clones to see how much collateral damage the new CRISPR/Cas9 and TALENs nucleus tools present. They found a very low amount of off-target mutations [7]

July, 2014- The CRISPR/Cas9 technology was used in haematopoietic cells and mice. A novel drug inducible lentiviral system was developed to deliver platform cells needed in the methods to cells allowing an easy and rapid way of genome engineering.

August, 2014- After combing the CRISPR/Cas9 and ChAP-MS, a new tool was made to see every protein of a specific genomic region[7].

August, 2014- The short guide RNA (sgRNA) directs the Cas9 to a specific target. The sgRNA was modified to make it reach a wider variety of locations in the genome [7].

August, 2014- Scientists applied ChIP-Seq to prove that Cas9 can sometimes cause off-target effect [7].

August, 2014- Patient's specifically induced pluripotent stem cells (iPSCs) are added to the CRISPR/Cas9 method. This allowed the system to meet more specific requirements when editing DNA [7].

Current Day Genome Editing- The main ways of genome editing are Zink-Finger Nucleus, TALENs, and CRISPR/Cas9. Zink-Finger was the first programmable genome editing tool that relies on proteins. It is has inconsistent results and can be negatively impacted by small uncontrollable activities that naturally occur in the human body [8]. The TALENs and CRISPR/Cas9 methods are relatively similar. However, the TALENs method is not as efficient as the CRISPR/Cas9 method because CRISPR/Cas9 uses RNA guides for precision DNA cutting, causing it to be more precise and safe. It is also able to be used with multiple cells at once.

History of CRISPR /Cas9 technology:

Ishino et al. initially discovered the CRISPR architecture in the 1980s when they noticed an "unusual structure" in the 3′ flanking region of the *Escherichia coli* iap gene. The region contained 5 highly homologous 29 base pair (bp) nucleotide sequences separated by 32 bp nucleotide variable regions. Over the next decade additional examples of CRISPR loci were identified as more and more bacterial genomes were sequenced. The CRISPR acronym itself was proposed in 2002 by Jansen and Mojica. It wasn't until 20 years after their initial discovery that the spacer sequences located within the CRISPR repeats were shown to confer resistance to specific bacteriophage introduced to the bacterial strain *Streptococcus thermophilus*, commonly used bacteria in the dairy industry.

These initial observations have since been confirmed in other organisms and now, the unique spacer regions in the CRISPR loci are understood to be a type of immune memory system to protect against invading phage or plasmid DNA. Through this system the bacteria are able to extract a short sequence from the invading DNA and file it away in the CRISPR locus where it can be accessed later by transcription.

Recent work utilizing CRISPR/Cas loci deficient Staphylococcus aureus transformed with the commonly used *Streptococus pyogenes* CRISPR locus has shown that multiple cas genes are important for the initial identification and excision of invading DNA. Structural analysis of different Cas proteins identified homology to known

endonuclease domains, suggesting a possible role in conferring viral resistance through the introduction of DSBs. During activation of the CRISPR response the unique spacer sequences are transcribed into short crRNAs. Garneau et al., was one of the first groups to show crRNA worked with Cas proteins to lead to DSBs in invading DNA.

The identification and characterization of different Cas genes in multiple bacteria and archaea lead to the classification of three major CRISPR types (I, II and III). Evidence that Cas9 was an RNA guided endonuclease with independent nuclease domains responsible for cutting both strands of DNA was presented later by Jinek et al. This was followed by the direct confirmation of this interaction through the solving of the crystal structure of the Cas9-guide RNA-target DNA complex [9]. A summary of the history of CRISPR/Cas technological development can be seen in **Fig. 2**.

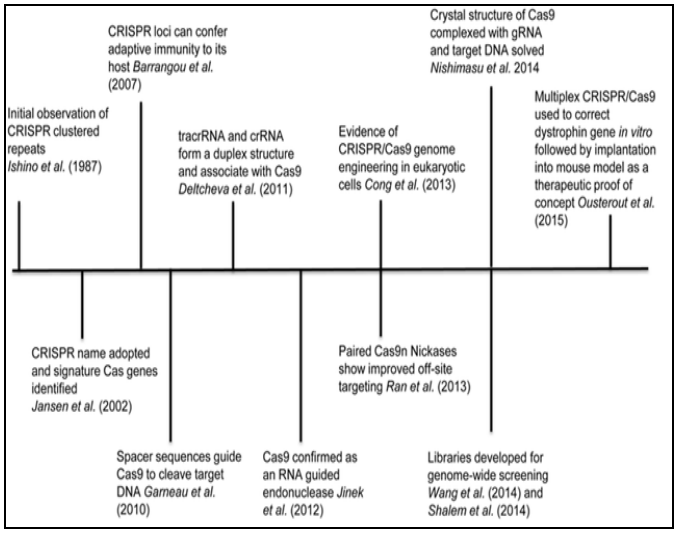

FIG. 2: HISTORY OF CRISPR/CAS DEVELOPMENTS

CRISPR/Cas9 system: [10]

The CRISPR-Cas mediated defense process can be divided into three stages. The first stage, adaptation, leads to insertion of new spacers in the CRISPR locus. In the second stage, expression, the system gets ready for action by expressing the cas genes and transcribing the CRISPR into a long precursor CRISPR RNA (pre-crRNA). The pre-crRNA is subsequently processed into mature crRNA by Cas proteins and accessory factors. In the third and last stage, interference, target nucleic acid is recognized and destroyed by the combined action of crRNA and Cas proteins.

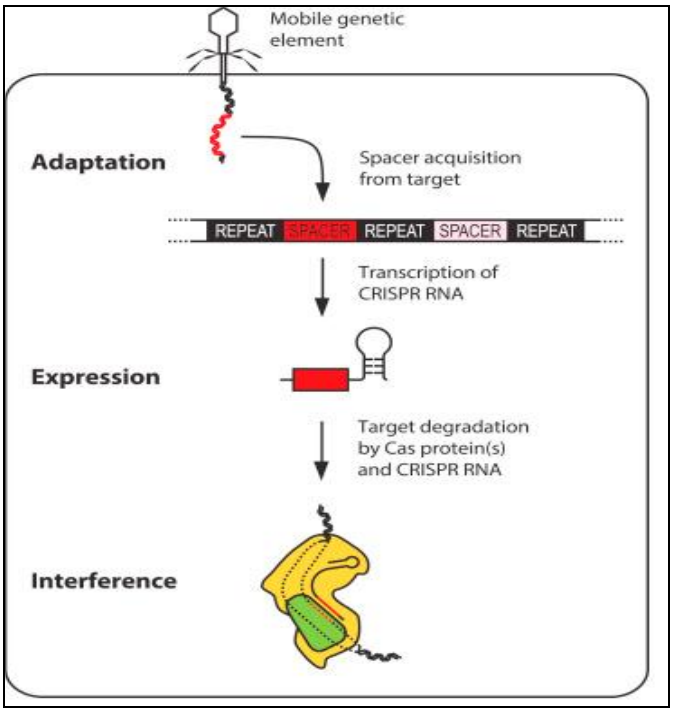

FIG. 3: THE KEY STEPS OF CRISPR-Cas IMMUNITY. 1) ADAPTATION: INSERTION OF NEW SPACERS INTO THE CRISPR LOCUS. 2) EXPRESSION: TRANSCRIPTION OF THE CRISPR LOCUS AND PROCESSING OF CRISPR RNA. 3) INTERFERENCE: DETECTION AND DEGRADATION OF MOBILE GENETIC ELEMENTS BY CRISPR RNA AND Cas PROTEIN(S).

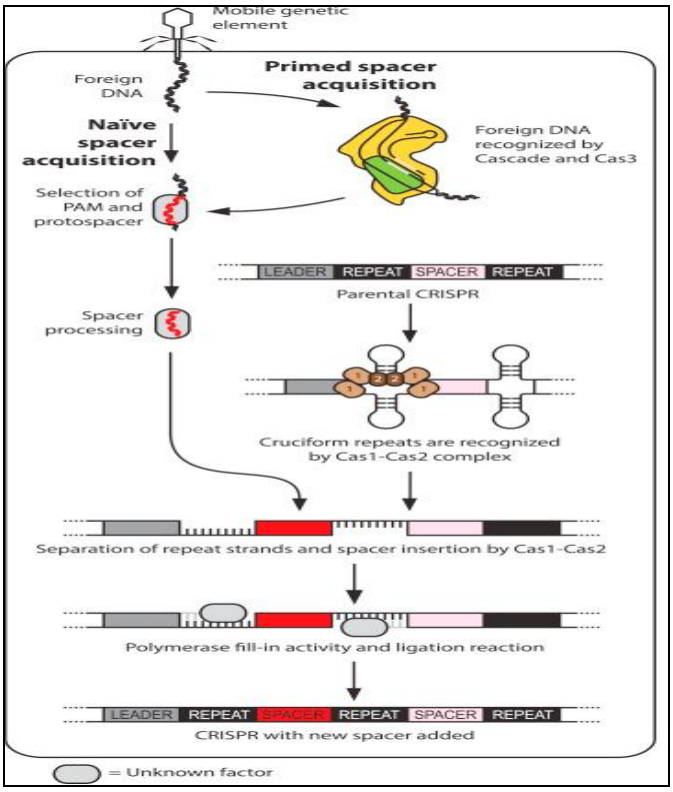

FIG. 4: MODEL OF THE ADAPTATION IN THE TYPE I-E SYSTEM.

There are two types of spacer acquisition, naïve and primed. Both require the presence of a PAM and are dependent on the Cas1–Cas2 complex. The Cas1–Cas2 complex recognizes the CRISPR and likely prepares it for spacer integration. Naïve spacer acquisition occurs when there is no previous information about the target in the CRISPR. Primed spacer acquisition requires a spacer in the CRISPR locus that matches the target DNA and the presence of Cas3 and the Cascade complex. Primed acquisition results in insertion of more spacers from same mobile genetic element. PAM = Protospacer Adjacent Motif.

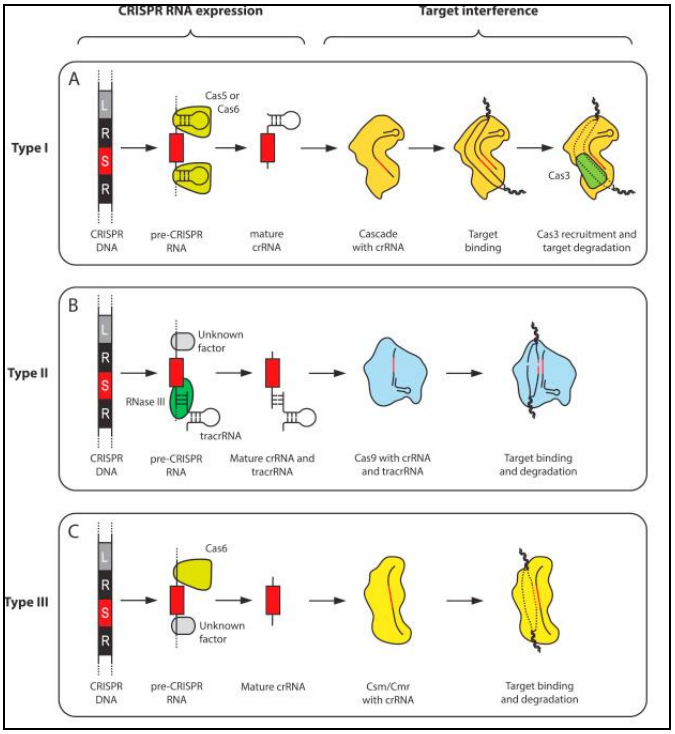

FIG. 5: MODEL OF crRNA PROCESSING AND INTERFERENCE. (A) IN TYPE I SYSTEMS, THE PRE-crRNA IS PROCESSED BY Cas5 or Cas6. DNA TARGET INTERFERENCE REQUIRES Cas3 IN ADDITION TO CASCADE AND crRNA. (B) TYPE II SYSTEMS USE RNase III and tracrRNA FOR crRNA PROCESSING TOGETHER WITH AN UNKNOWN ADDITIONAL FACTOR THAT PERFORM 5′ END TRIMMING. Cas9 TARGETS DNA IN A crRNA-GUIDED MANNER. (C) THE TYPE III SYSTEMS ALSO USE Cas6 FOR crRNA PROCESSING, BUT IN ADDITION AN UNKNOWN FACTOR PERFORM 3′ END TRIMMING. HERE, THE TYPE III Csm/Cmr COMPLEX IS DRAWN AS TARGETING DNA, BUT RNA MAY ALSO BE TARGETED.

Metagenomic, Structural and Functional Diversity of Cas9:

Cas 9 is exclusively associated with the type II CRISPR locus and serves as the signature type II gene. Based on the diversity of associated Cas genes, type II CRISPR loci are further subdivided into three subtypes (IIA–IIC). Type II CRISPR loci mostly consist of the cas9, cas1, and cas2 genes, as well as a CRISPR array and tracrRNA. Type IIC CRISPR systems contain only this minimal set of cas genes, whereas types IIA and IIB have an additional signature csn2 or cas4 gene, respectively. Subtype classification of type II CRISPR loci is based on the architecture and organization of each CRISPR locus. For example, type IIA and IIB loci usually consist of four cas genes, whereas type IIC loci only contain three cas genes.

However, this classification does not reflect the structural diversity of Cas9 proteins, which exhibit sequence homology and length variability irrespective of the subtype classification of their parental CRISPR locus. The length distribution of most Cas9 proteins can be divided into two populations centered on 1,100 and 1,350 amino acids in length. It is worth noting that a third population of large Cas9 proteins belonging to subtype IIA, formerly called Csx12, typically contains around 1500 amino acids. Despite the apparent diversity of protein length, all Cas9 proteins share similar domain architecture consisting of the RuvC and HNH nuclease domains and the REC domain, a helix-rich region with an Arg-rich bridge helix. Unlike type I and III CRISPR systems, which are found in both bacteria and archaea, type II CRISPRs have so far only been found in bacterial strains.

The majority of Cas9 orthologs in fact belong to the phyla of *Bacteroidetes*, *Proteobacteria*, and *Firmicutes*. The length difference among Cas9 proteins largely results from variable conservation of the REC domain, which associates with the sgRNA and target DNA. For example, the type IIC *Actinomyces naeslundii* Cas9, which is more compact than its *Streptococcus pyogenes* ortholog, has a much smaller REC lobe with substantially different orientation[11].

Protospacer Adjacent Motif: Cas9 target range and search mechanism:

A critical feature of the Cas9 system is the protospacer-adjacent motif (PAM), which flanks the 30 end of the DNA target site and dictates the

DNA target search mechanism of Cas9. In addition to facilitating self versus non-self discrimination by Cas9, because direct repeats do not contain PAM sites, biochemical and structural characterization of SpCas9 suggested that PAM recognition is involved in triggering the transition between Cas9 target binding and cleavage conformations. Single-molecule imaging indicated that Cas9-crRNAtracrRNA complexes first associate with PAM sequences throughout the genome, allowing Cas9 to initiate DNA strand separation via unknown mechanisms. DNA competitor cleavage assays additionally suggested that formation of the RNA-DNA heteroduplex is initiated at the PAM site before proceeding PAM distally by interrogating the target site upstream of the PAM for guide sequence complementarily.

Binding of the PAM and a matching target then triggers Cas9 nuclease activity by activating the HNH and RuvC domains, supported by the observation of HNH domain flexibility within the Cas9-sgRNA-DNA ternary complex. The complexity of the PAM sequences also determines the overall DNA targeting space of Cas9. For example, the 50-NGG of SpCas9 allows it to target, on average, every 8 bp within the human genome. Additionally, SpCas9 can target sites flanked by 50-NAG PAMs, albeit at a lower efficiency, further expanding its editing versatility. The PAM is specific to each Cas9 ortholog, even within the same species, such as 50-NNA GAAW for *Streptococcus thermophilus* CRISPR1 and 50-NGGNG for *Streptococcus thermophilus* CRISPR3. Another Cas9 from *Neisseria meningitidis* with a 50-NNNNGATT PAM requirement was recently applied in human pluripotent stem cells. Computational or metagenomic analysis of bacteria and archaea containing CRISPR loci could lead to the discovery of Cas9 nucleases with additional PAMs to expand the targeting range of the Cas9 toolkit.

Delivery of multiple Cas9 proteins with different PAM requirements facilitates orthogonal genome engineering, in which independent but simultaneous functions are applied at different loci within the same cell or cell population. NmCas9 and SpCas9, for example, can be employed for independent transcriptional repression and nuclease activity. PAM specificity can also be modified. For instance, orthologous replacement of the PAM-interacting (PI) domain from the *Streptococcus thermophilus* CRISPR3 Cas9 with the corresponding domain from *Streptococcus pyogenes* Cas9 successfully altered PAM recognition from 50-NGGNG to 50-NGG. PAM engineering strategies could also be exploited to generate short Cas9 orthologs with flexible 50-NGG or 50-NG PAM domains[11].

Comparision between Genome Editing Tools [12]:

ZFNs and TALENs function as dimers and only protein components are required. Sequence specificity is conferred by the DNA-binding domain of each polypeptide and cleavage is carried out by the FokI nuclease domain. In contrast, the CRISPR/Cas9 system consists of a single monomeric protein and a chimeric RNA. Sequence specificity is conferred by a 20-nt sequence in the gRNA and cleavage is mediated by the Cas9 protein.

The design of ZFNs is considered difficult due to the complex nature of the interaction between zinc fingers and DNA and further limitations imposed by context-dependent specificity. Commercially available ZFNs generally perform better than those designed using publicly available resources but they are much more expensive. TALENs are easier to design because there are one-to-one recognition rules between protein repeats and nucleotide sequences, and their construction has been simplified by efficient DNA assembly techniques such as Golden Gate cloning. However, TALENs are based on highly repetitive sequences which can promote homologous recombination in vivo. In comparison, gRNA-based cleavage relies on a simple Watson–Crick base pairing with the target DNA sequence, so sophisticated protein engineering for each target is unnecessary and only 20 nt in the gRNA need to be modified to recognize a different target.

ZFNs and TALENs both carry the catalytic domain of the restriction endonuclease FokI, which generates a DSB with cohesive overhangs varying in length depending on the linker and spacer. Cas9 has two cleavage domains known as RuvC and HNH, which cleave the target DNA three

nucleotides upstream of the PAM leaving blunt ends.

ZFNs can theoretically target any sequence but in practice the choice of targets is limited by the availability of modules based on the context dependent assembly platform. A functional ZFN pair can be prepared for every ~100 bp of DNA sequence on average using publicly available libraries. TALEN targets are limited by the need for a thymidine residue at the first position, but not all TALENs work efficiently in vivo and some pairs therefore fail to generate the anticipated mutations, which mean that each TALEN pair must be experimentally validated.

In contrast, the only theoretical requirement of the *S. pyogenes* CRISPR/Cas9 system is the presence of the NGG (or NAG) PAM motif downstream of the target sequence. However, imperfectly matched spacer sequences can result in cleavage at off-target positions, which means that gRNA sequences must be chosen carefully to avoid such artifacts thus reducing the number of targets that can be used in practice.

In terms of comparative performance, the first studies using the CRISPR/Cas9 system for genome editing in mammalian cell lines and zebrafish embryos showed that the technology was at least as efficient as ZFNs and TALENs targeting the same sites and in some cases even higher. Although the CRISPR/Cas9 system is generally efficient, some gRNAs achieve higher mutation rates than others, and this does not always depend on the local accessibility of the nuclease complex. Some guidelines are emerging to predict the efficiency of gRNAs. For example compared several gRNAs targeting the same gene in a human cell line and looked for trends associated with targeting efficiency. They found that gRNAs with an unusually high or low GC content tended to be less effective than those with an average GC content, and that gRNAs targeting the transcribed strand were less effective than those targeting the non-transcribed strand. They also found that Cas9 preferentially binds to gRNAs containing purine residues in the last four positions of the spacer sequence, and that the efficiency of cleavage is influenced by the affinity between the gRNA and Cas9.

TABLE 1: COMPARISON OF DIFFERENT PROGRAMMABLE NUCLEASE PLATFORMS

	Zinc finger nuclease	TALEN	Cas9	Meganuclease
Recognition site	Typically 9–18 bp per ZFN monomer, 18–36bp per ZFN pair	Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair	22 bp (20-bp guide sequence + 2-bp protospacer adjacent motif (PAM) for *Streptococcus pyogenes* Cas9); up to 44 bp for double nicking	Between 14 and 40 bp
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated	Small number of positional mismatches tolerated
Targeting constraints	Difficult to target non-G-rich sequences	5′ targeted base must be a T for each TALEN monomer	Targeted sequence must precede a PAM	Targeting novel sequences often results in low efficiency
Ease of engineering	Difficult; may require substantial protein engineering	Moderate; requires complex molecular cloning methods	Easily re-targeted using standard cloning procedures and oligo synthesis	Difficult; may require substantial protein engineering
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold; FokI is derived from bacteria and may be immunogenic	Unknown; protein derived from *Xanthamonas* sp.	Unknown; protein derived from various bacterial species	Unknown; meganucleases may be derived from many organisms, including eukaryotes
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of *in vivo* delivery	Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors	Difficult due to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors	Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologs exist	Relatively easy as small size of meganucleases allows use in a variety of viral vectors
Ease of multiplexing	Low	Low	High	Low

Similar conclusions were drawn during the development of a web tool to design gRNAs for the effective targeting of mouse and human genes. As in mammals, the CRISPR/Cas9 system has been shown to achieve high mutation rates in plants, matching or exceeding those obtained with ZFNs and TALENs. For example, a comparison of TALENs and CRISPR/Cas9 targeting the same sites in maize showed 13.1% efficiency with CRISPR and 9.1% with TALENs. Furthermore, the reported mutation rate can depend on the sensitivity of the analytical method (e.g., T7 endonuclease I or Surveyor assay, PCR/restriction analysis or PCR/direct sequencing). Therefore, it is not surprising that reported mutation rates vary even within the same species.

Finally, CRISPR/Cas9 activity is greatly dependent on the cell type and delivery method, as is the case for other nucleases. For example, PEG-mediated transfection of *N. benthamiana* mesophyll protoplasts resulted in a mutation rate of 37.7% in the PDS gene whereas the same constructs delivered by agro infiltration into whole leaves achieved a mutation rate of 4.8% in the same gene. It is unclear whether this ~10-fold change represents differences in transfection efficiency, gRNA/Cas9 expression levels or DNA repair mechanisms in the distinct cell types.

Applications of CRISPR/Cas9 Technology:

CRISPR-Cas9 in the generation of animal models:

Gene targeting based on homologous recombination and embryonic stem cells has been used as the typical approach for animal genome modification, which has played indispensable roles in making a causal link between genomic mutations and phenotypes during development and in disease. However, gene targeting has limited applications in some organisms due to time-consuming procedures and the lack of available embryonic stem cells. Many recent studies have shown that CRISPR-Cas9 technology could be used for rapidly generating targeted genome modifications in the germ lines of various model organisms[13-27], which will significantly advance the functional genomics. Microinjection of Cas9-encoding mRNA and customizable sgRNA into one-cell stage zebrafish embryos is able to efficiently modify the target genes in vivo in a simple, rapid and scalable manner [13, 14]. Co-injection of Cas9 mRNA and sgRNAs targeting different genes into mouse zygotes generates mutant mice with biallelic mutations, confirming that CRISPR/Cas-mediated gene editing could be used for the simultaneous disruption of multiple genes with high efficiency [15].

Gene knock in mice carrying precise point mutations of two genes can be obtained by co-injection of Cas9 mRNA/sgRNAs together with mutant oligos [16]. The following study demonstrates that reporter and conditional mutant mice can also be generated in one step by co-injecting mouse zygotes with Cas9 mRNA and different sgRNAs, as well as DNA vectors of different sizes. Additionally, mice with the predicted deletions have been generated using sgRNAs targeting two separate sites in the gene [15].

Multiplexed activation of endogenous genes can be achieved by injecting a two-component transcriptional activator including a nuclease-dead Cas9 protein fused with a transcriptional activation domain and sgRNAs targeting gene promoters[17]. These previous studies have demonstrated that CRISPR-Cas9 technology can be used for efficient one-step generation of various sophisticated mutant mice, including mice carrying gene insertions, deletions, conditional alleles and endogenous reporters at different loci. A recently established Credependent Cas9 knock in mouse may further facilitate the generation of genetic modified mutant mice by simply injecting sgRNA [18].

CRISPR-Cas9 technology has been used for efficient genome engineering in many other model organisms, including Drosophila [19, 20], *Caenorhabditis elegans* [21], Axolotl [22], *Xenopus tropicalis* [23, 24], rat [25] and pig [26]. Significantly, the CRISPR-Cas9 system has been shown to be an efficient and reliable approach for targeted modification of cynomolgus monkey genomes [27]. The application of CRISPR-Cas9 technology for genome editing in a wide range of organisms will promote our understanding of development and disease and help develop animal models and therapeutic strategies for human diseases.

CRISPR-Cas9 in somatic genome editing:

Rapid progress in genome engineering based on the CRISPR-Cas9 system enables fast functional characterization of putative disease genes in various mouse models via somatic genome editing [28-30]. A CRISPR plasmid DNA expressing Cas9 and sgRNAs can be delivered to the liver through hydrodynamic injection, and CRISPR mediated Pten mutation with or without p53 mutation phenocopies the effects of PTEN and p53 gene knockout using Cre-LoxP technology [28]. Previous studies have also shown that an activated mutant β-catenin gene could bedelivered into hepatocytes by co-injection of Cas9 plasmids expressing sgRNAs targeting the β-catenin gene and a DNA oligonucleotide donor carrying β-catenin activating point mutations [28].

This previous study demonstrated that the CRISPR-Cas system could be used for directly mutating tumor suppressor genes and oncogenes in somatic tissues, providing a new approach for developing new types of disease models. The CRISPR-Cas9 system has also been used to induce a specific chromosomal rearrangement, the Eml4-Alk inversion, in somatic cells of adult animals to generate a mouse model of Eml4-Alk-driven lung cancer [29]. The resulting tumors exhibit the typical histopathological and molecular features of ALK(+) human non-small cell lung cancer (NSCLC), which is sensitive to ALK inhibitors [29]. Interestingly, using a lentiviral-based delivery system, a recent study demonstrated that CRISPR-induced genome editing of tumor suppressor genes together with Cre-dependent somatic activation of oncogenic Kras(G12D) causes lung adenocarcinomas with different histopathological and molecular features [30].

Using the Cas9 gene knockin mice, lung adenocarcinoma models can be generated by simultaneously introducing a single AAV vector carrying loss-of-function mutations in p53, Lkb1 and Kras(G12D) mutations in the lung [18], suggesting that Cas9 gene knockin mice could be widely used for somatic genome editing. The rapid somatic genome engineering approach will greatly help to systematically identify critical genes underlying disease initiation and progression in many well-established disease mouse models.

CRISPR-Cas9 in functional genomics screening:

Functional genomics screening is largely used for identifying the essential genes for a specific cellular process. RNA interference (RNAi) [31] has been dominantly applied for genome-wide screening; however, the off-target effects of RNAi have limited its applications [32-34]. In addition, RNAi could not be used for silencing RNAs located in nucleus. The CRISPR-Cas9 system has been successfully used in various genome-scale loss of function screening [35-38]. Using a genome-scale lentiviral sgRNA library, all expected genes of the DNA mismatch repair pathway have been identified in screening for resistance to the nucleotide analog 6-thioguanine, and numerous genes corresponding to fundamental processes have been obtained with a negative selection screening for essential genes [35].

A genome-scale CRISPR-Cas9 knockout (GeCKO) library has been developed and successfully used for screening genes essential for cell viability in cancer and pluripotent stem cells and for genes associated with the resistance to vemurafenib, a drug for late-stage melanoma [36]. A CRISPR-Cas-based knockout library has been applied to identify the host genes mediating the cellular responses to anthrax and diphtheria toxins [37].

A recent study has shown that saturation editing of genomic regions could be achieved by coupling CRISPR-Cas9 technology with multiplex homology-directed repair using a complex library of donor templates, facilitating high-resolution functional screening of both cis-regulatory elements and trans-acting factors in the genome [38].

A series of studies has demonstrated that CRISPR mediated repression (CRISPRi) and CRISPR-mediated activation (CRISPRa) are powerful tools for functional genomic screening. A CRISPRi system consisting of a catalytically inactive Cas9 and a guide RNA has been shown to specifically and efficiently repress the transcription of target genes in Escherichia coli and mammalian cells [39, 40], whereas a catalytically inactive Cas9 fused with a transcriptional activation domain has been used to activate the expression of specific endogenous genes [41-43]. Genome-scale CRISPRi and CRISPRa libraries that specifically target transcriptional

repressors or activators to endogenous genes have been successfully used for screening essential genes for growth, tumor suppression, differentiation regulation, and cellular sensitivity to a cholera-diphtheria toxin, suggesting that CRISPRi and CRISPRa are valuable tools for mapping complex pathways [44].

A very recent study has shown that CRISPRCas9 complexes with synergistic activation mediators are able to achieve robust, single sgRNA-mediated gene upregulation at endogenous genomic loci. When used with an sgRNA library, the engineered Cas9 activation complexes can activate multiple genes simultaneously, upregulate long intergenic non-coding RNA transcriptsand identify genes conferring resistance to a BRAF inhibitor through a genome-wide dCas9-based transcription activation screening in a melanoma model[45]. These results demonstrate that CRISPR-Cas9 technology can be a promising functional genomic screening tool for discovering essential genes in various biological processes.

CRISPR-Cas9 in correction of genetic disorders:

One of the most exciting applications of the CRISPR-Cas9 is the possibility of curing genetic diseases. The CRISPRCas9 system has been shown to efficiently correct a dominant Crygc gene mutation in a cataracts mouse model by co-injecting Cas9 mRNA and sgRNA targeting the mutant Crygc allele into zygotes [46]. A very recent study has shown that the CRISPR-Cas9 system can be used to modify an EGFP transgene or the endogenous Crygc gene in spermatogonial stem cells (SSCs).

The modified SSCs carrying a corrected Crygc mutation can undergo spermatogenesis and produce offspring with the corrected phenotype at an efficiency of 100% [47]. The injection of Cas9, sgRNA, and homology-directed repair template into mouse zygotes has been shown to correct the dystrophin gene mutation responsible for muscular dystrophy in the germ line and prevent the development of muscular dystrophy in mutant mice[48]. Interestingly, a similar strategy using the CRISPR-Cas9 technology has successfully corrected the cystic fibrosis transmembrane conductor receptor (CFTR) locus by homologous recombination in cultured intestinal stem cells of cystic fibrosis human patients [49], demonstrating that primary adult stem cells derived from patients with a single-gene hereditary defect could be corrected by CRISPR/Cas9 mediated homologous recombination, suggesting a promising strategy for gene therapy in human patients.

CRISPR-Cas9 in the treatment of infectious diseases:

Considering that the CRISPR-Cas system originally functions as an antiviral adaptive immune system in bacteria, this system could be used for treating infectious diseases by eradicating pathogen genomes from infected individuals. Recently, studies have shown that the CRISPR-Cas9 system can eliminate the HIV-1 genome and prevent new HIV infection [50, 51]. When transfected into HIV-1 provirus-integrated human cells, a sgRNA expression vector targeting the long terminal repeats (LTR) of HIV-1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression.

In addition, this system has been shown to delete viral genes from the host cell chromosome [50]. The high specificity of Cas9/sgRNAs in editing the HIV-1 target genome has also been recently demonstrated [51]. Cas9/sgRNAs efficiently inactivate HIV gene expression and replication in latently infected cells, including microglial, promonocytic and T cells. Significantly, Cas9/sgRNA mediated genome editing has been shown to immunize cells to prevent HIV-1 infection[51]. These results indicate that the CRISPR-Cas9 technology can serve as a potential tool for clinical applications to cure infectious diseases.

CONCLUSION: The CRISPR-Cas9 technology, an efficient, inexpensive, fast-to-design, and easy-to-use genomic editing tool, has been rapidly applied in many fields, ranging from basic biology to translational medicine. The innovative applications of the CRISPR-Cas9 system will accelerate our understanding of the mechanisms underlying development, physiology and disease. CRISPR is still a young system and more research must be completed in order to rectify its problems. While there are many challenges ahead before

CRISPER/Cas9 can be utilized as a safe and reliable gene therapy, these challenges do not seem insurmountable. Research in the area of CRISPR/Cas9 is gaining speed and this system could very well be the solution to many medical issues we face today.

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How to cite this article:
Benazir F and Abhinayani G: CRISPR/Cas9 Technology- A New Boon in Genome Editing. Int J Pharm Sci Res 2016; 7(8): 3336-47.doi: 10.13040/IJPSR.0975-8232.7(8).3336-47.