Arabian Journal of Chemical and Environmental Research

Vol. 04 Issue 2 (2017) 127–136

CRISPR-Cas systems: The 21th century Biorevolution

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Received 18 July 2017, Revised 25 Dec 2017, Accepted 28 Dec 2017

Abstract

The development of gene editing tools becomes an important field of biotechnology research. In 2012, a prokaryotic repetitive DNA clusters became an important tool in Eukaryotic cells gene editing known as Clustered Regularly Interspaced Short Palindromic repeats (CRISPR). CRISPR-associated proteins (Cas) make functional structures know as CRISPR-Cas systems involved essentially in prokaryotic adaptative immunity and highly efficient gene editing tool for precision-cuts used in basic and applied research. CRISPR-Cas systems are now a well democratized tool sold as kits to the applied research laboratories and used as routine technique. Beginning with the Bacterial transformation aiming to enhance the brewing processes, passing through crops genetic modification to enhance the productivity and landing on biomedical research, the CRISPR technology is being tested on different laboratory models and on humans to enhance the therapeutic approaches regarding many diseases. However, the democratization of CRISPR technology is encouraging the rise of the transhuman ideology boosted by the "CRISPR auto-users" aiming to modify human genetic patrimony to enhance the body's performance. This intensifies the ethical debate about the respect of the biodiversity and the necessity of its conservation, shedding light on the importance of regulating the access to CRISPR kits. In conclusion, CRISPR technology is a hope to bypass many bioproblems at the molecular level but could also be a lethal weapon to destroy the biodiversity if the ethical window is closed

Keywords: CRISPR Technology, Gene editing, Ethical Debate, Therapeutic Molecular Tool, Ethical threads, gene therapy

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1. Introduction

Throughout the historical timeline of science, human being always seeks to control nature and living things behavior. By the end of World War II, the research machinery became more productive in all fields especially in biology. In 1953, [1] first published a paper on the molecular structure of the deoxyribonucleic acid (DNA). This discovery made an inflection point of the biological research field.

ISSN : 2458-6544

The transition from the biochemical to the molecular studies was of great impact on the discovery of gene editing tools that could be helpful for basic and applied research, drug discovery, and diseases control and management. It all started by the publication of the seminal work of development of homologous recombination principle discovered independently by [2, 3]. Furthermore, ZFNs (Zinc finger nucleases) in Xenopus oocytes [4] and TALENs (transcription activator-like effector nucleases) in xanthomonas bacteria [5] are now considered as laboratory routine tools based on programmable nucleases activity that make gene editing more precise and accurate. Most recently, a new technological revolution made the precise gene editing hope possible by the discovery of clustered regularly interspaced palindromic repeats (CRISPR) systems by the hybrid team of Doudna and Charpentier [6]. CRISPR are linked to a set of nucleases proteins called CRISPR associated nucleases (Cas) which make the CRISPR-Cas systems functional and ready to precise gene cutting.

The present paper reviews the CRISPR technology discovery shedding light on the possible applications in both basic and applied research and reminding the possible ethical issues to be considered to avoid free and overwhelming biodiversity transformation.

2. Brief history of CRISPR systems discovery

While studying the gene involved in isozyme conversion of the alkaline phosphatase in E. Coli, Yoshizumi Ishino, a member of Atsuo Nakata research Team (Osaka University), first detected repetitive clusters in the bacterium genome [7]. The molecular function of this highly organized prokaryotic genome clusters was hardly predictable at this time. In 1993, the same genomic organization pattern was observed in *Haloferax meterannei* (archea) by Mojica when studying the archaeon behavior at different salinites [8]. In a seminal work published twenty years after the first CRISPR description, Haft team gave insight on the possible biological function of the CRISPR as a part of bacterial immune system against bacteriophages [9]. Barrangou studied the CRISPR immune function by inducing a bacterial viral challenge and studying the viral genome integration in the invaded bacterium [10]. At this time the automatic and accurate cut and paste ability of the CRISPR associated nuclease was outstanding (Figure 1). In 2011, Charpentier and Doudna collaboration aimed to develop a universal genomic editing tool exploiting Type II CRISPR-Cas system from Streptococcus pyrogens [6].

This work is considered as the most influencing one in the CRISPR technology development. The discovered versality of CRISPR-Cas systems announced the begin of a new era of universal precise gene editing tool and made flex in the number publications related to CRISPR technology research (Figure 1). However, the CRISPR democratization opened the gates toward mammalian genome editing with an emerging case of controversial and unethical first edited human embryos in china [11]. One year later, UK and US authorities licensed research teams to use CRISPR Technology for editing human embryos

and for clinical testing in hope to treat some critical diseases [12, 13].

Table 1. Historical timeline of Clustered regularly Interspaced Palindromic repeats CRISPR-Cas systems discovery

Date	Discovery description	*Team affiliation*	Reference
1987	The CRISPR first observed in *E. coli*	*Osaka University*	[7]
2000	DNA clustered repeats identified in bacteria and archaea and named Short Regularly Spaced Repeats (SRSR)	*University of Alicante, University Miguel Hernandez*	[14]
2002	Term CRISPR published for first time	*Utrecht University*	[15]
2005	Researchers identified families of Cas genes involved in protecting bacteria against invading viruses	*The Institute for Genomic Research*	[9]
2007	Experimental demonstration of the role of CRISPR together with Cas9 genes in protecting bacteria against viruses	*Danisco USA Inc*	[10]
2008	DNA, not RNA, is the molecular target of most CRISPR-Cas systems	*Danisco USA Inc*	[16]
2011	Emmanuelle Charpentier and Jennifer Doudna collaboration to investigate Cas9 enzyme	*University of California Berkeley, Umea University*	[17]
2012	Publication of new gene editing method that exploits the CRISPR-Cas9 system	*University of California Berkeley*	[6]
2015	First genes edited in human embryos ignited global ethical debate about gene editing technology	*Sun Yat-sen University*	[11]
2016	First licence to edit human embryos using CRISPR-Cas 9 delivered to Dr. Niakan K.	*Crick Institute*	[12]
2016	NIH authorizes first clinical trial using gene editing tool CRISPR/Cas 9 to treat patients	*University of Pennsylvania*	[13]

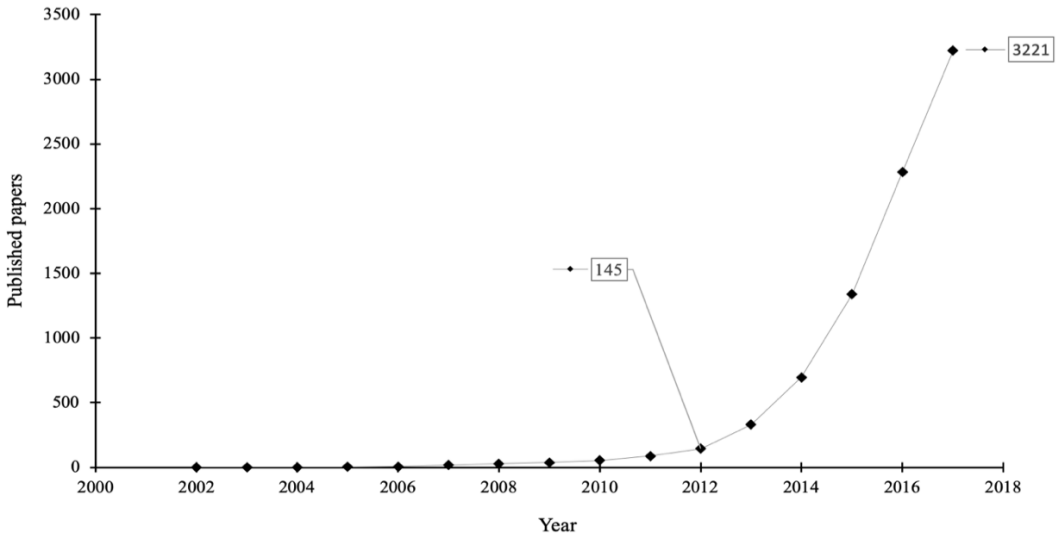

Figure 1. Evolution of CRISPR related papers (data extracted from Pubmed)

3. Basic principle of CRISPR Systems gene editing

The CRISPR-Cas9 structure and functional behavior are shown in Figure 2. The system requires essentially two complexed components: a Cas 9 DNA nuclease and a single guide RNA (sgRNA) that is complementary to the target DNA.

The Cas 9 protein is the most studied CRISPR associated nuclease. The electron microscopy and X-ray crystallography studies showed a bilobed structure and-state dependent conformation of this nuclease. Cas 9 double strand break (DSB) activity is not constitutive and requires the presence of the gRNA to be expressed in target site [6,18,19]. Further conformational changes follow the Cas9-target DNA interaction via the g-RNA. This sequence of events may occur simultaneously with target-DNA unwinding and g-RNA strand invasion [20]. In the other hand, mechanistic investigations showed the crucial role of a specific target DNA sequence know as Protospacer Adjacent motif (PAM) for the initial binding to the DNA. Absence of the PAM sequence makes the target DNA sequence not recognized by the CRISPR-Cas system even if the g-RNA is fully complementary to the target DNA [21].

When introduced to eukaryotic cells, Cas9-sgRNA induces a double strand break (DSB) at the target DNA sequence. This molecular maneuver is immediately repaired via an error-prone repairing pathways: non-homologous end joining (NHEJ) (consisting of ligation of the DSB resulting in addition or deletion of nucleotides in target site); or homology directed repair (HDR) (when the repairing machinery replace the cut sequence by a second copy of it) [22,23] (Figure 2). The implication of these repair mechanisms made the gene editing using CRISPR technology easier in research laboratories.

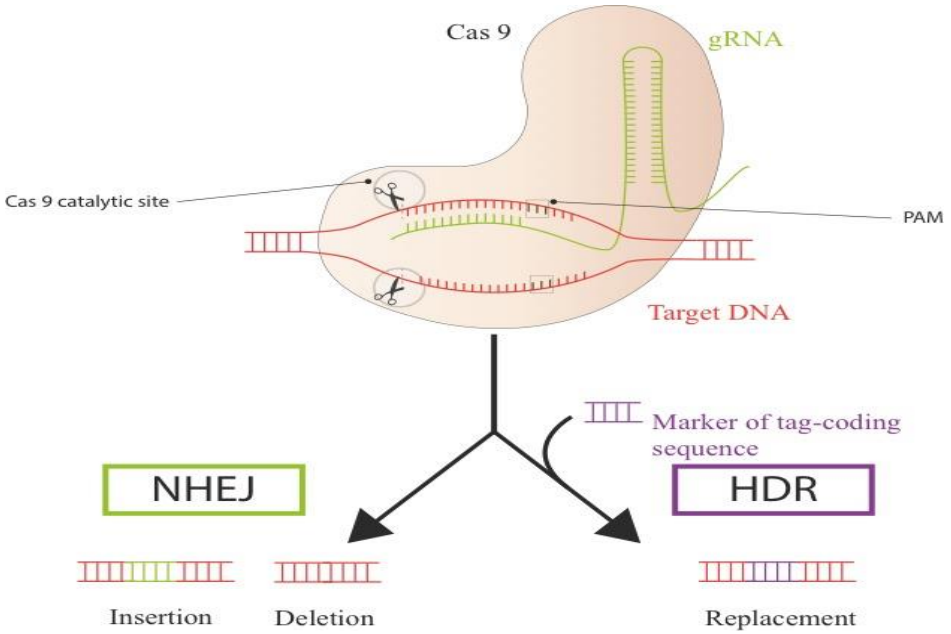

Figure 2. Clustered regularly interspace palindromic repeats associated protein 9 (Cas 9) and the guide RNA (gRNA) complex binding to the target DNA double strand. The RNA-DNA interaction in made possible because of the Protospacer Adjacent Motif (PAM) in the target DNA. After the precise cut at the target site, two possible mechanisms of repairing could start: Non-homologous End Joining (NHEJ) and Homology directed repair (HDR).

The rising problem of CRISPR-Cas system gene editing is the off-target editing. Inducing a gene editing in other loci out of the targeted sequence could induce problematic mutations in the studied cell or organisms or even if this technology in translated to clinical application. However, J. Doudna and E. Charpentier state that "*Active Cas9 rarely cleaves the DNA at off-target binding sites, implying*

decoupled binding and cleavage events in which nearly perfect complementarity between the guide RNA and the target site are necessary for efficient DNA cleavage. These observations are consistent with results obtained for Cas9–guide RNA complexes in single-molecule experiments" Claims by Sternberg work published in 2014 [20, 21]

4. *CRISPR technology and basic research*

The discovery of CRISPR-Cas systems removed the technical and financial barriers to use gene editing tools in basic laboratory research [24-26]. The advent of this novel technology has drastically revolutionized the landscape of genetically engineered laboratory models development that are translatable to humans for disease treatment studies [27-30].

4.1.*CRISPR-Cas plasmid*

The plasmid vectorized CRISPR-Cas technology has been used in research laboratories because of the technical feasibility and the affordable and ready to use transfection technology. This technique has been used in a wide range of laboratory models and aims on the transfection or injection of a plasmid-carried CRISPR-Cas 9 to the target cell (Figure 3A). In studies conducted on *D. melanogaster*, Gokzade team proposed a bicistronic simple plasmid injection of Cas9/sgRNA vectors in embryos [31]. This versatile technique allowed a rapid isolation of knock-out / knock-in alleles after 2 months of the plasmid injection. In another study, a humanized Cas9/sgRNA has been inserted in PX330 plasmid and injected in mouse zygote [32]. This experiment yielded 52.9±22,3 % of target mutation in adult mice making the technique applicable for large scale mammalian mutagenesis. However, Y. Fujihara and M. Ikawa, in their study conducted in Osaka university (japan), reported that a Cas9/gRNA integration to the pCAG-EGxxFP plasmid permits a better integration and reproducibility of the mutagenicity in mice [33] .

4.2.Single cell fertilized zygote injection

Direct transfection or nucleic acid microinjection is one the classic and dominating techniques used in transgenesis laboratories. It consists of direct integration of the nucleic acid molecule (DNA/RNA) to the target cell (zygote) via an injection pipet to the pronucleus of a developing zygote avoiding the disruption of its membrane (Figure 3B). [34]. In a study conducted by Xie *et al*, the efficiency of direct injection of CRISPR-Cas in inducing the required edition is better when the injection is done prior oocyte fertilization in zebrafish [35]. In other work, a cytoplasmic injection has been described by Horri and Hatada in a paper on use of *in vitro* transcribed Cas 9/ gRNA to edit a fertilized mouse zygote [36]. The application of this technique in laboratory animals made it translatable to large mammalians [37].

In 2016, a Chinese team induced a human like porcine laboratory model of Duchene muscle dystrophy that could be used to study the therapeutic possibilities of this genetic disease in humans [38]. In an original work conducted in Guangzhou Medical University in China, tripronuclear (non-viable) human

embryos (3PN) had been edited by co-injecting CRISPR-Cas 9 technology and donor DNA to introduce a mutation in the C-C chemokine type 5 reception (CCR5Δ32) allele [39, 40].

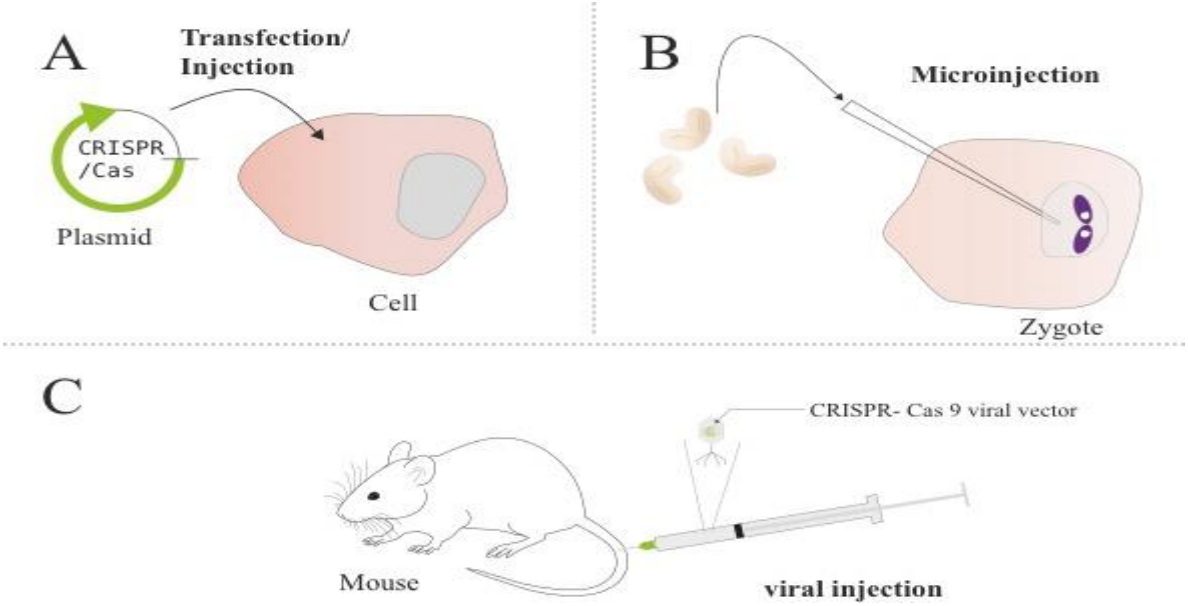

Figure 3. Techniques used for CRISPR-Cas system integration to different systems.

The triploid human embryos edition has been successful and opening the gates toward a possible diploid (viable) human embryos edition. The first procedure of the CRISPR-Cas9 technology on diploid human embryos has been conducted by Tang *et al.* in Beijing Proteome Research Center, China. In this controversial work, the researcher successfully edited viable human embryos to correct mutation in HBB (hemoglobin subunit beta) gene and the G6PDH (Glucose-6-phosphate dehydrogenase) gene. [41] However, this secret germline manipulation sparked a blazing commentary by S. Hohman in the *editor's commentary of Molecular Genetics and Genomics* published on March 2017.[42].

1.1. Virus-vectorized CRISPR reagents

The CRISPR-Cas system could be also delivered to organisms via a natural or synthetic viral vector (Figure 3C). Kratzer and Kreppel produced and purified the first generation adenovirus, based on type 5 adenovirus, expected to be used in CRISR-Cas vectorization for genetic edition. [43]. In a paper authored by Nishiyama et al., a combination of CRISPR-case mediated DNA cleavage and Adenovirus-mediated donor template delivery has been used to tag an endogenous protein in an *in vitro* primary and organotypic neuronal culture as well as *in vivo* developing, adult, aged and pathological mouse brains [44]. Among the adeno viruses, artificial "core-shell" viruses could be used in delivery the CRISPR cas 9 to ensure a more accurate cell targeting and gene edition.[45]

2. CRISPR Technology and applied research

Clinical gene therapy is a wide field of basic and translational research that emerged after the discovery of ZFNs TALENs but had been always stopped by the high cost and the technical difficulty during the

application. However, the hope of democratizing this therapeutic procedure raised up after the discovery of the universal possible use of the CRISPR-Cas systems. In their narrative review, Cai *et al.* cited the principal works that had been conducted on disease specific iPS human cells for a possible therapeutic use of the CRISPR technology against Barth syndrome effects on the heart, Duchenne muscular dystrophy, hemophilia, β-Thalassemia, and cystic *fibrosis.* [46]. Other works had been dedicated to discuss the application of the technology to treat ß-globulinopathies [47] and genetic/non genetic eye disease [48] . CRISPR Clinical Trials are led by Chinese research teams. Actually, 5 clinical trials are running in different Chinese institutes (Table 2).

Table 2: CRISPR technology clinical trials in different Chinese institutions. Data collected from https://clinicaltrials.gov (2017)

Molecule	*Study Title*	*Condition*	*Affiliation*	*Country*	*Trial ID*
Cas9	Programmed cell death protein 1 (PD-1) knockout	Metastatic cell lung cancer	Peking University	China	NCT02793856
Cas9	Programmed cell death protein 1 (PD-1) knockout	Stage IV bladder cancer	Peking University	China	NCT02863913
Cas9	Programmed cell death protein 1 (PD-1) knockout	Metastatic renal cell carcinoma	Peking University	China	NCT02867332
Cas9	Programmed cell death protein 1 (PD-1) knockout	Hormone refractory prostate cancer	Peking University	China	NCT02867345
Cas9	Programmed cell death protein 1 (PD-1) knockout	EBV-positive, advanced stage malignancies	Nanjing Drum Tower Hospital of Nanjing University Medical School	China	NCT03044743
Cas9	Programmed cell death protein 1 (PD-1) knockout	esophageal cancer	Hangzhou Cancer Center	China	NCT03081715
Cas9	CCR5 knockout	HIV	Affiliated Hospital to Academy of Military Medical Sciences	China	NCT03164135

3. Ethical debate

The discovery of the precise gene editing properties of CRISPR-Cas system induced an avalanche of ethical commentaries in hope of regulating this novel technology. At First, the risk/benefit of this technology must be evaluated before any application in the key industrial areas such as biomedicine, agriculture and biotechnology. Here we emphasize the possible benefits in efficiently treating genetic disorders, enhancing the crop resistance to parasites, and enhancing bacterial performance in agri-food industry. However, those benefits are challenged by many risks in almost all areas of CRISPR/Cas application. In biomedical industry, the possibility of using CRISPR technology in germline editing is the most anchoring ethical point about this technology. This arises another social problem related to

eugenics and ethnical selection. In the other hand, CRISPR application in agriculture and crops development is alarming and could be detrimental to the natural occurring species and then perturbates ecosystems worldwide [49]. Similarly, the open access to CRISPR technology and its unregulated democratization could be of high risk to the transhuman society aiming to develop high-level humans by maybe autoinjecting CRISPR/Cas systems to target cell senescence and intelligence genes. The second bold point of the CRISPR ethical debate is the race behind patenting the CRISPR/Cas system. An international debate raised about the patentability of this molecular technology. While DNA is considered as a "Product of Nature" it could not be patented. However, CRISPR/Cas system, in essence, is not comparable to the DNA molecule alone and other questions may appear "is this a novel system?" "is it only product of nature?" "is it an invention of only nature discovered?". [50, 51]. Technically, CRISPR/Cas systems had been discovered in bacteria and archaea but had been subject to laboratory modification to be functional in animal and human cells. Hence, the subsequent modifications are patentable. Here, the US Patent and Trademark Office awarded the Broad Institute team led by Zhang the first patent rights to CRISPR/Cas technology. Another Us Team led by DOUDNA from University of California filed an interference claim against the patent award challenging the date in which each team adapted CRISPR technology to work in cells others than bacteria [51]. As matter of fact, CRISPR /Cas technology application in different fields, if based on fundamental moral and deontological reasons, could be of high benefits to human beings around the world.

Conclusion

The CRISPR technology has now become a leading specific molecular guided gene editing tool that could be applied to almost all bioediting processes through basic of applied approaches. However, living organisms' gene editing is a problematic topic pulled bilaterally by the necessity of bypassing many genetic problems and the possibility of inducing ecochaotic events that put *Homo sapiens* in the dilemma of being human.

Conflict of interest-The author declares no conflict of interest.

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