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RESEARCH ARTICLE

CRISPR DELIVERY SYSTEMS AND ITS APPLICATIONS

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ABSTRACT

Discovery of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), the mechanism of the CRISPR based prokaryotic adaptive immune system and its repurposing into a potent gene editing tool has revolutionized the field of molecular biology and generated excitement for new and improved gene therapies. Additionally, the simplicity and flexibility of the CRISPR /Cas 9 site specific nuclease system has led to its widespread use in many biological research areas including development of model cell lines, discovering disease mechanisms, development of transgene plants and animals. In this review we present a CRISPR delivery systems including physical delivery methods like microinjection, hydrodynamic delivery and viral vector delivery methods like Adeno Associated Virus (AAV), Lentivirus (LV) and Adenovirus (AdV) and its applications in different fields.

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INTRODUCTION

The features of the most widely used systems for delivery of CRISPR/Cas9 components. Delivery can be broken into two major categories: cargo and delivery vehicle. Regarding CRISPR/Cas9 cargoes, there are three approaches that are commonly reported: (1) DNA plasmid encoding both the Cas9 protein and the guide RNA, (2) mRNA for Cas9 translation alongside a separate guide RNA, and (3) Cas9 protein with guide RNA (ribonucleoprotein com-plex). The delivery vehicle used will often dictate which of these three cargos can be packaged, and whether the system is usable in vitro and/or in vivo. As an example, Cas9 protein is positively-charged, but oligonucleotides and Cas9: sgRNA RNP are negatively charged (Sun *et al*., 2015). Vehicles used to deliver the gene editing system cargo can be classified into three general groups: physical delivery, viral vectors, and non-viral vectors.

The most common physical delivery methods are microinjection and electroporation, while methods such as hydrodynamic delivery are currently under investigation. Viral delivery vectors include specifically engineered adeno-associated virus (AAV), and full-sized adenovirus and lentivirus vehicles. Especially for in vivo work, viral vectors have found favor and are the most common CRISPR/Cas9 delivery vectors. Non-viral vector delivery is not as prominent as viral-based delivery; however, non-viral vectors possess several advantages over viral vectors and area bourgeoning area of research. Non-viral vector systems include systems such as lipid nanoparticles, cell-penetrating peptides (CPPs), DNA 'nano clews', and gold nano particles. There are additionally many delivery technologies that have not been demonstrated in the literature as suitable to CRISPR/Cas9 delivery, though they appear to naturally lend them-selves to the application.

Four such technologies are streptolysin O, multifunctional envelope-type nanodevices (MENDs), lipid coated mesoporous silica particles, andother inorganic nanoparticles.

Physical delivery methods

Microinjection: Microinjection is considered the 'gold standard' for introducing CRISPR components into cells, with efficiencies approaching 100% (Yang *et al*., 2013; Horii *et al*., 2014). In this method, either plasmid DNA encoding both the Cas9protein and the sgRNA, mRNA encoding Cas9 and sgRNA, orCas9 protein with sgRNA, can be directly injected into individual cells. Using a microscope and a 0.5–5.0 l m diameter needle, a cell membrane is pierced and cargoes are delivered directly to a target site within the cell. This process circum-navigates barriers associated with delivery through extra cellular matrices, cell membranes, and cytoplasmic components. Further, microinjection is not limited by the molecular weight of the cargo, which is a significant limiting factor with viral vector delivery systems. This method also allows for the con-trolled delivery of known quantities of the cargo, improving control over off-target effects. Naturally, microinjection is best suited for in vitro and ex vivo work only, as the use of a microscope to target individual cells (and precisely inject car-goes to specific locations within them) precludes the use of microinjection in a true in vivo setting.

Nucleic acids are by far the most common cargo for microinjection delivery. There are three primary methods for injection of these components: (1) as DNA directlydelivered to the cell nucleus, (2) as in vitro-transcribed mRNA molecules delivered to the nucleus, or (3) as in vitro transcribed mRNA molecules delivered to the cytoplasm. These different methods have benefits and drawbacks. By placing the DNA encoding both Cas9 and the sgRNA into the nucleus, the cell is free to transcribe and translate the components. This method is preferred by some groups, such as Chuang *et al.,* 2017 and Nakagawa *et al.,* 2015, due to the ability to omit lengthy in vitro transcription reactions from the overall process. However, single-stranded DNA is prone to random integration into the host genome, which may disrupt genes, result in constitutive expression of Cas9, and lead to greater off-target effects. Even circularized plasmid DNA can undergo this phenomenon (Yang *et al*., 2013). When delivering mRNA, the ideal case is to deliver the sgRNA directly to the nucleus and the Cas9-encoding mRNA to the cytoplasm, facilitating translation and shuttling of Cas9 to the nucleus. Unfortunately, microinjection is a technically challenging and laborious process, making two different microinjections into a single cell impractical. Further, two microinjections, even when separated by several hours, typic-ally results in non-viable cells (Yang *et al*., 2013). Therefore, microinjections of CRISPR mRNA components often occurs directly into the cytoplasm of the cell; for some examples see Crispo *et al*., 2015, Raveux *et al*., 2017, and Sato *et al*.,2015. This method has the advantage of putting the Cas9mRNA directly into the cytoplasm, where it can be translated by the cell. sgRNA in the cytoplasm is then bound by Cas9 while being shuttled into the nucleus, allowing for modification of the host DNA. The vast majority of studies using microinjection to deliver CRISPR use this approach, including simultaneous knock-out of four genes from a single injection into rat zygotes (Ma *et al*., 2014), disruption of two genes in cynomolgus monkeys from a single injection into one-cell-stage embryos (Niu *et al*., 2014), correction of a cataract-causingmutation in mice

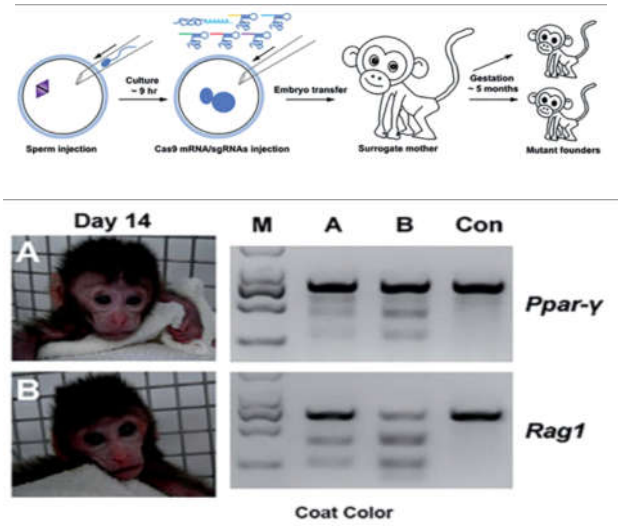

Figure 2.1. Microinjection disrupting two genes (Ppar-c and Rag1) in Cynomolgus monkeys from a singleinjection into one-cell-stage embryos. Photographs of Founder Monkeys A and B, PCR products of the targeted loci from genomic DNA of A and B, and a control wild-type Cynomolgus monkey (Con). Adaptedwith permission from Nui *et al.* (2014)

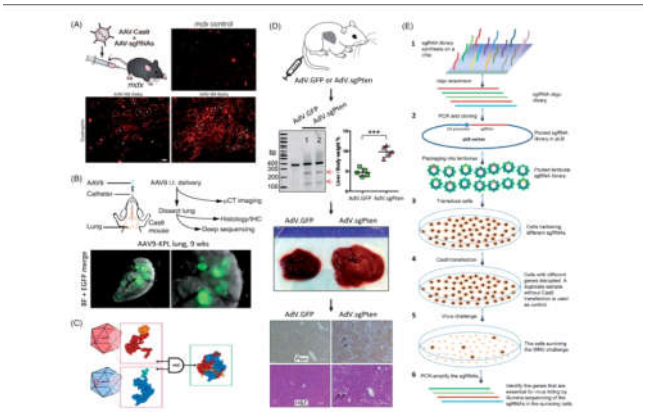

Figure. 3 Viral vector methods for delivery of CRISPR. (A) AAV delivery of Cas9 and sgRNAs disrupting mutations in the Dmd gene in adult mdx mice, resulting in improvement of muscle biochemistry and function. Adapted with permission from Long *et al*., (2016). Copyright 2016 American Association for the Advancement of Science. (B) AAV intratracheal instillation delivery of sgRNAs in Cre-dependent Cas9 knock-in mice, resulting in lung adenocarcinoma (EGFP-positive tumors). Adapted with permission fromPlatt *et al.,* (2016). Copyright 2014 Elsevier Inc. (C) A split Cas9 system in which the Cas9 C-terminal is packaged into one AAV vector and the Cas9 N-terminal is packaged into a second AAV vector. Reconstitution results in a fully functioning Cas9. Reprinted from Truong *et al*., (2015). Copyright 2014 TheAuthors (CC BY license). (D) AdV delivery of Cas9 and sgRNA targeting the Pten gene in mouse liver resulting in Pten mutation (see arrows by gel), and massive hepatomegaly and features of NASH in infected livers. Immunohistochemistry shows loss of Pten staining (arrows) one month after AdV

(Wu *et al*., 2013), and correction of a Duchenne muscular dystrophy (DMD)-causing mutation in mice (Long *et al*., 2014). With some exceptions, microinjection of CRISPR/Cas9 RNA components into cells results in a finite duration ofaction of the system, owing to the natural decay of mRNA within eukaryotic cells (Ross, 1995). This is often desirable as it reduces off-target effects. Microinjection is also the most commonly used method for generating animal models.

Injection of the gene editing cargo into zygotes allows for efficient germline modification. In addition, there is evidence that injection of Cas9 mRNA and sgRNA into the zygote cytoplasm is the most efficient method for yielding normal embryos and full-term mouse pups harboring the desired modification (Horii *et al*., 2014). Microinjection can also be useful for CRISPR a and CRISPR i to provide transient up- or down-regulation of a specific gene within the genome of a mature cell. Microinjection is a well established technology andits use is widespread, as evidenced by the ability to custom-order microinjected mouse zygotes from facilities such as the Genome Modification Facility at Harvard University (https://gmf.fas.harvard.edu/talen-or-crispr-microinjection).

CONCLUSION

The CRISPR–Cas system is a unique technology for gene editing. Studies summarized in this review represent only the first steps in the CRISPR–Cas era of genetic engineering. Indeed, the CRISPR–Cas system brings high-quality, desired benefits like never before. Fields of application for this technology also appear to be limitless. CRISPR–Cas, a highly precise genome editing tool, allows us to improve our quality of life. Our food will become more nutrient-dense without the presence of toxins or pathogens. The CRISPR-mediated improvement of quality and quantity, and resistance to viruses, herbicides, drought, salt, and cold have already been reported in several crops. However, the technology will bring a completely new generation of crops including novel varieties. The CRISPR revolution will affect the production of biofuels, new materials, and more. The CRISPR technology also bears the potential to revive extinct species in the future and even to create completely new species. However, misuse of CRISPR–Cas for gene editing could be a risk and danger; therefore, ethical discussion about CRISPR in the scientific community is important. Despite all risks, webelieve that the application of CRISPR is a great opportunity for humanity and that exactgene editing will bring us a bright future. An age of CRISPR has already started.

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