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Delivery methods for CRISPR/Cas9 gene editing in crustaceans

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Abstract

In this mini-review we provide an up-to-date overview of the delivery methods that have been used for CRISPR/Cas9 genomic editing in crustacean species. With embryonic microinjection as the main workforce for delivering CRISPR/Cas9 reagents, biologists working with crustacean species have to tackle the technical challenges involved in microinjection. We use examples of three crustacean species (the branchiopod *Daphnia*, amphipod *Parhyale hawaiiensis*, and decapod *Exopalaemon carinicauda*) to provide a technical guide for embryonic microinjection. Moreover, we outline two potentially useful new techniques for delivering CRISPR/Cas9 components into crustaceans, i.e., Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) and electroporation.

Keywords

microinjection; electroporation; vitellogenesis; *Daphnia*; *Parhyale hawaiiensis*; *Exopalaemon carinicauda*

Introduction

The development of Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR)/Cas-based RNA-guided endonucleases as a genomic editing tool has revolutionized the field of life science research (Cho et al. 2013; Cong et al. 2013; Jinek et al. 2012; Mali et al. 2013a; Mali et al. 2013b). Much enthusiasm has been ignited for using CRISPR/Cas9 in treating human disease (e.g., Eyquem et al. 2017; Xiong et al. 2016), understanding mechanisms of genetic diseases (e.g., Findlay et al. 2014; Yin et al. 2014), and using various model organisms to understand gene functions (e.g., Dickinson et al. 2013).

A critical component of successful genetic modification is the delivery of the CRISPR/Cas9 components into the cell and nucleus. A delivery vehicle and cargo are the major

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Authors' contributions

SX, TP, and SN reviewed literature and wrote the manuscript. The manuscript has been approved by all authors.

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Conflicts of interests

The authors declare no conflicts of interests.

Animal and human rights statement

No animal and human rights are involved in this article.

components of the delivery system. The CRISPR/Cas9 cargos usually consist of: (1) DNA plasmid encoding both the Cas9 protein and the guide RNA (gRNA), (2) mRNA for Cas9 translation with a separate gRNA, or (3) Cas9 protein with guide RNA [ribonucleoprotein (RNP) complex]. The delivery vehicle consists of physical delivery, viral vectors, and non-viral vectors (Lino et al. 2018). For well-established model organisms (e.g., mice, zebra fish, *Caenorhabditis elegans*, and *Drosophila*) and cell lines, the delivery methods are also well developed. For *in vivo* genetic editing, microinjection into embryos of *Drosophila* or into the gonad area of *C. elegans* provides a major reliable means for genetic editing. Furthermore, electroporation allows a relatively high throughput approach for delivering CRISPR/Cas9 machinery into a larger number of embryos at the same time (Kaneko and Mashimo 2015).

Nonetheless, for emerging model organisms, the delivery methods are not well developed and often involve significant technical challenges. For crustaceans, which are a major part of aquatic ecosystems and contain many species of economic importance, CRISPR/Cas9 gene editing has been successfully carried out in a few key model species such as the branchiopod *Daphnia* (Hiruta et al. 2018; Nakanishi et al. 2014), amphipod *Parhyale hawaiiensis* (Martin et al. 2016), and decapod *Exopalaemon carinicauda* (Gui et al. 2016). Here we review the delivery techniques for crustacean gene editing and discuss some of the relevant techniques that could be used for delivery with high potential.

Microinjection

Microinjection is the most common method for all the crustacean species where CRISPR/Cas9 has been successfully performed. To perform microinjection, a micromanipulator and a stereomicroscope/inverted microscope are essential. The reader should consult the original papers for the specific models of micromanipulators, which are usually capable of manipulation on micrometer level. Moreover, microinjection needles are also critical for the success of creating genetically engineered crustaceans. Some of the studies cited here employ custom-made needles from microcapillaries using needle pullers, whereas others employ commercially available needles. Manufacturers of needle pullers often provide user guides for how to make microinjection needles for different applications. Readers are encouraged to consult those resources for preparing microinjection needles.

For all the current works on CRISPR/Cas9 genetic editing on crustacean species, embryos are the main targets of microinjection. It is impossible to perform pro-nuclear injection as the embryos often are not completely transparent. A significant challenge with microinjecting into embryos is to minimize damaging them so that they are still able to hatch after microinjection. We will see below in the work done on *Daphnia* that sucrose solution was used to balance the osmotic pressure between the embryo and external culture medium (Hiruta et al. 2018). More importantly, the developmental stage of embryos is critical for hatching success after microinjection, e.g., within 1 hour of ovulation in *Daphnia* (Nakanishi et al. 2014).

Daphnia magna* and *D. pulex

Daphnia is capable of reproducing asexually, producing directly developing embryos that hatch into genetically identical daughters. Thus, these asexually produced embryos are ideal

for genetic editing. The microinjection system in *D. magna* was first developed by Kato et al (2011) for RNAi, and the same microinjection system was successfully used for CRISPR/Cas9 gene editing (Nakanishi et al. 2014). Importantly, asexually produced embryos should be collected immediately after ovulation from adult females. Specifically, the females in the middle of ovulation are transferred to ice-cold culture medium and, once the ovulation is finished, the embryos can be dissected from the brood pouch (Kato et al. 2011; Toyota et al. 2016). It is found that the membrane plasticity of embryos at this stage is elastic so embryos would remain intact after microinjection (Kato et al. 2011). Moreover, to prevent any embryo leakage, microinjection is carried out in 80 mM sucrose M4 medium (M4 medium solution is a common growth medium for *Daphnia*) for *D. magna*. The injected embryos are also stored in 80 mM sucrose M4 medium for hatching at 18 °C. Regarding making suitable microinjection needle, Toyota et al (2016) provided a set of parameters that can be used on a Model P-97 needle puller (Sutter Instrument, Novato, CA, USA).

A very similar microinjection has been developed for *D. pulex*, where microinjection was first used for RNAi (Hiruta et al. 2013) and then successfully extended to CRISPR/Cas9 genetic editing (Hiruta et al. 2018). A notable difference for *D. pulex* is that 60 mM sucrose M4 medium is used for microinjection due to the osmotic pressure specific to the embryo of this species. Additionally, a 2% agar plate with 60 mM sucrose M4 solution is used for hatching the injected embryos (Hiruta et al. 2018). Embryos usually hatch out in 2-3 days.

Microinjection of CRISPR/Cas9 gene editing for *Daphnia* has been done with both plasmids encoding Cas9 and gRNA, and Cas9 enzyme with separate gRNA (Hiruta et al. 2018; Kumagai et al. 2017; Nakanishi et al. 2014). Nakanishi et al. (2014) also performed a comparison of CRISPR/Cas9 gene knockout efficiency with different concentrations of Cas9 enzyme and sgRNA. These authors found that the highest gene knockout efficiency reached ~50% with 50 ng/μL gRNA and 500 ng/μL Cas9.

Parhyale hawaiiensis

As an emerging model system in developmental biology, *P. hawaiiensis* has a diverse array of genomic editing tools such as transposon- and integrase-mediated transgenesis (Kontarakis et al. 2011; Pavlopoulos and Averof 2005), CRISPR/Cas9 (Kontarakis and Pavlopoulos 2014), and RNA-level gene expression knockdown tools such as RNAi and morpholino-based gene knockdown (Liubicich et al. 2009; Ozhan-Kizil et al. 2009). These techniques all critically rely on microinjection, which is performed on sexually produced embryos. For details of how to set up mating pairs of *P. hawaiiensis* and dissect embryos out of the females, see Kontarakis and Pavlopoulos (2014). To increase the likelihood of creating stable transgenic animals, early-stage embryos are desirable for microinjection. The optimal stage for injection is the 1-cell-stage, which corresponds to the first 4 hours post-fertilization.

Since the microinjection needle is critical for the success, Kontarakis and Pavlopoulos (2014) provide instructions for how to make suitable needles. Furthermore, an injection pad (i.e., agarose steps) is also necessary in order to facilitate the injection process described by Kontarakis and Pavlopoulos (2014), although different labs may use different kinds of set-up to serve similar purposes (Farboud et al. 2018). Unlike *Daphnia*, the microinjection of *P.*

hawaiensis embryos only requires artificial sea water without any additional additive for balancing osmotic pressure.

For CRISPR/Cas9 gene editing, Farboud et al. (2018) recommend 2 μM Cas9, 4-8 μM gRNA, and 0.05% phenol red in a volume of 1.5 μL RNP for injection. For 2-cell embryos (i.e., 4-6 hours post-fertilization), both blastomeres should be injected. After injection, the embryos are incubated in filter-sterilized oxygenated seawater at 26°C for 12 hours-light dark cycles.

Exopalaemon carinicauda

This species is an economically important shrimp. Stable germline mutants have been successfully established through CRISPR/Cas9 and microinjection into embryos (Gui et al. 2016). The microinjection was carried out at the 1-cell embryo stage with a commercially available microinjection needle, and the mRNA of Cas9 (200 ng/ μL) and gRNA (100 ng/ μL) were injected into the embryos. The survival rate of embryos after microinjection was ~15%. Notably, about 50% of the hatched embryos carried mutations in the target chitinase gene (Gui et al. 2016).

Future perspectives

As we have seen from the examples above, microinjection is currently the main workhorse for gene editing in crustaceans. However, it is a time consuming and tedious experimental procedure, and often requires extensive training and practice. More importantly, the survival rate of injected embryos and rate of successful genetic editing tend to be relatively low. Because of these commonly identified disadvantages associated with microinjection, some alternative approaches for CRISPR/Cas9 genomic editing have been developed in other species. Although we have not seen the application of these newer techniques in crustaceans, these methods can potentially significantly increase the efficacy of genetic editing with CRISPR/Cas9 in crustaceans.

Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control)

Chaverra-Rodriguez et al. (2018) identified ligands derived from arthropod yolk protein precursors (YPPs) that bind to RNP complex (Cas9 complexed with a sgRNA). Taking advantage of the fact that during ovary and egg maturation (i.e., vitellogenesis) most female oviparous animals deliver protein material to their developing ovaries, these authors tested whether YPPs can be used as a vehicle for delivering RNP to developing oocytes in order to facilitate successful genomic editing (Chaverra-Rodriguez et al. 2018). This procedure does not require embryo microinjection but rather the injection of YPP-RNP complex into the hemolymph of the animals (e.g., mosquitoes in this study). The authors performed deletion analysis to identify a small region of the YPP peptide (41 amino-acid long fragment) that can successfully deliver RNP to the ovary through Receptor-Mediated Endocytosis (RME). Another component of this system was a suitable endosomal release reagent (ERR) because following RME, the delivered cargo of RNP is confined to endosomes and would not result in gene editing unless released. The ERR identified in this study was chloroquine: other candidates included ammonium chloride, saponin, and monensin (Chaverra-Rodriguez et al.

2018). The co-injection of ERR and YPP-RNP complex resulted in an efficiency as high as 0.3 mutant offspring per injected mosquito. Furthermore, the delivery system designed for one mosquito species was also effective for multiple related mosquito species.

The high efficiency of this method and the low demand of equipment (i.e., no specialized microinjection equipment) make it an attractive alternative for CRISPR/Cas9 gene editing. To apply this technique to crustaceans, identifying suitable candidates of YPP and ERR is pivotal. Because the proteins used for RME in closely related species are highly conserved, it is possible that once developed in one crustacean species, little modification would be necessary for applying the same procedure to others.

Electroporation

Electroporation is a widely used technique for delivering reagents into cells for genetic modification. It uses high voltage for creating temporary pores on cell membrane through which DNA or other charged molecules can pass to get into the cell. To date, we have not seen any examples of electroporation for CRISPR/Cas9 gene editing in crustacean species, although it has been used for introducing foreign DNA into *Daphnia magna* (Watanabe et al. 2010).

Some recent advances in the development of electroporation for CRISPR/Cas9 gene editing suggest its potential use in crustacean species. In rodents, electroporation of Cas9 mRNA with gRNA results in high efficiency of knock-out (67-88%) and knock-in (33%) in mice and rats (Kaneko and Mashimo 2015). The electroporation method has also been used with Cas9-sgRNA RNP in mice (Troder et al. 2018). It should be noted that a special buffer is often used in electroporation experiments to provide appropriate conductivity. For example, Kaneko and Mashimo (2015) used PBS solution as an electroporation buffer. The high salinity of the buffer may be problematic for the survival of the embryos of some crustaceans, especially freshwater species. For marine species, it seems that artificial sea water may be sufficient for electroporation (see below). Another advantage of electroporation is the number of embryos that can be accommodated in one round of electroporation, which can range from 5 to 10, depending on the sizes of embryos and the electroporation chamber. The ease of getting the chemical components of Cas9 and gRNA, and the higher throughput of embryos compared to microinjection, make this method an attractive alternative to embryonic microinjection. It should be noted that some of these protocols may only work for RNP complexes.

Electroporation for CRISPR/Cas9 has also been done with plasmid DNA, as described in the protocol for the sea squirt *Ciona* (Sasaki et al. 2014; Stolfi et al. 2014). In this case, artificial sea water was used as electroporation buffer, making this method potentially relevant for marine crustacean species. This protocol should be of interest to researchers that plan to use plasmid DNA for CRISPR/Cas9 gene editing.

Conclusions

Although current CRISPR/Cas9 genomic editing in crustaceans relies heavily on microinjection, we see great potential in adopting technologies developed in other model

organisms to achieve more efficient genomic editing in crustaceans. As the delivery method for CRISPR/Cas9 gene editing is a rapidly developing field, the alternative methods that we have suggested may serve as a good starting point for interested researchers. However, future research may result in the development of other novel methods that may provide even greater potential solution to the delivery of CRISPR/Cas9 reagents for genomic editing in crustaceans.

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