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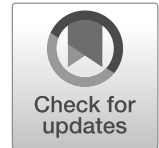
SHORT COMMUNICATION



Targeted Gene Disruption in Pacific Oyster Based on CRISPR/Cas9 Ribonucleoprotein Complexes

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Abstract

The Pacific oyster (*Crassostrea gigas*) is a representative bivalve mollusc that is widely cultured in the world. In recent years, it has become an important model species for ecological, evolutionary, and developmental studies because of its ability to survive in extreme environmental conditions as a sessile filter feeder and its classical mosaic pattern of development. Although the complete genome sequence of *C. gigas* is available and omics data have been rapidly generated for the past few years, the genetic tools for gene functional studies have thus far been limited to RNA interference technology. In this study, we developed a gene editing system for *C. gigas* based on CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 ribonucleoprotein complexes. Two candidate genes, *myostatin* (MSTN) and Twist, were selected as targets. After microinjecting CRISPR/Cas9 ribonucleoprotein complexes into fertilized eggs, CRISPR-induced indel mutations were detected in the target genes. The CRISPR/Cas9-induced mutations were predominantly small indel mutations ranging in size from 1 to 24 bp in these two target genes. These results demonstrate that CRISPR/Cas9 can be successfully used as an effective targeted gene editing system in *C. gigas*. The method reported here provides a powerful tool for gene functional studies in oysters and other marine bivalves, and potentially as a new technology for genetic engineering to improve oyster traits for aquaculture.

Keywords CRISPR · Cas9 · *Crassostrea gigas* · Gene editing

Introduction

The Pacific oyster, *Crassostrea gigas,* has been studied for over a century because of its importance in aquaculture and marine ecosystems, and their unique developmental patterning. Pacific oyster is a major global aquaculture species in many regions of the world. According to a recent report by Food and Agriculture Organization of the United Nations (FAO), the global production of Pacific oyster reached almost 0.6 million tons in 2016 (FAO 2018). As an efficient filter feeder thriving in coastal zones, *C. gigas* plays an important role in coastal ecosystem (Walles et al. 2015). In addition, as a sessile bivalve species living in intertidal regions, *C. gigas* has been used as a model organism for studying immunological, ocean acidification and stress responses (Wang et al. 2016; Song et al. 2017). Moreover, *C. gigas* also serves as an interesting model system for developmental studies owing to its unique model of indirect development including trochophore and veliger larvae as well as metamorphosis (Zhang et al. 2012).

The completion of the entire *C. gigas* genome in 2012 opened new avenues for exciting genomic and genetic studies (Zhang et al. 2012). Together with the recent development of other omics analyses, we have gained increasingly valuable insights into understanding the unique biological characteristics of *C. gigas* (Zhao et al. 2016; Huang et al. 2017; Riviere et al. 2017; Yue et al. 2018). However, one major challenge in oyster research lies in the bottle neck of gene functional analysis. The genetic studies in *C. gigas* have been very limited due to the lack of effective and target gene-specific tools in gene knockout and editing.

Hong Yu and Huijuan Li contributed equally to this work.

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Reverse-genetic technologies utilizing gene knockout and knockdown have played a critical role in uncovering gene functions in many model and non-model systems. In *C. gigas,* most of the genetic studies have been carried out using the RNA interference (RNAi) technology to investigate the genetic regulation of germ cell development, reproduction, and salinity tolerance (Fabioux et al. 2009; Huvet et al. 2015; Zhao et al. 2017). However, there are a number of caveats associated with the RNAi approach. First, due to the fact that RNAi acts by knocking down the targeted gene translation rather than a complete loss-of-function knockout, in many cases a phenotype may not be detectable owing to insufficient knockdown. Second, the knockdown effects are usually transient and highly variable among individuals, and the off-target effect is also a potential problem with RNAi technology (Bono et al. 2015).

Recently, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system has been developed into a powerful gene editing tool, which enables researchers to perform various genetic experiments involved in modifying genomes at a precise position such as generating loss-of-function mutations. With the advantages of highly efficient targeted gene editing, this tool has been widely used in both model and non-model organisms such as zebrafish, mouse, fruit fly, Atlantic salmon, jellyfish, and sea urchin. (Doudna and Charpentier 2014; Momose and Concordet 2016; Taning et al. 2017). Based on the nature of cell types and organisms, several methods have been utilized for delivery of CRISPR/Cas9 reagents into cells and organisms, including microinjection, electroporation, viral infection, and lipofection. Microinjection into early-stage embryos or unfertilized eggs has been mostly used. It is the first choice in both model and non-model animals (Momose and Concordet 2016). However, microinjection poses a challenging problem for organisms with small size egg and embryo, such as *C. gigas*. There is only one report on successful gene editing with CRISPR/Cas9 system in molluscs, marine gastropod *Crepidula fornicate,* to date (Perry and Henry 2015). Microinjection into *C. gigas* eggs at a size < 50 μm in diameter is technically challenging. In addition, because of the small volume of injection, the concentrations of sgRNA and Cas9 have to be optimized to achieve a high efficiency of genome editing.

In this study, we investigated the possibility of delivering CRISPR/Cas9 ribonucleoproteins (RNPs) into *C. gigas* eggs by microinjection and evaluated the optimal doses of sgRNA and Cas9 in inducing targeted genetic mutations. We report here the efficient induction of DNA mutations in two target genes in *C. gigas* after microinjection of an optimized combination of sgRNA and Cas9 protein complexes. To our knowledge, this is the first successful report on gene editing using CRISPR/Cas9 system in Pacific oyster to date. The establishment of CRISPR/Cas9 genome editing technology in *C. gigas* provides a solid basis for future studies of gene function using the reverse-genetic approach. The method reported here provides a powerful tool for genetic engineering and gene functional studies in oysters and other marine bivalves, and potentially as a new technology for genetic engineering to increase beneficial traits of oyster for aquaculture.

Materials and Methods

In Vitro Fertilization of Pacific Oysters and Embryo Production

Mature *C. gigas* were purchased from a local oyster farm in Rongcheng, China. The oysters were dissected, and their gametes were obtained by gonad striping. In vitro fertilization was carried out as described in our previous reports (Li et al. 2011; Wang et al. 2012). The embryos were cultured in filtered seawater at 22 °C.

Target Site Selection and Synthesis of sgRNAs

The second and third exons of *C. gigas Twist* (LOC105325904) and *myostatin* (MSTN, LOC105337024) genes, which are predicted to be single-copy genes based on the genome sequence, were respectively scanned for potential CRISPR target sites using the CRISPR Design Tool (http://crispr.mit.edu). A CRISPR target sequence was identified for each gene and named MSTN-sgRNA and Twist-sgRNA, respectively. They are characterized by including the first two nucleotides as GG or GA sequence at the 5′, having fewer potential off-target sites, and ending with the NGG PAM sequence at the 3′ (Fig. 1). DNA templates for synthesizing MSTN-sgRNA and Twist-sgRNA were generated by PCR using the pDR274 plasmid as a template as described (Cai et al. 2018). The PCR was performed using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with a gene target-specific forward primer and a common reverse primer (Table 1). The target gene-specific forward primers include a common 6-nt protective base, the T7 promoter sequence, a 20-nt gene-specific target sequence, and the 12-nt pDR274 5′ sequence. PCR products were purified using a SanPrep Column PCR Product Purification Kit (Sangon Biotech).

All sgRNAs were generated by in vitro transcription of the sgRNA PCR templates using the MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The synthesized sgRNAs were purified by phenol-chloroform extraction and precipitated in 0.5 M ammonium acetate with two volumes of 100% ethanol. The precipitated RNA was dissolved in RNase-free water and stored at − 80 °C until use. RNA quality was assessed using a NanoDrop (Thermo Fisher Scientific) and by electrophoresis on a 2% agarose gel.

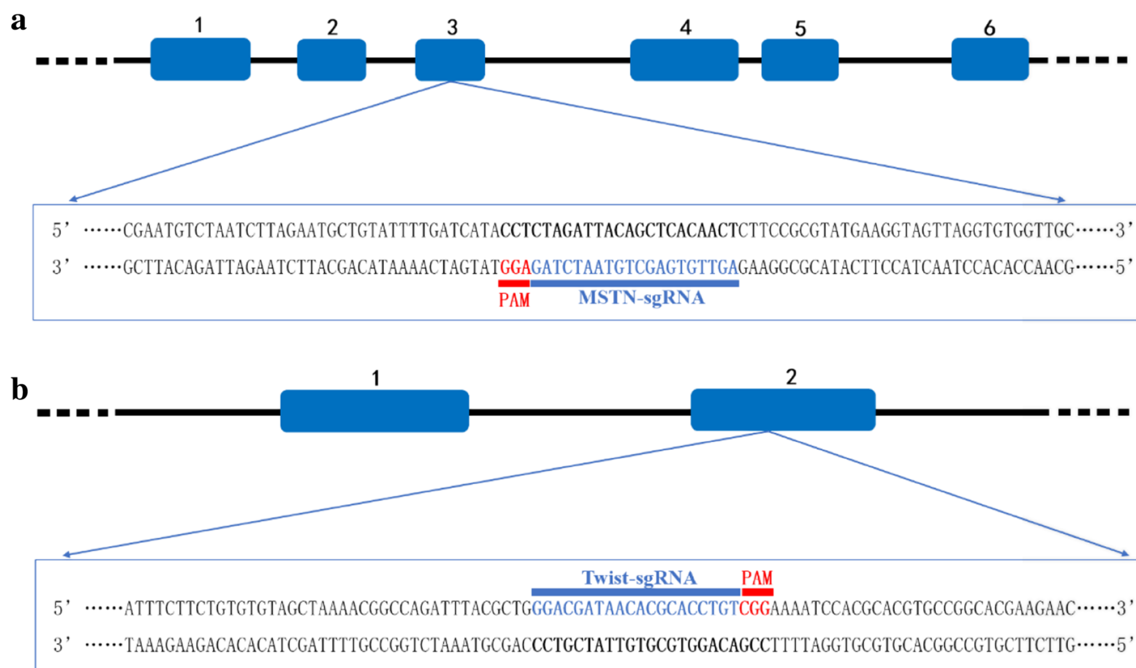

Fig. 1 Schematic diagram of the MSTN-sgRNA and Twist-sgRNA target sites and sequences. The PAM sequences were shown in red, and the sgRNA target sequences were shown in blue. **a** MSTN-sgRNA was targeted to the exon 3 (the antisense strand). **b** Twist-sgRNA was targeted to the exon 2 (the sense strand)

In Vitro Evaluation of sgRNA Efficacy

The gene editing efficacy of the sgRNA/Cas9 was tested in vitro using the Guide-it™ sgRNA Screening Kit (Clontech) according to the manufacturer's instructions. Briefly, a 600–900 bp DNA fragment containing the sgRNA target sequence was amplified by PCR with the specific primers (Table 1). PCR products were purified using a SanPrep Column PCR Product Purification Kit (Sangon Biotech), and 100–250 ng amplicons were digested with sgRNA (50 ng) and Cas9 nuclease (250 ng) in a 15 μl reaction volume at 37 °C for 1 h. The reaction was terminated by incubating at 80 °C for 5 min and stored at 4 °C for later use. Analysis of the enzymatic digestion was performed by electrophoresis on a 1.5% agarose gel.

Microinjection in Oyster Embryos

The sgRNA/Cas9 was delivered into oyster embryos by microinjection. An Olympus inverted microscope IX73 equipped with two micromanipulators for holding and microinjection pipettes (Narishige) was used. Holding and injection

Table 1 The primers used in this study

Primer name	Sequence (5′-3′)
MSTN-sgRNA-F	GATCACTAATACGACTCACTATAGGTTGTGAGCTGTAATCTAGGTTTTAG AGCTAGAAAT
Twist-sgRNA-R	GATCACTAATACGACTCACTATAGGACGATAACACGCACCTGTGTTTTAG AGCTAGAAAT
sgRNA-common-R	AAAAGCACCGACTCGGTGCC
MSTN-sequencing-F	AACCCAACCTAGATTTGCGGAC
MSTN-sequencing-R	TACGCACCAACGTTGAAGGTGA
Twist-sequencing-F	GACGGAATCCTGTCTTCTTGG
Twist-sequencing-R	TAGGAAAGAAACCTGACCATTTC
MSTN-sgRNA-verify-F	GACGAGATGCATGTTTCAATCGAT
MSTN-sgRNA-verify -R	TGCTGTAATCTTTCATAGGATGGAAC
Twist-sgRNA-verify-F	GAAACTTCAACACCCTTCACCT
Twist-sgRNA-verify-R	CAAACATACAAGGAGCAAGAATAGG

The underlined sequences in MSTN-sgRNA-F and Twist-sgRNA-R are 6-nt protective base. The red sequence in MSTN-sgRNA-F and Twist-sgRNA-R are the gene-specific target sequences within the respective sgRNAs

pipettes were prepared from Narishige glass capillaries with filament (GD-1, Narishige) using a Sutter P-97 micropipette puller (Sutter). The holding pipettes were further polished with a MR-900 microforge (Narishige), and the injection pipettes were further processed on an EG-401 microgrinder (Narishige). Microinjection was carried out using a PLI-100A Pico-Injector microinjector (Warner Instruments).

Alexa Fluor 488 phalloidin (AAT Bioquest) was injected at a concentration of 33 μg/μl. Approximately 0.1 nl of the florescent dye was microinjected in each oyster embryo. The sgRNAs and Cas9 protein (Guide-it™ Recombinant Cas9, Clontech) were mixed in equal volume and diluted in the injection buffer (20 mM HEPES, 150 mM KCl, and 0.5% phenol red) and then incubated for 15 min at 37 °C before use. Various concentrations of mstn-sgRNA and Twist-sgRNA were tested (Table 2). Different final concentrations of sgRNA and Cas9 protein were co-injected into 1–2-cell stage oyster embryos (Table 2). Approximately 0.1 nl of the sgRNA/Cas9 protein mixture was microinjected in each oyster embryo. The injected embryos were cultured in filtered seawater at 22 °C.

DNA Isolation and Genotyping by DNA Sequencing

The genomic DNA was isolated from trochophores at approximately 12 h after fertilization using a proteinase K digestion method modified from Cai et al. (2018). Briefly, 20 μl of lysis buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8, 50 mM KCl, 0.5 mg/ml gelatin) was added to each larva in a 200-μl tube. The larvae were incubated at 94 °C for 10 min and 4 °C for 10 min. 2.5 μl of proteinase K (20 mg/ml) was added into each sample, and the samples were digested at 55 °C for 1 h, denatured at 94 °C for 10 min, and cooled down to 4 °C. The DNA samples were centrifuged, and 1 μl of the supernatant was used for each PCR reaction.

The genomic region covering the target sites and their flanking sequences were amplified using the respective gene-specific primers (Table 1). Amplicons were purified and sequenced directly using the respective MSTN-F or Twist-F primers (Sangon Biotech). In addition, the PCR amplicons were cloned into pEASY vector. Thirty clones were randomly selected for plasmid isolation and DNA sequencing using the M13-F primer.

Results

In Vitro Testing of sgRNAs

To evaluate the efficacy of MSTN-sgRNA and Twist-sgRNA in guiding Cas9-induced gene editing at their respective target sites, we first tested these sgRNAs in vitro prior to microinjection into oyster embryos. DNA fragments containing the MSTN-sgRNA or Twist-sgRNA target sequences were amplified by PCR. The PCR products were incubated with MSTN-sgRNA/Cas9 or Twist-sgRNA/Cas9 ribonucleoprotein (RNP) complexes. The DNA products of the enzymatic digestion were analyzed by agarose gel. The results showed that incubating MSTN-sgRNA/Cas9 or Twist-sgRNA/Cas9 with their respective targets resulted in the production of two DNA bands with the expected size. In contrast, no DNA cleavage was detected when the PCR fragments were incubated with either Cas9, MSTN-sgRNA, or Twist-sgRNA alone (Fig. 2). Together, these results indicate that both MSTN-sgRNA and Twist-sgRNA could effectively guide Cas9 protein to cut DNA at their respective target sites.

Embryo Microinjection and Toxicity Testing of Cas9 Protein and sgRNAs

To assess the delivery of sgRNA/Cas9 RNP complex into oyster embryos by microinjection, we first tested microinjection of a florescent dye (Alexa Fluor 488 phalloidin) into

Table 2 Summary of injections with different concentrations of Cas9 protein and sgRNAs

Cas9 protein concentration (ng/μl)	sgRNAs concentration (ng/μl)	Survival (%)		Editing efficiency (%)	
		MSTN	Twist	MSTN	Twist
750	0	9/45 (20%)	8/40 (20%)	0/9 (0%)	0/8 (0%)
750	1500	8/40 (20%)	10/50 (20%)	8/30 (26.7%)	6/33 (18.2%)
750	375	8/41 (19.5%)	8/39 (20.5%)	0/8 (0%)	0/8 (0%)
375	0	9/37 (24.3%)	8/33 (24.2%)	0/9 (0%)	0/8 (0%)
375	1500	13/48 (27.1%)	12/46 (26.1%)	0/13 (0%)	0/12 (0%)
187	0	39/49 (79.6%)	35/40 (87.5%)	0/16 (0%)	0/16 (0%)
187	1500	40/50 (80%)	38/50 (76%)	0/16 (0%)	0/16 (0%)
0	1500	36/45 (80%)	34/40 (85%)	0/16 (0%)	0/16 (0%)
0	0	50/54 (92.6%)	45/50 (90%)	0/10 (0%)	0/10 (0%)

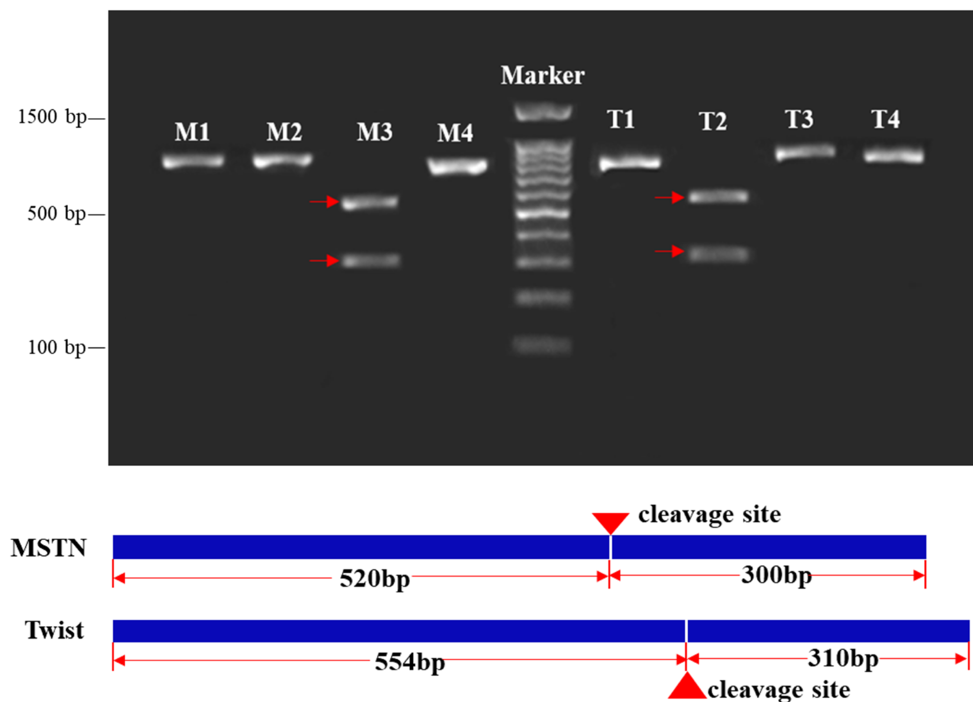

Fig. 2 In vitro cleavage assay for testing the MSTN-sgRNA and Twist-sgRNA guided DNA cleavage by Cas9. M1 represented the mixture of MSTN-sgRNA and MSTN cleavage templates; M2 was the mixture of Cas9 protein and MSTN cleavage templates; M3 was the mixture of MSTN-sgRNA, Cas9 protein, and MSTN cleavage templates; and M4 represented the mixture of MSTN-sgRNA, Cas9 protein, and Twist cleavage templates. T1 represented the mixture of Twist-sgRNA and Twist cleavage templates; T2 was the mixture of Twist-sgRNA, Cas9 protein, and Twist cleavage templates; T3 was the mixture of Cas9 protein and Twist cleavage templates; and T4 was the mixture of Twist-sgRNA, Cas9 protein, and MSTN cleavage templates. The red arrow indicted the cleavage fragments

fertilized oyster embryos at 1–2 cell stages. 0.1 nl of Alexa Fluor 488 phalloidin was microinjected into each embryo. The injected embryos were allowed to develop for 1 h and examined under a fluorescence microscope. A fluorescence signal was clearly detected in the injected embryos, and conversely the non-injected embryos showed no fluorescence signal (Fig. 3). The results indicated that reagents could be successfully injected into Pacific oyster embryos.

To investigate whether Cas9 protein or sgRNA is deleterious to oyster embryos, we decided to test the effect of injecting sgRNA or Cas9 protein alone on embryonic development and survival. The MSTN-sgRNA or Twist-sgRNA sgRNA was diluted at the final concentration of 1500 ng/μl, and 0.1 nl of the sgRNA was microinjected into each embryo. The injected embryos showed a survival rate of 80–85% to trochophores stage (Table 2). In contrast, injection of Cas9 protein at 375 ng/μl and 750 ng/μl resulted in a high mortality rate. Approximately 80% of the injected embryos died at the trochophores stage (Table 2). Reducing the Cas9 concentration to 187 ng/μl significantly improved the embryo survival to 79.6–87.5% (Table 2). Collectively, these data indicate that sgRNA and Cas9 RNPs can be delivered into oyster embryos by microinjection. The oyster embryos could tolerate sgRNA whereas a high dose of Cas9 protein is deleterious to the embryos.

Injection of CRISPR/Cas9 Induced a Dose-Dependent Targeted Gene Mutations in Oyster Embryos

To determine whether microinjection of sgRNA/Cas9 could induce genetic mutations at their target genes in oyster, MSTN-sgRNA or Twist-sgRNA was mixed with a varying amount of Cas9 protein and injected into oyster embryos. The genomic regions surrounding the CRISPR targets were amplified by PCR and sequenced directly to investigate the successful rate of genome editing. The results revealed a clear dose-dependent effect of sgRNAs/Cas9 injection. At low doses of sgRNAs (375 ng/μl) and Cas9 protein (187 ng/μl and 375 ng/μl), no genetic mutation was observed in either *myostatin* or *Twist* genes in the injected embryos. However, injection of sgRNA at a higher concentration (1500 ng/μl) with a ninefold molar excess over Cas9 protein (750 ng/μl) successfully induced mutations in the target genes (Fig. 4), suggesting Cas9 RNPs induced mutations in a dose-dependent manner. Interestingly, we noted that injection of Cas9 protein at a higher concentration (750 ng/μl) and sgRNAs at a low concentration (375 ng/μl) resulted in no detectable mutations, suggesting that the molar ratio of sgRNAs to Cas9 protein is critical for efficient editing using the CRISPR/Cas9 system.

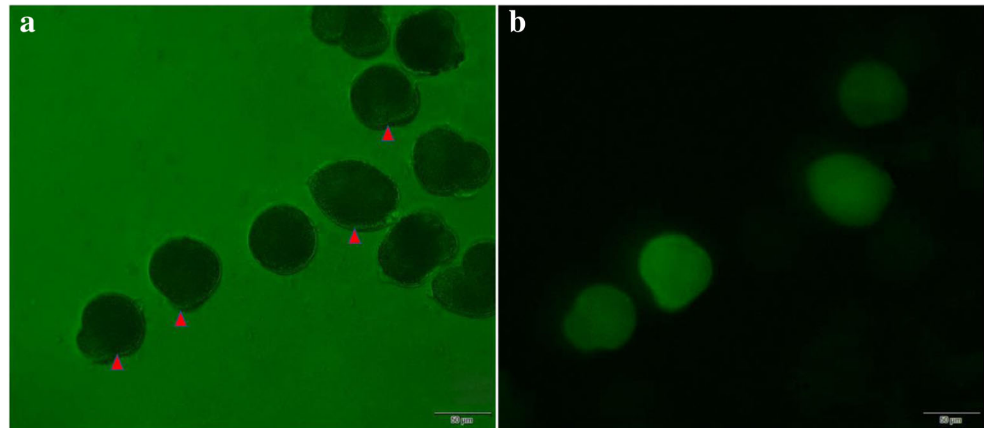

Fig. 3 Microinjection of Phalloidin 488 into oyster embryos. Phalloidin 488 was injected into oyster embryos. The injected and un-injected embryos were monitored under a fluorescence microscopy. **a** Bright field microscopy picture of embryos, and the red triangle indicated the injected embryos. **b** Fluorescence microscopy pictures of the injected embryos

To determine the successful rate of gene editing in the sgRNA (1500 ng/μl)/Cas9 (750 ng/μl) injected embryos, 30 larvae from the MSTN-sgRNA/Cas9 and 33 larvae from the Twist-sgRNA/Cas9 injected groups were selected for PCR and sequencing. Of the larvae sequenced, 26.7% and 18.2% of the injected individuals contained mutations at the target sites in MSTN and Twist genes, respectively. We noted that a higher concentration of sgRNA/Cas9 injection increased the rate of mutated embryos. However, this was associated with a reduced embryonic viability (~ 20%, Table 2).

Characterization of CRISPR/Cas9-Induced Indel Mutations in the Injected Embryos

To characterize the indel mutations induced by CRISPR/Cas9-mediated genome editing, we cloned the PCR amplicons from the injected larvae that displayed mutations on the sequencing chromatograph. Thirty clones were randomly selected for sequencing. Sequencing analysis showed eight MSTN clones and six Twist clones contained DNA mutations at the target sites. As expected, various types of indel mutations were detected in MSTN and Twist genes, respectively (Fig. 5). The CRISPR/Cas9-induced mutations in the two target genes were predominantly small indel mutations ranging in size from 1 to 24 bp. Some of these mutations are frame-shift mutations that can disrupt the protein translation, and thus expected to be null mutations.

Discussion

In this study, we have established an effective approach to deliver sgRNA/Cas9 RNP complexes into *C. gigas* embryos by microinjection. We demonstrated that injection of sgRNA/Cas9 RNPs induced a dose-dependent mutations at the target genes. To our knowledge, this is the first report on gene editing in the Pacific oyster using the CRISPR technology. This approach should provide a solid basis for future

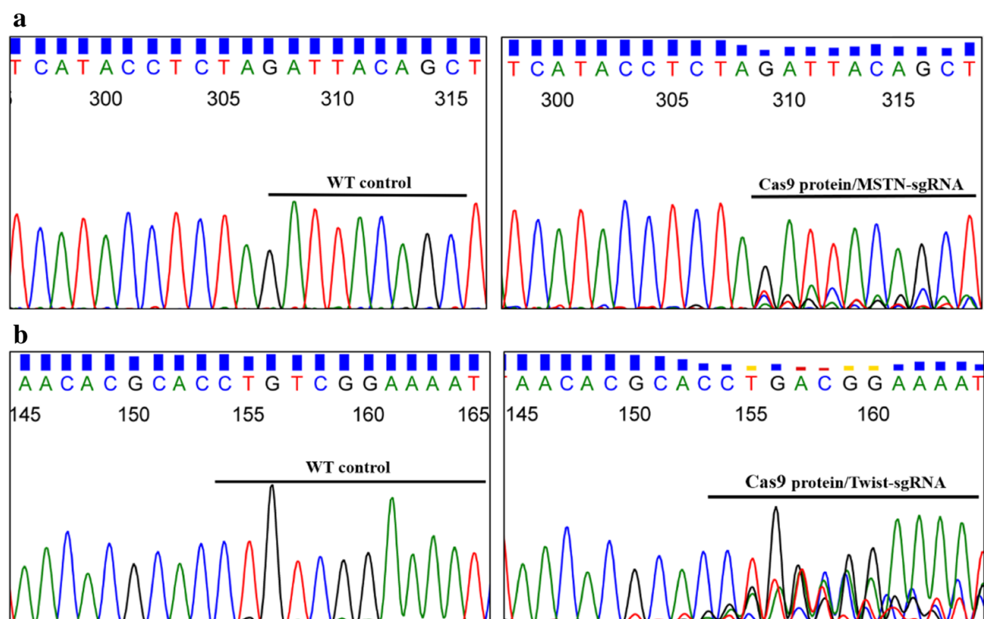

Fig. 4 Injection of MSTN-sgRNA/Cas9 or Twist-sgRNA/Cas9 RNPs induced DNA mutations at the target sites. MSTN-sgRNA/Cas9 or Twist-sgRNA/Cas9 RNPs were injected into oyster embryos. Thirty and 33 individual embryos were randomly selected for DNA isolation and PCR amplification of the DNA fragment covering the targeted regions. Representative DNA sequences of a control and a Cas9 RNPs injected embryos. Scrambled sequences were observed at − 3 position of the MSTN-sgRNA target site (**a**) or Twist-sgRNA target site (**b**). WT meant sequence analysis of control embryos

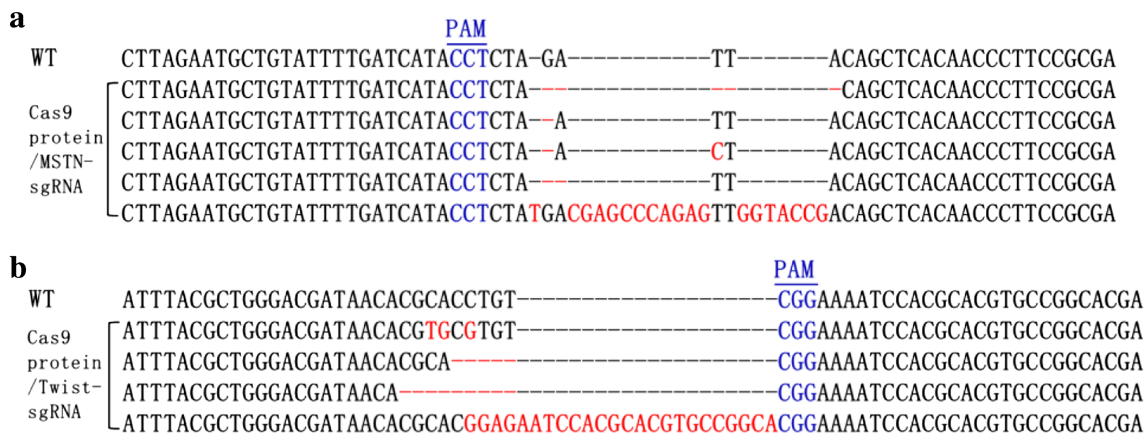

Fig. 5 Characterization of indel mutations in the injected embryos. PCR amplicons from the MSTN-sgRNA/Cas9 or Twist-sgRNA/Cas9 RNPs injected embryos were cloned into the pEASY vector. Thirty clones were randomly selected for DNA sequencing. Alignments of the DNA sequences surrounding the targeting sites revealed five clones with indel mutations in MSTN (**a**) and 4 clones with mutations in Twist genes (**b**). The wild-type (WT) sequence was in the top line, and mutant sequences were in the subsequent lines. The PAM sequences were in blue. The inserted and deleted nucleotides were shown in red

CRISPR/Cas9 studies of gene function in oysters and other bivalves, and potentially as a powerful tool for genetic engineering to improve oyster traits for aquaculture.

Delivery of sgRNA/Cas9 into Oyster Embryos by Microinjection

There are three major methods to introduce the CRISPR/Cas9 system into cells or organisms: (1) introduction of Cas9 and sgRNA as an all-in-one DNA plasmid expression vector, (2) introduction of Cas9 and sgRNA as in vitro transcribed RNAs, and (3) introduction of Cas9 protein and in vitro transcribed sgRNA as sgRNA/Cas9 ribonucleotide protein complexes (Hiruta et al. 2018). The third method via the delivery of sgRNA/Cas9 RNPs was found to be a simple and effective approach in CRISPR/Cas9 mediated gene editing. Direct delivery of sgRNA/Cas9 RNPs has been reported to yield a high editing efficiency in several different organisms such as mouse, zebrafish, water flea, and fungi (Aida et al. 2015; Burger et al. 2016; Hiruta et al. 2018; Wang et al. 2018). In addition, direct delivery of sgRNA/Cas9 RNPs by microinjection has several advantages. First, there is no need to consider the promoter and codon usage. Second, Cas9 and sgRNA are able to form a stable ribonucleoprotein in vitro, and the pre-loaded sgRNAs are possibly protected from degradation (Burger et al. 2016). Third, using the in vitro assembled sgRNA/Cas9 RNPs alleviates the possibility of integration of genetic material to the host genome (Wang et al. 2018). Consistent with these studies, we successfully applied the sgRNA/Cas9 RNPs system in targeted gene editing in the Pacific oyster.

Various methods, such as microinjection, electroporation, and lipofection, have been utilized for delivery of CRISPR/Cas9 into different organisms. Each method has its own advantages and disadvantages. For example, electroporation allows the simultaneous delivery of CRISPR/Cas9 systems into a larger number of embryos. However, this method can cause significant cell death, and may not work for some marine species which are not tolerant to isotonic solution with low ionic strength for electroporation (Liu et al. 2017; Momose and Concordet 2016). Lipofection is relatively safe and easy to prepare, but low delivery efficiency has hampered its application. Microinjection is the direct injection of CRISPR/Cas9 into embryos with a high reproducibility and with minimal reagents using a micropipette at a microscopic level. Because of its simplicity and accuracy, microinjection has been used mostly so far. Certainly, there are several disadvantages of microinjection. Microinjection requires a high level of sophistication and manual skills and is only suitable for a limited number of embryos (Momose and Concordet 2016).

Our studies here support the idea that microinjection is an effective approach to deliver CRISPR/Cas9 in both model and non-model animals. However, there are several challenges for microinjection in *C. gigas*. Although a large number of eggs can be produced, the small size (< 50 μm in diameter) and opacity of the oocytes make the introduction of sgRNA/Cas9 RNPs by microinjection technically difficult. The first key factor for microinjection is the immobilization of eggs. In zebrafish, embryos can be easily held in wedge-shaped troughs made with 1.5% agarose. However, it is not suitable for oyster eggs because they are too small to be arranged in wedge-shaped troughs. In sea urchin, zygotes can be adhered to microinjection dishes coated with protamine sulfate or poly-L Lysine (Stepicheva and Song 2014; Hirsinger et al. 2015). We were not successful with this approach in oysters (data not shown here). In our study, the oyster eggs were held in place with a holding glass pipette by gentle suction. The next challenge is the tip size of the injection needle. Due to the small size of oyster eggs, the diameter of the needle tip must be very small (~ 1 μm), which makes it easily clogged by oocyte cytoplasm and viscous injection solution especially at

the high Cas9 concentration. Therefore, a sharp and beveled pipette tip is preferable. Although microinjection is feasible as a practical method to deliver sgRNA/Cas9 RNPs into oyster embryos, it is, however, time-consuming and technically demanding to produce mutant oysters on a large scale. High throughput methods still need to be explored for applying gene editing technologies to improve oysters for aquaculture.

Balance Between Mutagenesis Rate and Embryo Survival

Our data indicated that higher concentration of sgRNA/Cas9 injection is a key factor in inducing efficient genetic mutations in *C. gigas* embryos. We found that the sgRNA and Cas9 concentrations used in oyster microinjection were tenfold higher than that used in zebrafish in order to produce mutations (Cai et al. 2018). This could be explained by the smaller volume of sgRNA/Cas9 injected into each *C. gigas* embryo. In zebrafish, a typical volume of microinjection is 2–3 nl per embryos. In contrast, only 0.1 nl could be injected into the *C. gigas* embryo. This could be related to the lower efficiency in gene editing compared with zebrafish. Although successful induction of genetic mutations has been routinely accomplished in mouse embryos via microinjection of sgRNA/Cas9 at a moderate concentration comparable with that used in zebrafish, it should be noted that this is done with pronuclear injection into mouse embryos. Direction injection of sgRNA/Cas9 into the pronucleus allows a high concentration of the reagents in the nucleus and easy access to the genomic DNA.

We noted that a higher concentration of sgRNA/Cas9 injection increased the rate of mutated embryos. We found that the molar ratio of sgRNAs to Cas9 protein is critical for efficient editing using the CRISPR/Cas9 system. This is consistent with a previous report that six-fold molar excess of sgRNA over Cas9 protein resulted in maximizing the on-target mutation frequencies when delivered into cultured human leukemia K562 cells (Kim et al. 2014). However, a higher concentration of shRNA/Cas9 injection is associated with a reduced embryonic viability. A similar observation has been reported in monkeys (Midic et al. 2017). One feasible solution is the deployment of multiple sgRNAs (Zuo et al. 2017). Therefore, in future studies, it will be important to consider the deployment of multiple sgRNAs targeting the same gene. In addition, more sgRNAs can be designed and tested, because different sgRNAs show different efficiencies.

The Characteristic of Indel Mutations in Oyster Embryos

We found that the CRISPR/Cas9-induced mutations were predominantly small indel mutations in these two target genes, ranging in size from 1 to 24 bp. This is very similar to the results obtained in other invertebrate species (Lin and Su 2016; Hiruta et al. 2018). Because several different types of indels were observed from a single injected larva, it suggested that the indel mutations might occur independently in different cells. Moreover, we showed that only 20–25% of the sequenced clones were found to be mutated, suggesting the existence of mosaicism, which is common in gene-edited animals using the CRISPR/Cas9 system in injected embryos (Mehravar et al. 2018). Mosaicism in gene editing is not a problem if mutants are identified in the F1, F2, and F3 generations and used for gene functional studies.

Author Contributions Conceived and designed the experiments: QL and SD. Performed the experiments: HL, RX, CY, and HY. Analyzed the data: HL, HY, and SD. Wrote and revised the paper: HY, QL, and SD.

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Compliance with Ethical Standards

All animal studies were carried out according to the guideline for the Care and Use of Laboratory Animals of Ocean University of China. The protocol was approved by the Institutional Animal Care and Use Committee of Ocean University of China.

Competing Interest The authors declare that there is no conflict of interest.

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