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ORIGINAL ARTICLE

CRISPR/Cas9 Mediated High Efficiency Knockout of Myosin Essential Light Chain Gene in the Pacific Oyster (*Crassostrea Gigas*)

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Abstract

Pacific oyster (*Crassostrea gigas*) is one of the most widely cultivated shellfish species in the world. Because of its economic value and complex life cycle involving drastic changes from a free-swimming larva to a sessile juvenile, *C. gigas* has been used as a model for developmental, environmental, and aquaculture research. However, due to the lack of genetic tools for functional analysis, gene functions associated with biological or economic traits cannot be easily determined. Here, we reported a successful application of CRISPR/Cas9 technology for knockout of myosin essential light chain gene (CgMELC) in *C. gigas*. *C. gigas* embryos injected with sgRNAs/Cas9 contained extensive indel mutations at the target sites. The mutant larvae showed defective musculature and reduced motility. In addition, knockout of CgMELC disrupted the expression and patterning of myosin heavy chain positive myofibers in larvae. Together, these data indicate that CgMELC is involved in larval muscle contraction and myogenesis in oyster larvae.

Keywords CRISPR/Cas9 · *Crassostrea gigas* · Myogenesis

Introduction

Pacific oyster (*Crassostrea gigas*) is one of the most widely cultivated shellfish in the world. Pacific oyster lives in the estuarine and intertidal zone. They have a complex life cycle that includes a free-swimming nauplius larva, a cypris larva and a permanently attached sessile juvenile and adult. *C. gigas* has become an animal model in aquaculture and scientific research because of its economic value and unique biology in transition from free swimming larvae to sessile juvenile during metamorphosis. *C. gigas* has been used for studies in development biology, immunology, and ecology. As a filter-feeding organism living in the coastal zones, *C. gigas* is also a unique model for studying ecotoxicology (Palmer et al. 2015), and adaptive response to climate changes (Li et al. 2018).

C. gigas larvae rely on retractor and adductor muscle contraction for swimming and feeding. Muscle contraction is triggered by a direct binding of Ca^{2+} to myosin protein complex. The myosin protein complex consists of two regulatory light chains (MRLC), two essential light chains (MELC) and two heavy chains (MHC) (Szent-Györgyi et al. 1999). The myosin essential light chain (MELC) is situated in the neck region of myosin, which provides the Ca^{2+} binding sites to trigger muscle contraction (Fromherz and Szent-Györgyi 1995). Most of the studies on MELC were carried out in vertebrates and invertebrate models such as mouse, fish, *C. elegans*, and Drosophila. Very little is known in molluscan except limited studies in gene cloning and expression analyses (Goodwin et al. 1987; Janes et al. 2000; Maita et al. 1987; Asakawa et al. 1981; Barouch et al. 1991; Yu et al. 2017). Sequence analysis revealed that *C. gigas* MELC is conserved protein that contains a conserved EF-hand calcium binding motif. Our previous studies showed that CgMELC is expressed in the larval velum retractors and adductor muscles (Yu et al. 2017). CgMELC is a muscle-specific gene showing a highest level of expression in adult adductor muscles. However, its

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function in molluscan musculature is completely unknown. This is mainly due to the lack of genetic tools for functional studies in mollusks.

CRISPR technology has become a promising gene editing tool in molluscan research. Our recent study demonstrated that injection of sgRNA/Cas9 protein complex into fertilized eggs induced efficient indel mutations at the target genes in *C. gigas* (Yu et al. 2019), suggesting that the CRISPR system is functional in molluscan. Here, we report the use of CRISPR/Cas9 technology to analyze the function of myosin essential light chain (CgMELC) during larval development in *C. gigas*. We showed that injection of sgRNAs/Cas9 resulted in extensive indel mutations at the targets sites of CgMELC gene. The mutant larvae showed defective musculature and reduced motility. In addition, knockout of CgMELC disrupted the expression and patterning of myosin heavy chain positive myofibers in larvae. Together, these data indicate that CgMELC is involved in larval muscle contraction and myogenesis in oyster larvae. Our research established CRISPR as a powerful tool for functional gene studies in *C. gigas*, facilitating the application of reverse genetics on gene functional studies in marine bivalves.

Materials and Methods

Fertilization and Rearing of *C. gigas*

The adult *C. gigas* were obtained from local oyster farm in Rongcheng, China. Mature female and male oysters were dissected, and their gametes were acquired by gonad striping. In vitro fertilization was performed as previously reported (Wang et al. 2012). The shell length and shell height of *C. gigas* were about 5 cm and 10 cm, respectively. After fertilization, the fertilized eggs were transferred to a 6 cm petri dish immediately for microinjection. The injected embryos were placed in a petri dish containing 10 ml of filtering seawater and cultured in an incubator at 22 °C.

Preparation of sgRNA and Cas9 mRNA

The third and fourth exons of CgMELC were selected for CRISPR-induced mutagenesis. The target sites were predicted using an online tool CRISPOR (http://crispor.tefor.net/). Four sgRNA were designed and named CgMELC-sgRNA-1, CgMELC-sgRNA-2, CgMELC-sgRNA-3, and CgMELC-sgRNA-4, respectively (Fig. 1). The DNA templates for synthesizing CgMELC-sgRNAs were generated by PCR using the DR274 plasmid as template. The PCR was performed with Phusion DNA polymerase (ThermoFisher), using the target specific forward primer and universal reverse primer (Table 1). PCR conditions included an initial denaturation step of 98 °C for 30 s, followed by 35 cycle of 98 °C for 10 s, 60 °C for 20 s, 72 °C for 20 s, following by a final extension at 72 °C for 5 min. The PCR fragments were purified by SanPrep Column PCR Product Purification Kit (Sangon Biotech). SgRNAs were generated by in vitro transcription using MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific). The synthesized sgRNAs were purified by phenol chloroform extraction and stored in aliquots at -80 °C.

The pT3TS-nCas9n plasmid was linearized by Xba I (NEB) and purified by MinElute PCR Purification Kit (Qiagen). The linearized plasmid was used as template for in vitro transcription to generate capped Cas9 mRNA using T3 RNA polymerase kit (Ambion). The synthesized Cas9 mRNA was purified and extracted using phenol-chloroform.

Plasmid Construction

To construct the pCgMHCS:EGFP reporter, the myosin heavy chain gene promoter containing 3000 bp sequence prior to ATG start codon was amplified using Tks Gflex™ DNA Polymerase (Takara). The PCR primers used for amplification were listed in the Table 1. The PCR fragments were purified by MinElute PCR Purification Kit (Qiagen). The CgMHCS promoter was cloned into the HindIII and BamHI sites of the pEGFP-1 vector. The pCgMHCS:EGFP construct was verified by DNA sequencing.

Microinjection of *C. gigas* Embryos

The sgRNAs and Cas9 mRNA were delivered into *C. gigas* embryos by microinjection. Microinjection was carried out by using a Warner PLI-100A Pico-Injector microinjector (Warner Instruments). Embryos were injected under inverted microscope IX73 (Olympus) by using two micromanipulators for holding and microinjection pipettes (Narishige). The Cas9 mRNA and sgRNAs were prepared in the injected buffer (0.5% phenol red, 20 mM HEPES, and 150 mM KCl) at 500 ng/μl or 1000 ng/μl, respectively. Approximately 0.1 nl of sgRNA/Cas9 solution was injected into one-cell stage *C. gigas* embryo. For co-injection with the pCgMHCS:EGFP reporter construct, the pCgMHCS:EGFP plasmid (500 ng/μl) was mixed with Cas9 mRNA (1000 ng/μl) and CgMELC-sgRNAs (1000 ng/μl) and co-injected into one-cell stage *C. gigas* embryos.

DNA Isolation and Genotyping by DNA Sequencing

Genomic DNA was extracted from D-shaped larvae at approximately 24 h after fertilization using Chelex®-100 method (Li and Kijima 2005). Briefly, 20 μl Chelex®-100, 0.5 μl proteinase (20 μg/ml), and 2 μl TE buffer were added to each larva in a 200 μl PCR tube. The samples were digested at 55 °C for 2 h, denatured at 99 °C for 10 min,

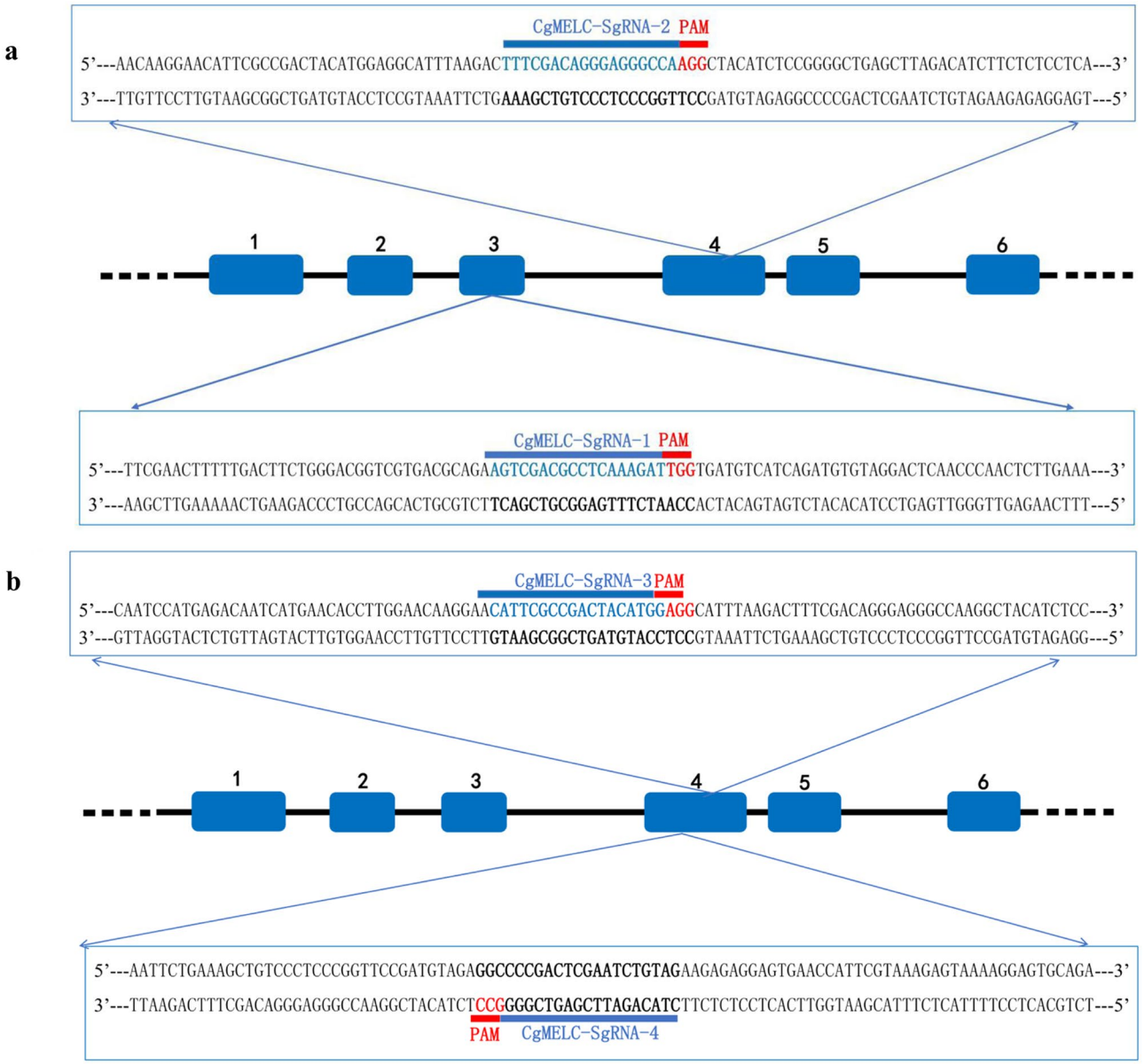

Fig. 1 Schematic diagram of CgMELC-sgRNAs. The PAMs are shown in red, and the sgRNA sequences are shown in blue. CgMELC-sgRNA-1 is targeted to exon 3; whereas CgMELCE-sgRNA-2, CgMELC-sgRNA-3 (the sense strand), and CgMELCE-sgRNA-2 (the antisense strand) are targeted to exon 4

and cooled down to 4 °C. The samples were centrifuged at 3000 g/min for 2 min, and 1 µl of the supernatant was used for PCR. The DNA region covering the target sites were amplified by PCR using the 2×Taq Plus Master Mix II (Dye Plus) (Vazyme) according to the manufacturer's instruction (Table 1). The PCR fragments were purified and sequenced using the forward primers (Sangon Biotech). To define the genetic mutations, the amplified fragments were then cloned into pCE2 TA/Blunt-Zero vector (Vazyme). Twelve clones were randomly selected for DNA sequencing using M13F primer. In the meantime, the possible off-target sites of sgRNAs were predicted using the online tool (http://crispor.tefor.net/). The off-target loci were amplified by PCR and identified by sanger sequence.

Characterization of Larval Musculature in *C. gigas*

Morphological analysis was examined on D-shaped larvae under an Olympus BX53 fluorescence microscope equipped with a DP80 camera (Olympus). To characterize the musculature in wild type and CgMELC knockout larvae, we performed phalloidin staining to analyze actin thin filament organization

Table 1 Primers for sgRNA synthesis, PCR analyses, and plasmid construction. The bold sequences in CgMELC-sgRNAs are the gene-specific target sequences within the respective sgRNAs. The underlined GG in sgRNAs were added by PCR primer for correct T7 transcription

Primer name	Sequence (5′-3′)	Usage
CgMELC-SgRNA-F1	GATCACTAATACGACTCACTATA**GGAGTCGACGCCTCAAAG AT**GTTTTAGAGCTAGAAAT	sgRNA synthesis
CgMELC-SgRNA-F2	GATCACTAATACGACTCACTATA**GGTTTCGACAGGGAGGGC CA**GTTTTAGAGCTAGAAAT	sgRNA synthesis
CgMELC-SgRNA-F3	GATCACTAATACGACTCACTATA**GGCATTCGCCGACTACATGG**G TTTTAGAGCTAGAAAT	sgRNA synthesis
CgMELC-SgRNA-F4	GATCACTAATACGACTCACTATA**GGGATGTCTAAGCTCAGC CC**G TTTTAGAGCTAGAAAT	sgRNA synthesis
SgRNA-R	AAAAGCACCGACTCGGTGCC	sgRNA synthesis
CgMELC-F1	GGAATCAGAGTTAATCAATTTCTG	Verify primer for CgMELC-SgRNA-F1
CgMELC-R1	ATGATTGTCTCATGGATTGGC	Verify primer for CgMELC-SgRNA-F2/3/4
CgMELC-F2	CAGATGTGTAGGACTCAACCCAAC	Verify primer for CgMELC-SgRNA-F2/3/4
CgMELC-R2	TAACCACTGCACCACGCTGT	Verify primer for CgMELC-SgRNA-F2/3/4
Off-target-F1	AAGTAAGCGTATTCCCTTCTATTGC	Off-target analyze (CGI_10010778)
Off-target-R1	CGACAGTAACCCAAAATGACACA	Off-target analyze (CGI_10010778)
Off-target-F2	GTAATTGTTCTAGACTTATGTTGGC	Off-target analyze (CGI_10018045)
Off-target-R2	GGCAGTCTCAACAGGTAAACAT	Off-target analyze (CGI_10018045)
Off-target-F3	GTATTGTTGGTTTCATCACACGGT	Off-target analyze (CGI_10005667)
Off-target-R3	GCAGTGCAAGTTTGATCCGC	Off-target analyze (CGI_10005667)
Promoter-CgMHCS-F	cccAAGCTTTCAAGCACATCCCACCCTCAA	Plasmid construction
Promoter-CgMHCS-R	cgcGGATCCGGGACCCAGCACATCTTCTTG	Plasmid construction

in muscle cells. D-shaped larvae were anesthetized with 7.5% $MgCl_2$ and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.3) for 3 h at room temperature (RT). Phalloidin staining was performed as previously reported (Li et al. 2018). Image acquisition and analysis were performed on an ultra-high-resolution laser confocal microscope Leica TCS SP8 STED 3X equipped with Leica Application Suite X software. Confocal image stacks were recorded with 1.5–3-µm step size along the *Z*-axis and merged as maximum intensity projections.

Results

Identification of Effective sgRNA and Testing Mutation Efficiency

To mutate gene encoding the essential myosin light chain in Pacific oyster, four sgRNAs were designed targeting to exon 3 and exon 4 of the MELC gene. Each of the 4 sgRNAs, CgMELC-sgRNA-1, CgMELC-sgRNA-2, CgMELC-sgRNA-3, and CgMELC-sgRNA-4, was mixed separately with Cas9 mRNA and injected into one-cell stage embryos. To assess the mutagenesis efficiency of each sgRNA/Cas9 combination, the genomic regions covering each sgRNA's target sites were isolated by PCR and sequenced. The data showed that CgMELC-sgRNA-1 and CgMELCE-sgRNA-2 were very effective in guiding Cas9-induced mutagenesis (Fig. 2). In contrast, CgMELC-sgRNA-3 and CgMELC-sgRNA-4 were ineffective, showing no multiple peaks near PAM sites of the target sequences (data not shown).

To test the dose effect on mutation efficiency, we compared the data from various injections of two different concentrations of Cas9/sgRNA. When CgMELC-sgRNA and Cas9 mRNA were injected at a final concentration of 500 ng/µl each, the mutation rates of CgMELC-sgRNA-1 and CgMELC-sgRNA-2 were 20% and 80%, respectively. The mutation rates of CgMELC-sgRNA-1 and CgMELC-sgRNA-2 increased to 80% and 90%, respectively, when CgMELC-sgRNA and Cas9 mRNA were injected at 1000 ng/µl. The injected embryos showed no significant difference in viability than un-injected embryos. These data indicate that there was a dose-dependent effect of sgRNA/Cas9 mRNA injection (Table 2).

Characterization of Indel Mutations in the Injected Embryos

To analyze the types of indel mutations induced by sgRNA/Cas9 mRNA injection, the PCR products at the targets were cloned and sequenced individually. Twelve clones were randomly selected for sequencing for each sgRNA. Sequencing results revealed that CRISPR/Cas9

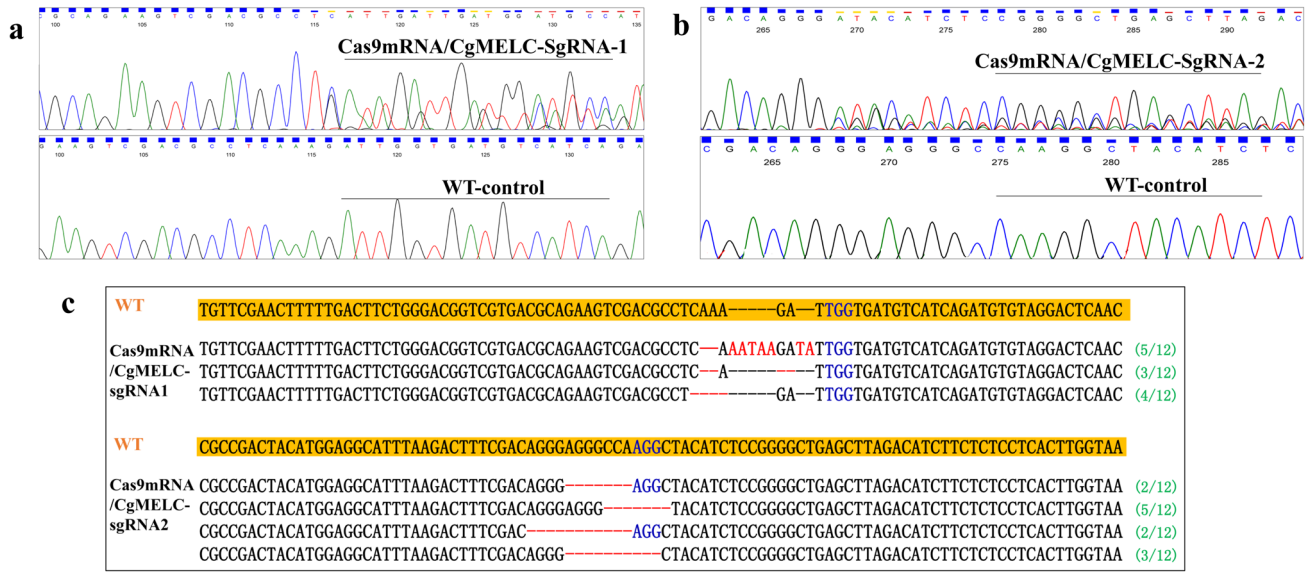

Fig. 2 Sanger sequencing of PCR products and characterization of indel mutations in the injected embryos. Embryos injected with CgMELC-sgRNA-1/Cas9 or CgMELC-sgRNA-2/Cas9 were sacrificed for PCR and sequencing. Induced mutations shown by the multiple peaks were detected at the respective target sites of CgMELC-sgRNA-1 **a** or CgMELC-sgRNA-2 **b**. WT means the wild type. DNA sequence analysis showed the presences of indels at the CgMELC-sgRNA-1/Cas9 and CgMELC-sgRNA-2/Cas9 **c**, PAM sequences were shown in blue, and the inserted and deleted nucleotides were shown in red

principally induced small indel mutations ranging in size from 1 to 11 bp. The mutation type in CgMELC-sgRNA-1 was mainly insertion and deletion (Fig. 2). The mutation type in CgMELC-sgRNA-2 was predominantly deletion (Fig. 2). All these indel mutations were frameshift mutations that alter the protein translation and disrupt the gene function. In addition, no data of mutagenesis was detected in the putative off-target loci (Attachment 1).

Defective Muscle Formation in CgMELC Knockout Larvae

The larval musculature was characterized in CgMELC knockout larvae by phalloidin staining that labels the F-actin thin filaments. The data showed that muscles of larval velum retractors, adductor, and larval retractors displayed various degrees of abnormality in CgMELC-sgRNA-1 and

Table 2 The indel mutation rate and phenotype mutation rate of different concentrations of Cas9 mRNA and sgRNAs

	Cas9 mRNA Concentration (ng/μl)	sgRNA concentration (ng/μl)	Injected embryos/survival D-shaped larvae (%)	Target point mutation larvae/sequenced larvae (%)	Phenotypic mutation rate (%)
Uninjected control	0	0	150/126 (84%)	—	—
Cas9mRNA injected	1000	0	148/117 (79%)	0	0
CgMELC-sgRNA-1/2 injected	0	1000	135/103 (76%)	0	0
Cas9mRNA/CgMELC-sgRNA-1 injected	500	500	169/136 (80%)	2/10 (20%)	—
Cas9mRNA/CgMELC-sgRNA-1 injected	1000	1000	125/95 (76%)	8/10 (80%)	18/60 (30%)
Cas9mRNA/CgMELC-sgRNA-2 injected	500	500	146/107 (73%)	8/10 (80%)	—
Cas9mRNA/CgMELC-sgRNA-2 injected	1000	1000	136/108 (79%)	9/10 (90%)	45/64 (70%)
Cas9mRNA/CgMELC-sgRNA-1/2 injected	1000	1000	179/144 (80%)	—	47/55 (85%)

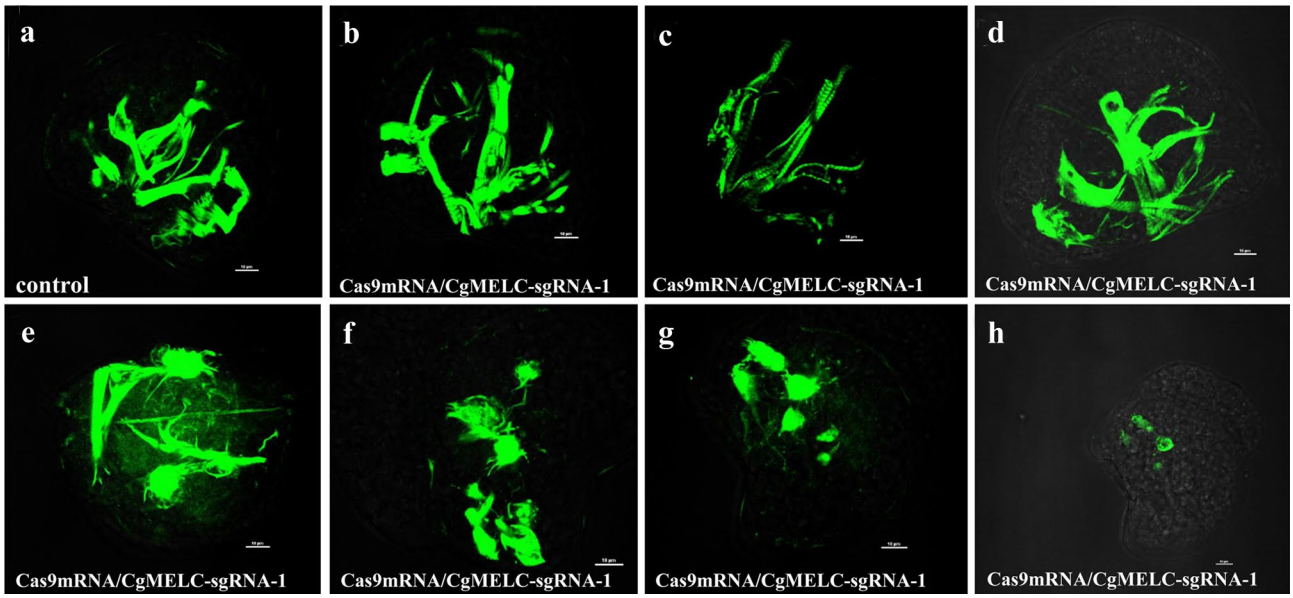

Fig. 3 The musculature of larvae injected with CgMELC-sgRNA-1/Cas9 mRNA. The larval musculature in figures **b–d** was not significantly different from that in control **a**. The larval musculature was disrupted in figure **e–h**

CgMELC-sgRNA-2 injected larvae. The sequence of larvae with muscle damage phenotype showed multiple peaks near PAM sites of the target sequences. When the embryos were injected with CgMELC-sgRNA-1 (1000 ng/μl) and Cas9 mRNA (1000 ng/μl), approximately 30% of the injected larvae showed defects in their musculature (Fig. 3). The proportion of larvae with muscle abnormality increased to 70% when CgMELC-sgRNA-2 (1000 ng/μl) was co-injected with Cas9 mRNA (1000 ng/μl) (Fig. 4). This is consistent with the finding that CgMELC-sgRNA-2 was more effective than CgMELC-sgRNA-1 in guiding Cas9-induced mutagenesis at their respective target sites.

It has been reported that co-injection of multiple sgRNAs redundantly targeting a single gene could dramatically increase the indel mutation efficiency in G0 zebrafish embryos (Wu et al. 2018). The injected G0 embryos provide a rapid system

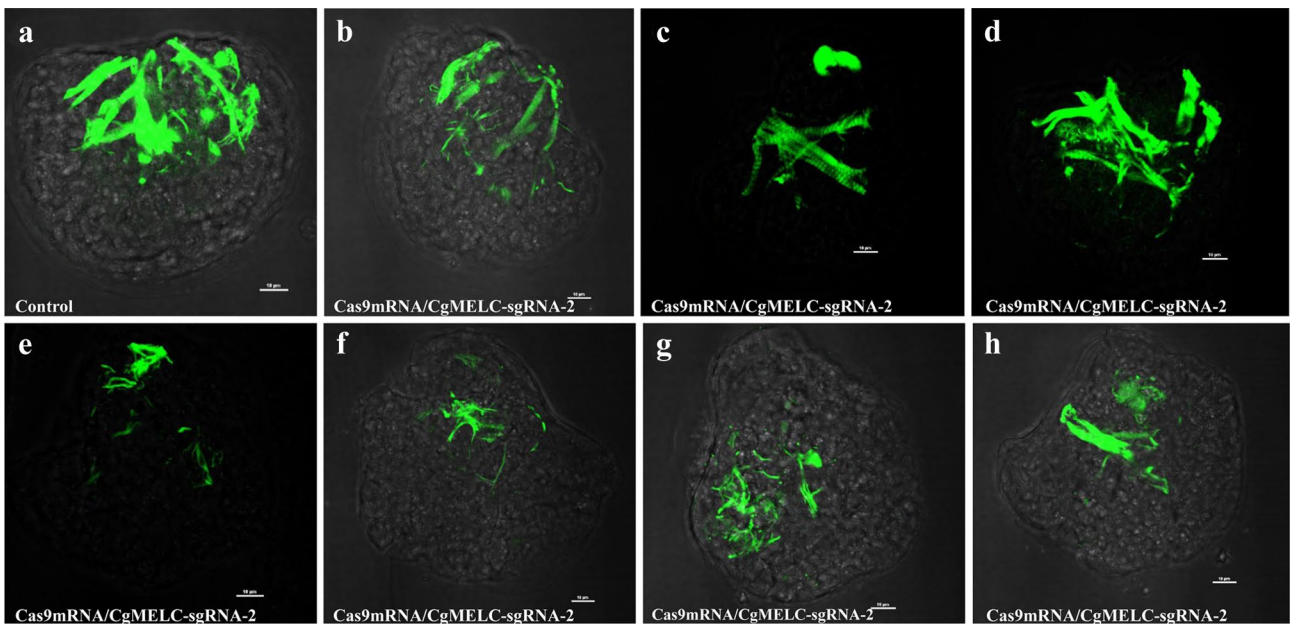

Fig. 4 The musculature of larvae injected with CgMELC-sgRNA-2/Cas9 mRNA. The larval musculature in figures **b–c** was not significantly different from that in control **a**.The larval musculature was disrupted in figure **d–h**

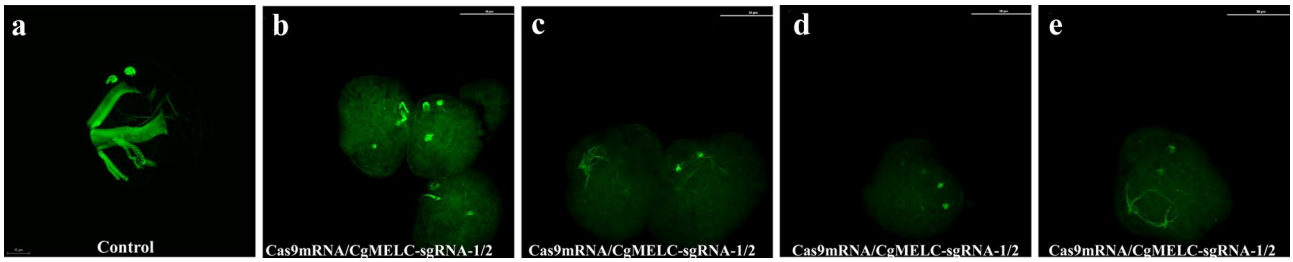

Fig. 5 The musculature of larvae injected with CgMELC-sgRNA-1/2 and Cas9 mRNA. The larval musculature including velum retractors and adductor muscle was disrupted severely in figures **b**–**e**. Dual sgRNAs (CgMELC-sgRNA-1 and CgMELC-sgRNA-2) injection not only led to increased number of larvae with muscle defects but also the severity of muscle damage

for directed gene knockout for genetic screening and phenotype analysis. To test whether co-injection of CgMELC-sgRNA-1 and CgMELC-sgRNA-2 could further enhance the mutation efficiency for phenotype analysis, we combined CgMELC-sgRNA-1 (1000 ng/μl) with CgMELC-sgRNA-2 (1000 ng/μl) and co-injected with Cas9 mRNA (1000 ng/μl) into fertilized eggs. The data showed that 85% of the injected larvae showed defects in their musculature (Fig. 5; Table 2). Moreover, dual sgRNA (CgMELC-sgRNA-1 and CgMELC-sgRNA-2) injection not only led to increased number of larvae with muscle defects but also the severity of muscle damage. In contrast, Cas9 mRNA or sgRNA injection alone had no significant effect on larval musculature compared with control. Together, these data indicate that CgMELC is required for muscle filament formation. Moreover, G0 injected embryos could be used for functional studies; especially, two sgRNAs were combined for injection.

Knockout of CgMELC Disrupted Myogenesis in the Larvae

To test whether loss of CgMELC could affect myogenesis of oyster larvae, we analyzed the patterning of MHC expression in CgMELC knockout embryos. A GFP reporter construct, pCgMHCS:EGFP, was generated that expressed EGFP driven by a 3-kb MHC gene promoter.

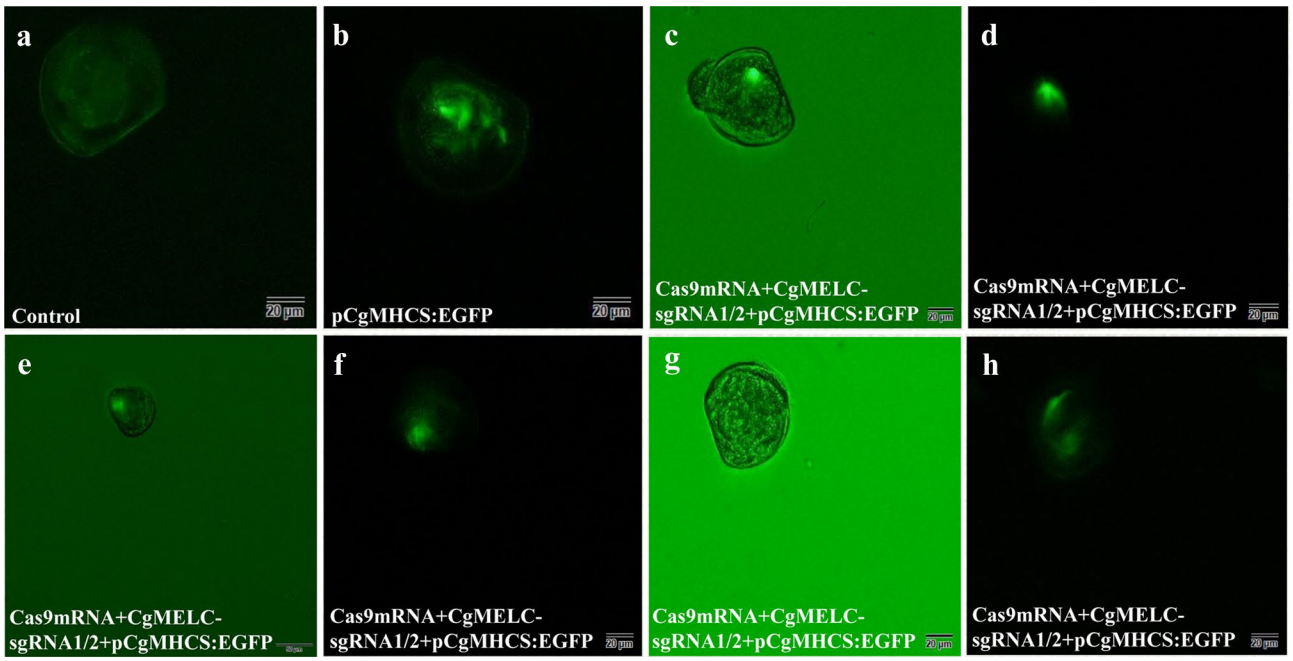

Fig. 6 The expression of pCgMHCS-EGFP of injected embryos. Embryos injected with pCgMHCS:EGFP alone showed a strong pattern of muscle-specific expression resembling the pattern of larval velum retractors **b**. Embryos co-injected with CgMELC-sgRNAs and Cas9 showed a significant decrease in pCgMHCS-EGFP expression **c**–**h**. The figures **c**, **e**, and **g** were the corresponding bright field pictures of figures **d**, **f**, and **h**

The pCgMHCS:EGFP construct was microinjected alone or together with CgMELC-sgRNAs and Cas9 into one-cell stage embryos. GFP expression was analyzed in the injected D-shaped larvae around 24 hpf. Embryos injected with pCgMHCS:EGFP alone showed a strong pattern of muscle-specific expression resembling the pattern of larval velum retractors (Fig. 6). In contrast, embryos co-injected with CgMELC-sgRNAs and Cas9 showed a significant decrease in pCgMHCS-EGFP expression. Collectively, these data indicate that CgMELC was required for normal expression and patterning of myosin heavy chain in oyster larvae.

To test whether larval locomotion was also affected in the sgRNA/Cas9 injected embryos, we carried out the locomotion assay at D-shaped stage when wild type larvae showed a fast and normal swimming. The result showed that knockout of CgMELC resulted in significant reduction of motor ability, mainly in swimming speed and distance (Attachment file 2). In contrast, Cas9 mRNA or sgRNA-injected embryos showed no significant difference in swimming ability (Attachment file 3). Collectedly, these data demonstrated that the myosin essential light chain was vital to muscle development and locomotion in pacific oyster larvae.

Discussion

Delivery of sgRNA/Cas9 mRNA into Oyster Embryos

There are three strategies to deliver CRISPR-Cas9 system to cells or organisms. The first method is to use a plasmid-based CRISPR/Cas9 system encoding Cas9 protein and sgRNA from same vector (Ran et al. 2013). The second is to induce the mixture of Cas9 mRNA and sgRNA (Niu et al. 2014). The third strategy is to deliver the complex of Cas9 protein and sgRNA (Hiruta et al. 2018). In our previous report, complex of Cas9 protein and sgRNA were successfully co-injected into fertilized oyster eggs and resulted in genetic mutations at the target sites (Yu et al. 2019). However, the Cas9 protein showed toxicity to the injected embryos leading to reduced viability of the injected larvae (Yu et al. 2019). In addition, high concentration of the Cas9 protein could block the injection needle. Here, we injected a mixture of Cas9 mRNA and sgRNAs into one-cell embryos and resulted in high efficiency of mutagenesis at the target gene. In contrast to Cas9 protein injection, Cas9 mRNA was less toxic to the injected oyster embryos. This result was consistent with the previous report that Cas9 mRNA showed low cytotoxicity in primary cell culture and cell lines (Li et al. 2014). In addition, Cas9 mRNA was less likely to block the injection needle, suggesting that Cas9 mRNA was an effective delivery way for microinjection into oyster embryos.

Relationship Between sgRNA Sequence and Indel Mutation Rate

Four sgRNAs were designed for CgMELC, each with a different mutation rate. The mutation rate of CgMELC-sgRNA-1 and CgMELC-sgRNA-2 was 80% and 90%, respectively, at the same concentration (1000 ng/μl). CgMELC-sgRNA-3 and CgMELC-sgRNA-4 showed no mutation at the target site. Current studies have shown that the mutation efficiency of sgRNA was related to its sequence composition. Mutation efficiency of sgRNAs with a very high (≥ 80%) or low (≤ 35%) GC contents were less effective (Wang et al. 2014). Here, the GC content in CgMELC-sgRNAs was all between 35 and 80%. The enrichment of guanine and the deletion of adenine could increase the stability and activity of sgRNA, thus increasing its mutation rate. The guanine could protect against 5′-directed exonuclease degradation. Eight guanines could fold into stable G-quadruplexes structure, which increased the stability of sgRNA (Moreno-Mateos et al. 2015). sgRNAs containing the TT- or GCC- motif generally had lower knockout activity (Graf et al. 2019). Among the four sgRNA sequences in CgMELC, there were six and nine guanines in CgMELC-sgRNA-1 and CgMELC-sgRNA-2, and six guanines in CgMELC-sgRNA-3 and CgMELC-sgRNA-4, respectively. CgMELC-sgRNA-2 with nine guanines showed the highest mutation rate, and it might be related to its high content of guanine.

The Ways of Reducing Mosaicism in the G0 Generation

CRISPR/Cas9 system is an effective technique in gene editing by microinjection into fertilized eggs. However, most of the injected embryos showed gene-functional mosaicism, with gene disruption occurs only in some cells, not in all (Yen et al. 2014). To analyze the knockout phenotype, gene functional studies are typically carried out in the F1 or F2 generations with complete genetic mutations in all cells. This multiple generation study was not practical for animal species with long breeding periods, especially when multiple genes are required to be knocked out simultaneously (Jennings et al. 2016). Various studies have attempted to produce gene-modified animals without mosaicism in a single step. Recent studies have reported that introduction of multiple sgRNAs into embryos synchronously could reduce the problem of mosaicism (Zhou et al. 2014). In mice and monkey, one-step generation of complete gene knockout was achieved by delivering two or more sgRNAs targeted to the same gene to embryos concurrently (Zhou et al. 2014; Niu et al. 2014; Zuo et al. 2017). Here, we showed that when CgMELC-sgRNA-1 and CgMELC-sgRNA-2 were injected alone with Cas9 into oyster embryos separately, 30% and 70% of the respectively injected embryos showed muscle phenotype. However, when the two sgRNAs were co-injected simultaneously with the Cas9 into oyster embryos, 85% of the

injected embryos showed the muscle phenotype, and moreover, the muscle phenotype appeared to be stronger. Therefore, our data are consistent with the notion that delivering two or more sgRNAs simultaneously was able to reduce mosaicism and improve phenotypic mutation rate. In addition, the time of embryo injection was closely related to mosaicism. To reduce the mosaicism, Sato et al. generated target gene knockout by injecting Cas9 mRNA and sgRNAs into the oocyte (Sato et al. 2015). In mouse, Hashimoto et al. generated non-mosaic mutants at two gene by electroporation of Cas9 protein/sgRNA into in vitro fertilized zygotes at the early pronuclear stage (Hashimoto et al. 2016). In our studies, we injected the Cas9 mRNA/sgRNA at the one-cell stage to reduce the mosaicism.

Functional Analysis of CgMELC in Larval Motor Ability and Musculature

The muscle contraction system in mollusks was triggered by a direct binding of Ca^{2+} to myosin. The Ca^{2+} binding sites were located in EF hand domains of MELC in molluscs (Szent-Györgyi et al. 1999). In our study, knockout of CgMELC caused the musculature which was the main motor organ of the larvae to lose its contractile function, resulting in reduced athletic ability of the larvae with CgMELC knockout. Our results suggested that the myosin essential light chain was necessary for the muscle contraction in *C. gigas*. This is consistent with previous report that MELC was of major significance for cardiac contraction in zebra fish (Meder et al. 2009). The larval velum retractor, larval retractor, and adductor muscle were disrupted in CgMELC knockout larvae, indicating that CgMELC was an indispensable gene in the larval myogenesis in *C. gigas*. The larval musculature was characterized in CgMELC knockout larvae by phalloidin staining that labels the myosin thin filaments. Together, these data suggested that disruption of CgMELC could result in defective organization of myosin thin filaments in *C. gigas*. Our results were consistent with previous studies showing that N-terminus of MELC interacted with actin, and the actin-myosin interaction played a vital role in myofibril assembly of thin filaments (Trayer and Trayer 1985; Robert et al. 1977).

The myosin consists of two myosin heavy chain (MHC), two essential light chain (MELC) and regulatory light chain (MRLC). Part of the myosin heavy chain and the two light chains formed two globular heads of myosin (Hooper 2005). MELC binds to MHC in the region of IQ motifs. Researches have showed that the primary function of MELC was stabilization of MHC α-helix in the regulatory domain of myosin head MELC (Logvinova et al. 2018). Association of myosin light chains with myosin heavy chains is necessary for the assembly and stability of myosin thick filaments, a key component in sarcomeres. Genetic mutations in the regulatory or essential light chain affect myosin motor function in skeletal and cardiac muscles (Chaudoir et al. 1999; Andersen et al. 2016). Here, knockout of CgMELC disrupted the pattern of CgMHCS-EGFP expressing myofibers in larvae, indicating that CgMELC was required for normal myogenesis in oyster larvae.

In conclusion, we demonstrated for the first time that CRISPR/Cas9 technology could be successfully applied to gene functional studies in *C. gigas*. The mutant phenotype could be analyzed in G0 generation of the injected embryos when multiple sgRNAs were co-injected to introduce more efficient mutagenesis. Our studies showed that CgMELC was essential in larval myogenesis and motor activities. The results provide foundation for gene functional studies in marine bivalves.

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Compliance with Ethical Standard

Competing Interest The authors declare that they have no competing interests.

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