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Original Article

CRISPR/Cas9-mediated gene knockout in the ascidian *Ciona intestinalis*

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Knockout of genes with CRISPR/Cas9 is a newly emerged approach to investigate functions of genes in various organisms. We demonstrate that CRISPR/Cas9 can mutate endogenous genes of the ascidian *Ciona intestinalis*, a splendid model for elucidating molecular mechanisms for constructing the chordate body plan. Short guide RNA (sgRNA) and Cas9 mRNA, when they are expressed in *Ciona* embryos by means of microinjection or electroporation of their expression vectors, introduced mutations in the target genes. The specificity of target choice by sgRNA is relatively high compared to the reports from some other organisms, and a single nucleotide mutation at the sgRNA dramatically reduced mutation efficiency at the on-target site. CRISPR/Cas9-mediated mutagenesis will be a powerful method to study gene functions in *Ciona* along with another genome editing approach using TALE nucleases.

Key words: ascidian, *Ciona intestinalis*, CRISPR/Cas9, knockout, mutagenesis.

Introduction

Ciona intestinalis is a representative species of urochordate ascidians for elucidating genetic functions in the simplified chordate body (Satoh 2003). A draft genome sequence of *Ciona intestinalis* was reported in 2002 (Dehal *et al.* 2002), and deep annotations of genes encoded by the genome have been carried out since then (Satou *et al.* 2008). The basic technologies for examining gene functions have been established in *Ciona*, that include introduction of exogenous DNA and RNA by means of microinjection (Imai *et al.* 2000), efficient introduction of reporter constructs by electroporation (Corbo *et al.* 1997), knockdown of genes by antisense morpholino oligonucleotides (Satou *et al.* 2001), and transposon-mediated germ cell transformation and mutagenesis (Sasakura *et al.* 2003, 2005). These technologies have supported our detailed and thorough analyses to reveal molecular and cellular mechanisms that underlie development of *Ciona*.

Gene knockout is the ultimate way to investigate gene functions by introducing mutations directly and specifically into targeted genes in the genome. Gene knockout was limited to very few model organisms until recently; however, technological innovations brought by the artificial nucleases such as zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) have enabled us to knockout genes in various organisms through quite simple approaches (Santiago *et al.* 2008; Ochiai *et al.* 2010, 2012; Miller *et al.* 2011; Watanabe *et al.* 2012; Ansai *et al.* 2013, 2014; Suzuki *et al.* 2013; Hayashi *et al.* 2014; Hosoi *et al.* 2014; Kondo *et al.* 2014; Sakane *et al.* 2014; Sakuma & Woltjen 2014; Sugi *et al.* 2014). In *Ciona*, our group have reported knockout of *enhanced GFP* (*eGFP*) gene inserted in the genome with ZFNs, and more recently, target mutagenesis of endogenous genes with TALENs (Kawai *et al.* 2012; Treen *et al.* 2014; Yoshida *et al.* 2014). In the latter two reports, we showed that TALENs have a high knockout efficiency in both somatic and germ cells of *Ciona*.

Although TALEN-mediated gene knockout is a powerful approach for addressing gene functions in *Ciona*, constructed TALENs do not always have sufficient mutation activity and therefore another approach that can compensate for inactive TALENs is necessary.

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Use of the clustered, regularly interspaced, short palindromic repeat (CRISPR) and Cas9-mediated genome editing is a relatively newer approach than TALENs for achieving targeted mutagenesis of genomes in various organisms (e. g. Hwang *et al.* 2013; Feng *et al.* 2013; Gratz *et al.* 2013; Nakayama *et al.* 2013; Friedland *et al.* 2013; Xie & Yang 2013; Daimon *et al.* 2014; Hisano *et al.* 2014; Mashiko *et al.* 2014; Mashimo 2014). CRISPR/Cas9 uses a short guide RNA (sgRNA) that contains 20-nucleotide stretch identical to the target DNA sequence, and an RNA-mediated nuclease Cas9 (Hwang *et al.* 2013; Mali *et al.* 2013). When sgRNA binds to its target site, Cas9 protein is recruited to the binding site, and then the nuclease induces a double-strand break at the target genomic region. The double-strand break is repaired by one of the two endogenous systems, homologous recombination or non-homologous end joining. The latter system is more dominant but more error-prone than the former, and therefore during the repair of the double-strand break insertions and/or deletions can accumulate at the target site. If a mutation is not introduced, the target site is again targeted by CRISPR/Cas9, and once mutations are introduced the region is no longer targeted because of the modification of the target DNA sequence. CRISPR/Cas9 could be a candidate that can compensate for TALENs, because their mechanisms that recognize target sites are quite different. In CRISPR/Cas9, the recognition of the target site is mediated by RNA, compared to the protein-DNA recognition in TALENs. Therefore, genomic regions that are resistant to one of the two methods could be sensitive to the other. An advantage of CRISPR/Cas9 compared to TALENs is easy construction of the components necessary for knockout. sgRNA is the only component that specifies target genes. As mentioned, sgRNA usually contains a 20-nt stretch that is specific to the target site. In addition, three nucleotides adjacent to the 3′ end of the target site should be NGG. This sequence is named the protospacer adjacent motif (PAM) (Wiedenheft *et al.* 2012). There is no critical restriction to select a target site other than the target length and the presence of a PAM in the CRISPR/Cas9 system. Because a 20-nt target site of sgRNA is quite short, the region can be easily substituted to another by conventional cloning techniques. In contrast, TALEN proteins are composed of about 20 repeats, each of which recognizes a single nucleotide, and the variable region of a TALEN is approximately 2000 bp long. Construction of highly repetitive TALENs requires special ligation methods (Cermak *et al.* 2011; Sakuma *et al.* 2013a,b). The easy construction of sgRNAs is an attractive advantage of CRISPR/Cas9 systems over TALENs.

In this study, we examined the efficiency of CRISPR/Cas9-mediated mutagenesis in *Ciona*. Our group is interested in the functions of *Hox* cluster genes in *Ciona* (Sasakura *et al.* 2012; Yoshida *et al.* 2014). With CRISPR/Cas9, we successfully introduced mutations in *Hox3* and *Hox5*, suggesting that CRISPR/Cas9 can be a powerful approach for addressing functions of genes in *Ciona*.

Materials and methods

Animals

Wild-type *C. intestinalis* were collected from or cultivated at Maizuru (Kyoto), Mukaishima (Hiroshima), Misaki (Kanagawa) and Usa (Kochi). Eggs and sperm were surgically collected. After fertilization, the embryos and larvae were cultured at 18°C.

Constructs

The cDNA of Cas9 was amplified from pMLM3613 (purchased from Addgene; Hwang *et al.* 2013) with the primers 5′-AAATATCACCGGATCCGATAATGGATAAGAAATACTC-3′ and 5′-GTTAGATATCGAATTTCATCCTGCAGCTCCACC-3′, and PrimeSTAR thermostable DNA polymerase (Takara Bio). The polymerase chain reaction (PCR) fragment was inserted into the *Bam*HI and *Eco*RI sites of pBS-HTB/N (Akanuma *et al.* 2002; Sasakura *et al.* 2010) by In-Fusion cloning technology (Clontech) to create pHTBCas9. The cDNA of Cas9 was amplified with the primers 5′-CGACTCTAGAGGATCGGATCCCCTTGCGGCCGCAATGGATAAGAAATACTCAATAG-3′ and 5′-CGCTCAGCTGGAATTGAATTCTCATCCTGCAGCTCCACCGCTC-3′, then the PCR fragment was inserted into the *Bam*HI and *Eco*RI sites of pSPeGFP (Sasakura *et al.* 2003) to create pSPCas9. Five base pairs at the multicloning site (underlined in the above primer sequence) were deleted by inverse PCR in order to adjust the reading frame, and then the *cis* element of *Ci-EF1α* (Sasakura *et al.* 2010) was inserted into the *Sal*I site of pSPCas9 to create pSPCiEF1α>Cas9. Primer sets for sgRNAs were designed with the ZiFiT program at the following URL (http://zifit.partners.org/ZiFiT/CSquare9Nuclease.aspx; Sander *et al.* 2007, 2010). The primer sequences are listed in Table S1. After annealing of the primer pairs, the DNA was inserted into the *Bsa*I site of pDR274 (Hwang *et al.* 2013) to create vectors for *in vitro* transcription of sgRNAs. The DNA encoding Hox5-sg1 was amplified with the primers 5′-GGCGACGACGGGTTAGGTAAG-3′ and 5′-TTGAATTCAAAAAGCACCGACTCGGTGCCAC-3′ from pDR274Hox5-sg1, digested with *Eco*RI, and then inserted into the *Eco*RV

and *Eco*RI sites of pSPU6EV (Nishiyama & Fujiwara 2008) to create pSPU6>Hox5-sg1. Other expression vectors for sgRNAs were created by identical procedures. Expression vectors of Hox3-sg3 that contain mismatched nucleotides were created by inverse PCR using pSPU6>Hox3-sg3 as the template and PrimeSTAR thermostable DNA polymerase (Takara Bio).

Microinjection and electroporation

pHTBCas9 was linearized with *Xho*I. mRNAs of Cas9 were synthesized using Megascript T3 kit (Ambion), poly (A) tailing kit (Ambion), and Cap structure analog (New England Biolabs). pDR274 vectors encoding sgRNAs were linearized with *Dra*I. sgRNAs were synthesized using Megascript T7 kit (Ambion). RNA was microinjected into unfertilized eggs according to a previously described method (Hikosaka *et al.* 1992). The volume of the injected media in an egg was approximately 30 pl. Electroporation of plasmids into 1-cell embryos was performed according to the previous report (Corbo *et al.* 1997; Treen *et al.* 2014). Forty micrograms of the expression vector of Cas9 and 20 μg of sgRNA expression vectors were simultaneously electroporated for each electroporation. After electroporation, the embryos were washed in filtered seawater three times to remove excess plasmid DNA.

Genome analyses

For bulked analyses, 20–40 Cas9 and sgRNA-expressing G0 embryos were gathered and genomic DNA was extracted using Wizard Genomic DNA isolation kit (Promega) according to the manufacturer's instructions. sgRNA targeted regions were amplified by PCR using Ex Taq thermostable DNA polymerase (Takara Bio). The primers used for PCR are shown in Table S1. After purifying PCR bands by electrophoresis, PCR products were subcloned into pGEM-T Easy vector (Promega) for sequencing analyses. Detection of mutations with Cel-I nuclease (Transgenomics) was done according to the previous report (Sakuma *et al.* 2013b; Treen *et al.* 2014) except that the amount of DNA was estimated by electrophoresis. The potential off-target sites of sgRNAs were searched for with the Blastn-based program (Altschul *et al.* 1990). The primers used for amplifying DNA fragments containing the potential off-target sites of Hox3-sg3 were 5′-AGA GTATACCAGCCCCATCTG-3′ and 5′-CCACAGCTTA GAAGAGTGCAG-3′ for off-target site 1, and 5′-GTAG CACCATAGATTGTAACGG-3′ and 5′- GATCTTGCAG CGTTGAACACC-3′ for off-target site 2.

Results

CRISPR/Cas9-mediated mutations of Hox *genes*

To knockout *Hox* genes of *Ciona*, we designed sgRNAs for *Hox3*, *Hox5* and *Hox12*. Eight sgRNAs were created in total for these genes (Table 1). They were microinjected into unfertilized *Ciona* eggs along with the mRNA encoding Cas9, then these eggs were fertilized with wild type sperm to start embryogenesis. After the embryos reached the larval stage, genomic DNA was isolated in bulk, and the DNA region including the target site of the injected sgRNA was amplified by PCR. The PCR fragments were treated with the surveyor nuclease Cel-I after denaturing and re-annealing processes. Cel-I nuclease cleaves mismatched sites of double stranded DNA. If the PCR product included nucleotide sequence variations, these variations could yield mismatched sites during the re-annealing. As a result the DNA fragments could be cleaved by Cel-I, and shorter bands could be detected by electrophoresis.

As shown in Figure 1A, Cel-I cleaved *Hox3* PCR product from larvae into which about 3.0 pg of a sgRNA for *Hox3* (Hox3-sg3 in Table 1) was introduced with 15 pg of Cas9 mRNA. The presence of Cel-I cleaved bands suggests that the PCR fragment contained nucleotide sequence variations, probably

Table 1. sgRNAs that target *Hox* genes in *Ciona intestinalis*

Target gene	Name of sgRNA	Sequence of the target site (5′–3′)†	PAM	Activity‡
Ci-Hox3	Hox3-sg1	GGCATACACAAACTCACAAT	TGG	Negative
	Hox3-sg3	GGCAGCCATAAGAGTCAACA	AGG	Active
	Hox3-sg4	GGTCTGCCTACCCAACACAC	GGG	Negative
	Hox3-sg5	GGTGTGGAACCGTAGTATAA	TGG	Negative
Ci-Hox5	Hox5-sg1	GGCGACGACGGGTTAGGTAA	CGG	Active
Ci-Hox12	Hox12-sg1	GGAACCAGATTTTAACTTGC	GGG	Negative
	Hox12-sg2	GGAAGAGCCCCTTCAAGTTC	CGG	Negative
	Hox12-sg3	GGCCGCTCGCTATCGACTTC	AGG	Negative

†DNA sequence of the target sites is shown. ‡Activity tested by the Cel-I assay.

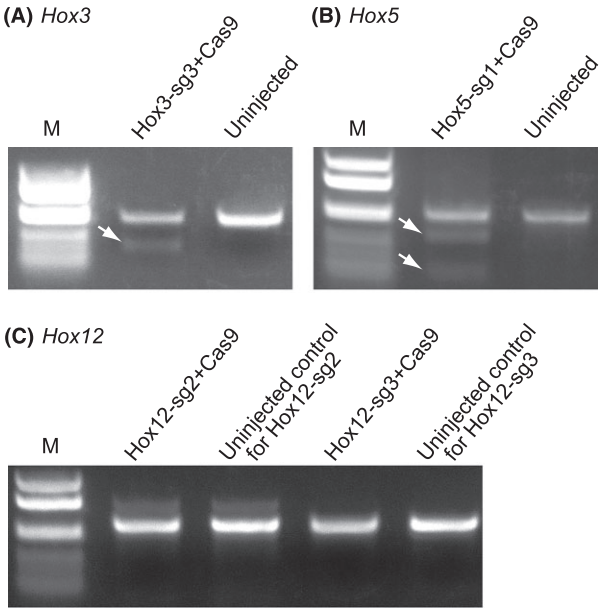

Fig. 1. CRISPR/Cas9-mediated mutations of *Hox* genes in *Ciona intestinalis*. (A) Cel-I assay of polymerase chain reaction (PCR) amplifications that contained the target site of Hox3-sg3. PCR bands were treated with Cel-I prior to electrophoresis. The "Hox3-sg3+Cas9" lane indicates the PCR product derived from larvae into which 3.0 pg of Hox3-sg3 RNA and 15 pg of Cas9 mRNA were microinjected. "Uninjected" lane indicates the PCR product derived from uninjected control larvae. M, marker lane. The arrow indicates the position of Cel-I cleaved band. (B) Cel-I assay of PCR amplifications that contained the target site of Hox5-sg1. 3.0 pg of Hox5-sg1 RNA and 15 pg of Cas9 mRNA were microinjected. (C) Cel-I assays of PCR amplifications that contained the target sites of Hox12-sg2 and Hox12-sg3. 3.0 pg of a sgRNA and 15 pg of Cas9 mRNA were microinjected. In both cases, Cel-I sensitive bands were not detected.

caused by mutations induced by the CRISPR/Cas9 system. Likewise, Cel-I cleaved *Hox5* the PCR product derived from larvae into which 3.0 pg of the sgRNA for *Hox5* (Hox5-sg1) was introduced with 15 pg of Cas9 mRNA (Fig. 1B). The other six sgRNAs designed to target *Hox3* or *Hox12* were not able to yield a Cel-I sensitive band (Fig. 1C), suggesting that appearance of Cel-I sensitive bands are dependent on the sequence of sgRNAs.

To further investigate whether Hox3-sg3 and Hox5-sg1 introduced mutations at their corresponding target sites, we performed sequence analyses of the PCR fragments that showed Cel-I sensitivity. As a result, deletions and/or insertions of nucleotides were seen in the *Hox3* target site derived from larvae into which Hox3-sg3 RNA and Cas9 mRNA were introduced (Fig. 2A). The frequency of the mutated *Hox3* clones among sequenced PCR clones was about 58% when 3.0 pg of Hox3-sg3 and 15 pg of Cas9 mRNA were injected. Likewise, *Hox5* in Hox5-sg1 and Cas9 mRNA-introduced animals had mutations at the target site (Fig. 2B), and the frequency of mutated PCR fragments was approximately 75.9%, when 3.0 pg of Hox5-sg1 and 15 pg of Cas9 mRNA were injected. These results suggest that CRISPR/Cas9 can induce insertion and/or deletion mutations at the target sites in the *Ciona* genome.

Influence of the quantity of CRISPR/Cas9 RNAs on mutation frequency

We next examined different conditions of sgRNA/Cas9 mRNA introduction in order to achieve efficient knockout by CRISPR/Cas9. For this purpose, we compared the mutation frequencies among different amounts of sgRNA and Cas9 mRNA being introduced into embryos by changing their concentration in the injection media. We first investigated this with Hox5-sg1, which showed a higher mutation frequency than Hox3-sg3 in the above experiments. There was a tendency that the more RNAs being introduced, the higher the observed mutation frequency (Table 2). When 0.3 pg of Hox5-sg1 was injected, the introduction of mutations was not detected by the Cel-I assay, suggesting that the mutation frequency was very low in this condition. Likewise, up to 3.0 pg of injected Cas9 mRNA was not able to introduce efficient mutations even though the quantity of injected sgRNA is high. When the amount of injected sgRNA and Cas9 mRNA was, respectively, adjusted to 1.5 and 15 pg, mutations were detected by Cel-I analysis, and sequencing of the PCR bands confirmed the occurrence of mutations at the target site (Table 2). Mutation frequency increased when the quantity of injected Hox5-sg1 was increased from 1.5 to 3.0 pg. However, increasing the quantity of injected Cas9 mRNA from 15 to 30 pg slightly reduced the mutation frequency. A similar tendency was seen when Hox3-sg3 was examined (Table 2). 0.3 pg of Hox3-sg3 or 3.0 pg of Cas9 mRNA did not cause efficient mutations that were detectable by Cel-I assay. We detected Cel-I sensitive bands when 1.5 pg of Hox3-sg3 and 15 pg of Cas9 mRNA were simultaneously microinjected. The difference of the mutation frequencies between 1.5 and 3.0 pg of Hox3-sg3 was not high, and increasing the injected Cas9 mRNA from 15 to 30 pg resulted in a 9% higher mutation frequency. Taking both results from Hox5-sg1 and Hox3-sg3 into consideration, we concluded that microinjection of 3.0 pg of sgRNA and 15 pg of Cas9 mRNA should be the standard condition due to its reproducible and high mutation activity, and there remains a possibility that the mutation frequency could be increased by further increasing the amount of sgRNA and Cas9 mRNA.

(A) *Hox3*

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Wild type   AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTCAACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTC-ACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGT---CAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTC-ACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAG----AACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAG--AACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTCACG-AGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTC-ACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTGC-----GCGAGGAACTAA
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGAGTAAATTCCACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAATCTATCGGCAGCCATAAGAGTCGAGGAACTAGCAAGAGAACAAGGCGAGGAACTAG
            AAACAGAGCCAAGAACCTATCGGCAGCCATAAGAGTCAAACAAGGCGAGGAACTAG
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(B) *Hox5*

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Wild type   AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGG----GGGTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGG-AACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTA----ACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAG-TAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGG-----GTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGG---ATTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGTTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTTTCTTTAAATAAAAAAAAAAAAAAAAAAAAAAAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGGTTAGTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGTTGTATGCGGCGACGACGGGTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGCCGCCGCATAGAGGTAACGGTTGTAATGA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGTCTATGCGGCGACGACGGGTTAACGGTTGTAATGAA
            AAAGTATGGGCGACCTCTATGCGGCGACGACGGGTTAGGTGCTATTTGTAACGGTTGTAATGAA
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Fig. 2. Insertions and/or deletions induced by microinjection of CRISPR/Cas9 RNAs. (A) Examples of mutations induced by simultaneous injection of Hox3-sg3 RNA and Cas9 mRNA. The quantity of injected RNAs was 3.0 pg of Hox3-sg3 RNA and 15 pg of Cas9 mRNA. "Wild type" indicates the un-mutated sequence. The recognition site of Hox3-sg3 was shown in red. Deleted nucleotides were shown by "-". Inserted nucleotides were shown in blue. (B) Examples of mutations induced by simultaneous injection of Hox5-sg1 RNA and Cas9 mRNA. The quantity of injected RNAs was 3.0 pg of Hox5-sg1 RNA and 15 pg of Cas9 mRNA.

Effect of Hox3 *and* Hox5 *knockout on embryogenesis by CRISPR/Cas9*

Expression patterns of *Ci-Hox3* and *Ci-Hox5* at early developmental stages have been described in previous reports (Gionti *et al.* 1998; Locascio *et al.* 1999; Ikuta *et al.* 2004). *Ci-Hox3* starts its expression at the late tailbud stage in the anterior tail epidermis and a part of the central nervous system (CNS). At the larval stage, expression in the CNS is restricted to the motor ganglion. *Ci-Hox5* starts its expression in the trunk lateral cells and tail nerve cord of early tailbud embryos, and the expression pattern persists until the late tailbud stage. At the larval stage, a weak expression of *Ci-Hox5* remains at the nerve cord.

We observed the morphology of larvae injected with Hox3-sg3 or Hox5-sg1 together with Cas9 mRNA. We did not recognize any morphological defect associated with knockout of these genes (Fig. 3A–C). This suggests that these genes may not be essential for forming the tadpole body of *Ciona*, as previous studies have pointed out (Ikuta *et al.* 2010; Yoshida *et al.* 2014).

By utilizing Hox3-sg3 and Hox5-sg1, we investigated the side-effect of expressing sgRNA and Cas9 mRNA

Table 2. Mutation frequencies induced by Crispr/Cas9 system

Name of sgRNA	Quantity of injected RNA		Mutation frequency (in %)	*n*†
	sgRNA (in pg)	Cas9 mRNA (in pg)		
Hox5-sg1	0.3	3.0	Cel-I negative	—
	0.3	15	Cel-I negative	—
	1.5	3.0	Cel-I negative	—
	3.0	3.0	Cel-I negative	—
	1.5	15	40.0	20
	3.0	15	75.9	31
	3.0	30	62.8	35
Hox3-sg3	0.3	15	Cel-I negative	—
	3.0	3.0	Cel-I negative	—
	1.5	15	54.5	22
	3.0	15	58.0	31
	3.0	30	67.7	31

†Number of sequenced polymerase chain reaction (PCR) clones. When the Cel-I assay did not yield cleaved band, sequenceing wan not performed.

on embryogenesis. For this purpose, we introduced 3.0 pg of sgRNAs and 30 pg of Cas9 mRNA, and compared the frequency of normally developed larvae with that of uninjected controls. As a result, the frequencies of larvae with normal morphology were almost identical between uninjected control larvae and larvae injected with sgRNA and Cas9 mRNA (Fig. 3D), suggesting that the toxicity of sgRNA and Cas9 is not significant at the highest quantity of introduced sgRNA and Cas9 mRNA in this study.

Mutagenesis with expression vectors of sgRNA and Cas9

Although RNA-based knockout is a powerful method, this method has some disadvantages over using expression vectors of sgRNA and Cas9. First, RNA-mediated methods require *in vitro* transcription after preparation of DNA constructs. This step can be skipped in the expression vector-mediated method. Second, microinjection is necessary to introduce sgRNA and Cas9 mRNA into eggs and/or embryos in RNA-based method. In *Ciona*, plasmid DNAs can be introduced into hundreds of 1-cell embryos simultaneously by electroporation (Corbo *et al.* 1997). Electroporation is an easy and fast method compared to microinjection. Taking these advantages into consideration, we decided to construct expression vectors of sgRNA and Cas9. For Cas9, conventional expression vectors for reporter genes can be applied because Cas9 is a protein-coding gene. In this study we used a promoter of the ubiquitously expressed gene *Ci-EF1α* (Fig. 4A; Sasakura *et al.* 2010; Treen *et al.*

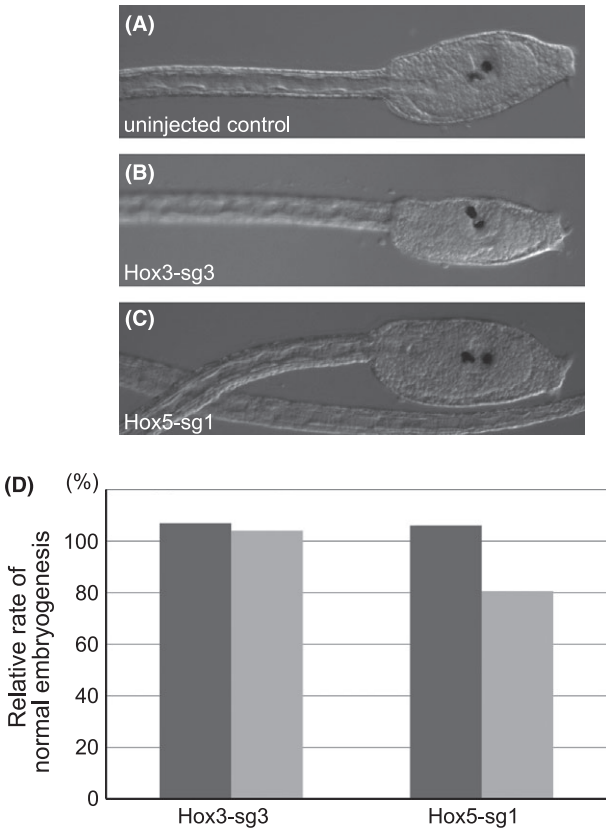

Fig. 3. CRISPR/Cas9 system does not have a strong side-effect on embryogenesis. (A–C) Morphology of larvae. (A) An un-treated control larva, lateral view. Anterior is toward right. (B) A larvae into which 3.0 pg of Hox3-sg3 and 30 pg of Cas9 mRNA was microinjected. (C) A larvae into which 3.0 pg of Hox5-sg1 and 30 pg of Cas9 mRNA was microinjected. (D) Relative rate of normally developed larvae. In each experiment, the % of normally developed larvae in the population injected with 3.0 pg of sgRNA and 30 pg of Cas9 mRNA was normalized with the score of the uninjected control populations. Therefore, "100%" indicates the rates of normal larvae were identical between two populations. ■, Experiment 1; ■, Experiment 2.

2014). By contrast, sgRNA requires a special promoter because sgRNA functions as a short RNA. For expressing sgRNA, we used U6 promoter of *Ciona intestinalis* that was reported in a previous study for expressing short palindromic RNAs (Fig. 4A; Nishiyama & Fujiwara 2008). We electroporated an sgRNA-expression vector and *EF1α*-driven Cas9 vector into 1-cell embryos. After the embryos were developed to the larval stage, genomic DNA was extracted in bulk and the occurrence of mutations were examined by Cel-I assay and sequencing analyses of the PCR products (Fig. 4B). We found that both *Ci-Hox3* and *Ci-Hox5* were mutated by, respectively, introducing Hox3-sg3 and Hox5-sg1 expression vector with Cas9 vector (Fig. 4C,D), although we were unable to

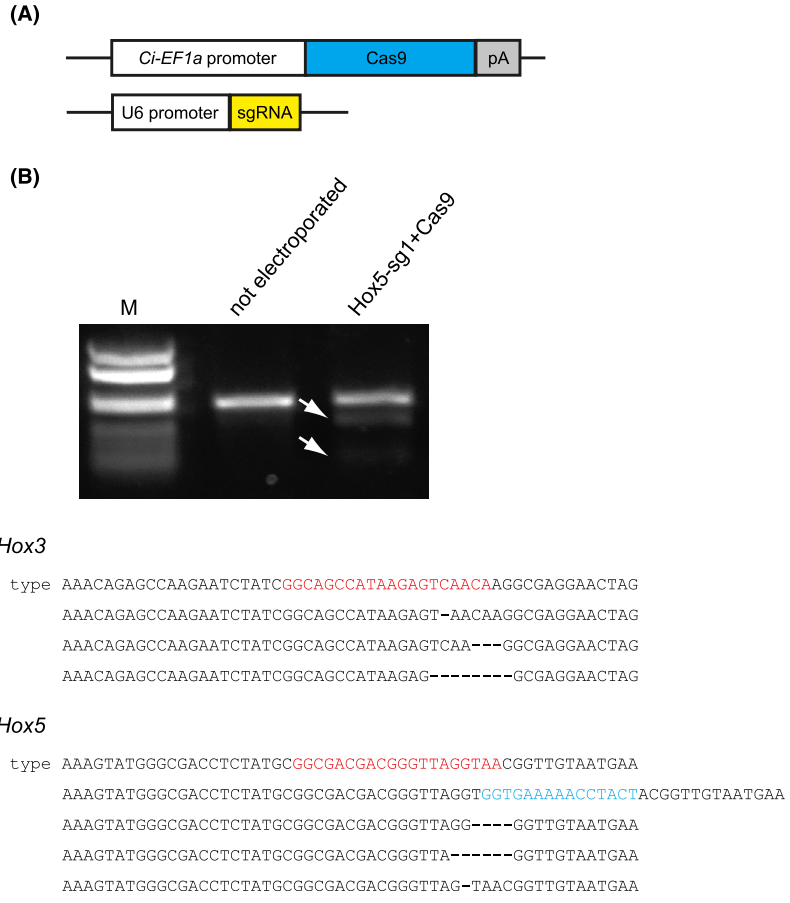

Fig. 4. Mutations induced by electroporation of expression vectors of Cas9 and sgRNA. (A) Schematic illustrations of expression vectors for Cas9 and sgRNA. pA, poly adenylation sequence. (B) Cel-I assay of polymerase chain reaction (PCR) amplifications that contained the target site of Hox5-sg1. "Not electroporated" lane indicates the PCR band from untreated control larvae. "Hox5-sg1+Cas9" lane indicates the PCR band from larvae electroporated with pSPCiEF1α>Cas9 and pSPU6>Hox5-sg1 vectors. Cel-I cleaved bands were shown by arrows. M, marker lane. (C) Examples of mutations induced by expression vectors of Cas9 and Hox3-sg3. "Wild type" indicates the un-mutated sequence. The recognition site of Hox3-sg3 was shown in red. Deleted nucleotides were shown by "–". (D) Examples of mutations induced by expression vectors of Cas9 and Hox5-sg1. Inserted nucleotides were shown in blue.

Table 3. Mutation frequency by electroporating expression vectors of sgRNA and Cas9†

Target gene	Name of sgRNA	Sequence of the recognition site in DNA‡	Mutation frequency (in %)	*n*§
Ci-Hox3	wt Hox3-sg3¶	GGCAGCCATAAGAGTCAACA	36.3	22
Ci-Hox5	wt Hox5-sg1	GGCGACGACGGGTTAGGTAA	46.2	26
	Hox5-sg1/-G	GCGACGACGGGTTAGGTAA	56.3	16
	Hox5-sg1/-GG	CGACGACGGGTTAGGTAA	0	15
	Hox5-sg1/+A	AGGCGACGACGGGTTAGGTAA	10.7	28
	Hox5-sg1/+TA	TAGGCGACGACGGGTTAGGTAA	3.1	32
	Hox5-sg1/+ATC	ATCGGCGACGACGGGTTAGGTAA	0	29
	Hox5-sg1/+ATCA	ATCAGGCGACGACGGGTTAGGTAA	0	14

†Twenty micrograms of sgRNA and 40 μg of Cas9 expression vectors were simultaneously electroporated. ‡Nucleotides added at the junction between U6 promoter were shown in red. §Number of sequenced polymerase chain reaction (PCR) clones. ¶wild type (wt) indicating the standard 20-nt recognition sites of the sgRNAs.

obtain a Cel-I sensitive band for Hox3-sg3. The mutation frequencies were 36.3% and 46.2% for *Hox3* and *Hox5*, respectively (Table 3).

While we have much experience for expressing protein-coding genes from *cis* regulatory elements, we have limited experience expressing small RNAs from a

U6 promoter. For this reason, we modified the junction sequence between U6 promoter and the DNA encoding sgRNA to see which variation could be acceptable. First, we deleted GG dinucleotides at the 5′ end of the sgRNA coding region, (this was previously required for constructing the T7 promoter in pDR274 vector, see the materials and methods section; Hwang *et al.* 2013). When one G was deleted, the modification did not decrease the mutation frequency (Table 3). However, when GG dinucleotides were deleted a significant reduction of mutation frequency was observed (Table 3). Next, we added a few nucleotides at the junction between U6 promoter and sgRNA coding region. The addition of even one nucleotide strongly impaired mutation frequency. Our conclusion is that (i) one G can be omitted from the 5′ end of sgRNA in the expression vector system, and (ii) additional nucleotides should not be added at the junction region, when constructing sgRNA expression vectors.

Target-specificity of CRISPR/Cas9 in Ciona

Several reports in other organisms have suggested that CRISPR/Cas9 shows lesser target specificity than other genome editing methods such as TALENs (Fu *et al.* 2013; Shen *et al.* 2014). We addressed specificity of target recognition by CRISPR/Cas9 in *Ciona* to see how effective CRISPR/Cas9 is in this organism. We used two strategies to approach this issue. First, we searched for the similar sequences to Hox3-sg3 and Hox5-sg1 on-target sites in the *Ciona* genome. These sites could be potential off-target sites. We found three sites that contained four-base mismatches to the target sequence of Hox3-sg3. We examined whether mutations could be introduced in these potential off-target sites by introduction of Hox3-sg3 and Cas9 mRNAs by means of microinjection. As a result, we did not detect any mutation in two putative off-target sites (Table 4), suggesting that a 4-bp mismatch is different from the on-target site enough for avoiding off-target mutations. We could not examine Hox5-sg1 because we were not able to find an off-target site of Hox5-sg1, which contains 4 bp or less mismatches.

Table 4. Mutation frequency at the putative off-target sites of Hox3-sg3†

Name of sites	Sequence of the target site in DNA‡	Mutation frequency (in %)	*n*§
On-target	GGCAGCCATAAGAGTCAACA	58.0¶	31
Off-target 1	GGCAcCCATAtcAGTCAACt	0	16
Off-target 2	GaCAaCCATAAGAGaCcACA	0	16
Off-target 3	tcCAGCCATAAGAGTCtAtA	Not examined	

†3.0 pg of sgRNA and 15 pg of Cas9 mRNA were microinjected. ‡Mismatched nucleotides are shown in lower case. §Number of sequenced clones. ¶The score is the same as that shown in Table 2.

In another approach, we created a series of Hox5-sg1 that contained single base mismatches at the recognition site, and examined their mutation capacity to the on-target site by PCR and sequencing analyses. We introduced these mismatched Hox5-sg1 series into *Ciona* embryos by microinjection of RNAs with Cas9 mRNA. As a result, these mismatched sgRNAs showed great reduction of the mutation frequency (Table 5). Two out of six mismatched sgRNAs introduced mutations at the on-target sites; however, the frequency was more than ten times lower than that of wild type Hox5-sg1. We performed a similar experiment with Hox3-sg3 to see whether this tendency is also true of other sgRNAs. In the case of Hox3-sg3, we used expression vector-based knockout. As shown in Table 5, we detected introduction of mutations with only one of the five mismatched Hox3-sg3, and the observed mutation frequency was about 40% of that of wild type Hox3-sg3. These results suggest that the recognition of the target site by sgRNAs exhibit high specificity in *Ciona* that could be significantly affected by even a single nucleotide difference, particularly when microinjections of sgRNA and Cas9 are performed.

Discussion

In this study, we demonstrated that CRISPR/Cas9 system could introduce mutations into endogenous genes in the *Ciona* genome. This suggests that CRISPR/Cas9 can be another powerful tool for investigating gene functions in this organism together with TALENs (Treen *et al.* 2014; Yoshida *et al.* 2014). In the following paragraphs we discuss the strengths and weaknesses of CRISPR/Cas9 in *Ciona*.

In this study we succeeded in the disruption of *Hox3* and *Hox5* with CRISPR/Cas9. For *Hox3*, we tested two pairs of TALENs but they failed to introduce mutations. This suggests that CRISPR/Cas9 can mutate genes that TALENs cannot, as suggested in the previous study (Hwang *et al.* 2013). The opposite situation also occurs, CRISPR/Cas9 failed to introduce mutations at the *Hox12* locus, which we successfully mutated with TALENs (Treen *et al.* 2014). This difference between the two methods may be derived from their different mechanisms of mutagenesis. In CRISPR/Cas9, guide RNAs determine target sites through formation of heteroduplex of targeted DNA and sgRNAs,

Table 5. Mutation frequency by sgRNAs that contain single base mismatches at the recognition site

Target gene	Name of sgRNA	Method of introduction	Sequence of the recognition site in RNA†	Mutation frequency (in %)	*n*‡
Ci-Hox5	wt Hox5-sg1§	Microinjection of 3.0 pg of sgRNA and 15 pg of Cas9 mRNA	GGCGACGACGGGUUAGGUAA	75.9¶	31
	Mismatch1		GGCGcCGACGGGUUAGGUAA	3.3	30
	Mismatch2		GGCGACGcCGGGUUAGGUAA	0	31
	Mismatch3		GGCGACGACGuGUUAGGUAA	0	28
	Mismatch4		GGCGACGACGGGUgAGGUAA	6.7	29
	Mismatch5		GGCGACGACGGGUUAGuUAA	0	30
	Mismatch6		GGCGACGACGGGUUAGGUAc	0	31
Ci-Hox3	wt Hox3-sg3§	Electroporation of 20 μg of sgRNA and 40 μg of Cas9 expression vectors	GGCAGCCAUAAGAGUCAACA	36.3¶	22
	Mismatch1		GGCAGCCcUAAGAGUCAACA	0	23
	Mismatch2		GGCAGCCAUAcGAGUCAACA	13.6	22
	Mismatch3		GGCAGCCAUAAGAuUCAACA	0	24
	Mismatch4		GGCAGCCAUAAGAGUCcACA	0	24
	Mismatch5		GGCAGCCAUAAGAGUCAACc	0	24

†Mismatched nucleotides are shown in lower case. ‡Number of sequenced polymerase chain reaction (PCR) clones. §wild type (wt) indicating perfect matching sgRNA. ¶The scores are the same as those shown in Tables 2 and 3.

and then Cas9 cleaves the sites by a RNA-mediated mechanism (Wiedenheft *et al.* 2012). By contrast, TALENs recognize their target sites by forming protein-DNA complexes, and they cleave the target sites by dimerization of the nuclease domains from a restriction enzyme *Fok*I (Christian *et al.* 2010). With this difference, the genomic loci that are resistant to one method could be sensitive to the other. Using CRISPR/Cas9 and TALENs to compensate for the weakness of each other, we will be able to knockout more genes than using a single method.

While CRISPR/Cas9 can be used as a substitute for TALENs in *Ciona*, our study has shown that designing sgRNAs that have high mutational activity is quite low compared to the cases reported in *Xenopus* and zebrafish (25% in this study versus >80% in *Xenopus* and zebrafish; Hwang *et al.* 2013; Guo *et al.* 2014). Therefore, we need extensive screening of sgRNA to destroy one genetic locus in *Ciona*. In the case of TALENs, our rough estimation of constructing a good TALEN pair exceeds 85%. Because of this disadvantage in CRISPR/Cas9, we currently use TALENs as the main tool for the knockout of *Ciona* genes. If several TALEN pairs fail to introduce mutations with good efficiency, then we consider CRISPR/Cas9. In future studies, the sequence information of good sgRNAs in *Ciona* will accumulate. By comparing their sequences we could deduce the rules to consistently design good sgRNAs for *Ciona*.

The recognition of the target sites by sgRNAs show high specificity in *Ciona* that could usually distinguish even a single nucleotide difference. This is in contrast to the reports in some other organisms like mammalian cell cultures and *Xenopus* (Fu *et al.* 2013; Guo *et al.* 2014). In *Xenopus*, mismatches at the core region of sgRNA named "seed" sequence significantly influenced the mutation frequency, but the other regions are basically resistant to single nucleotide mismatches. One possible explanation for the high specificity of CRISPR/Cas9 in *Ciona* is that the complex of sgRNA and Cas9 protein formed on the genome DNA may be less stable than those formed in other organisms, and is therefore less tolerant of individual mismatches. The high specificity of the target recognition is advantageous for analyzing gene functions, because this feature may reduce the occurrence of off-target effects, as we have shown that high quantities of sgRNA and Cas9 mRNA introduction do not have strong side effects on embryogenesis. The low off-target effect was also reported in zebrafish (Hruscha *et al.* 2013), suggesting that off-target effects of CRISPR/Cas9 systems are highly dependent on the conditions that differ among organisms.

Like other organisms (Feng *et al.* 2013; Kondo & Ueda 2013), sgRNAs as well as Cas9 mRNA can be supplied from expression vectors in *Ciona*. There are two applications of this method. One is examining the effectiveness of newly designed sgRNAs. As we mentioned above, the probability of designing an sgRNA with good mutational activity is not high in *Ciona*. Therefore, extensive screening of sgRNAs is necessary to mutate a gene. An electroporation-mediated method is an easier and faster way compared to microinjection of mRNA, and therefore this method is more suitable for screening of sgRNAs. The other application is thorough analysis of gene functions in the G0 generation, as we demonstrated with TALENs (Treen *et al.* 2014). In TALENs, we established the

method of tissue-specific knockout of genes by expressing TALENs in tissue-specific cell lineages. We will be able to apply CRISPR/Cas9 to perform conditional gene knockouts by inducing expression of Cas9 with tissue-specific promoters.

In the present study, we have shown that the *Ciona* genome can be mutated by the CRISPR/Cas9 system. Although we were unable to address whether the mutations introduced by the system can be reflected by phenotypes, we think this is highly probable, based on the mutation frequency done by TALENs (Treen *et al*. 2014). Therefore, CRISPR/Cas9-mediated mutagenesis will be another powerful approach in addition to TALENs to investigate functions of genes in *Ciona intestinalis*, the excellent model for elucidating molecular mechanisms underlying formation of the chordate body plan and its evolution.

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Author contributions

Y.S. designed research. H.S., K.Y., A.H. and Y.S. performed research. H.S., K.Y. and Y.S. analyzed data. Y.S. wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Primer list for construction and genomic analyses.