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PROKARYOTIC ENZYME SYSTEM CRISPR/CAS9 IN GENOME EDITING OF *CIONA INTESTINALIS*

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Abstract: Excluding genes with CRISPR (clustered regularly interspaced short palindromic repeats) / Cas9 is a new approach to studying the functions of genes in different organisms.

Cas9 enzyme is isolated from the prokaryotic organisms and is used as molecular scissors for cutting of specific regions of DNA. Cas9 is a bacterial RNA-guided endonuclease that uses base pairing to recognize and cleave target DNAs with complementarity to the guide RNA. The programmable sequence specificity of Cas9 has been harnessed for genome editing and gene expression control in many organisms.

The CRISPR method consist of a highly effective set of molecular "scissors" - can be easy to use but is not perfect. These "scissors" can cut more than necessary by cutting DNA into unexpected and unwanted sites. In early experiments, scientists have found that side effects can occur at some sites in DNA at approximately the same frequency as the target areas. This method includes three major components: the Cas9 enzyme that cuts a portion of the DNA; single-stranded RNA sequence (a series of nucleotides in its molecule) that gives Cas9 the exact place to cut; a new template DNA to repair the cut ("repair" is a DNA double strand recovery). Using CRISPR/Cas9, double-strand fractures are induced in the genome of *Ciona intestinalis.* CRISPR/Cas9 can mutate endogenous *Ciona intestinalis* genes, a great model for clarifying molecular mechanisms for building a horizontal plan of the body. CRISPR/Cas9 is sufficiently effective and specific to generate a large number of embryos carrying mutations in the target gene of interest, which allows rapid screening of the gene in *Ciona intestinalis.*

INTRODUCTION

Many S*treptococcus pyogenes* strains harbor CRISPR-Cas loci that encode for the RNA-guided nuclease Cas9. This nuclease is widely used to introduce genetic modifications in a variety of cells and organisms, from bacteria (Jiang, *et al.,*2013) and yeast (DiCarlo, *et al.,* 2013) to monkeys (Niu, *et al.,* 2014) and human cell lines (Cong, *et al.,* 2013). The high efficiency and simplicity of the Cas9 genome editing technique has accelerated the possibilities of human gene therapy. The nuclease Cas9 is a central player of the adaptive immunity that is provided by clustered regularly interspaced short palindromic repeats (CRISPR) loci (Marraffini, 2016). These loci consist of short repetitive sequences (30-40 bp) that are intercalated by equally short sequences of viral (bacteriophage) and plasmid origin DNA called "spacers." The presence of a spacer sequence that matches the genome of a bacteriophage or conjugative plasmid prevents the host from becoming infected by these genetic invaders.

Recent advances use the CRISPR / Cas system (Clusters for introduced short palindrome repeats / CRISPR related proteins) for purposeful genome editing (Ran et al., 2013). This prokaryotic immune system functions through a short RNA that directs the CAS enzyme to foreign DNA (Barrangou et al., 2007, Brouns et al., 2008). Cas9 (CRISPR associated protein 9) is an RNA-guided DNA endonuclease enzyme associated with the CRISPR adaptive immunity system in Streptococcus pyogenes.

Modified variants of this system have been used for genomic editing applications in various organisms (Cong *et al.,* 2013, Dickinson *et al.,* 2013). Ascidian of the genus *Ciona* (Cionidae, Tunicata, Chordata) are model organisms for the development of the thoracic structure of the string (Satoh, 2014). These are the lower horde animals. The entire genome of *Ciona intestinalis* was found that despite the small size, less than 1/20 of the human genome, it contained an almost complete set of genes characteristic of vertebrates. Hundreds of synchronized *Ciona* embryos can be simultaneously electroporated with plasmid DNA for high-yield transgenesis (Corbo *et al.,* 1997).

This technique is used in experiments to acquire functions for the re-expression of genes encoding protein (Stolfi and Christiaen, 2012). Target mutagenesis in *Ciona* is performed using effectors similar to transcriptional activators TALEs. TALEN (Transcription activator-like effector nucleases) has high knockout efficiency in both somatic and germ cells of *Ciona*) and zinc finger nucleases (Kawai *et al.,* 2012; Treen et *at. al.,* 2014). The use of CRISPR/Cas9 to induce changes (DSBs) in the genome of *C. intestinalis* is described here.

MATERIALS AND METHODS

Mutagenesis with expression vectors of sg RNA (single guide RNA) ***and Cas9:***

Although RNA-based knockout (removal of genes) is a potent method, this method has some drawbacks for use with the expression vectors of sg RNA (single guide RNA) and Cas9. First, mediated-RNA, the method requires in vitro transcription after the preparation of DNA constructs. This step may be omitted in the method mediated by an expression vector. Secondly, with microinjection, it is necessary to introduce sg RNA and Cas9 mRNA (generated by in vitro transcription that is coated and poly A-packaged). This mRNA should be used together with purified lead RNA in eggs and / or embryos in an RNA-based method. *Ciona* may be plasmid DNA introduced into hundreds of unicellular embryos simultaneously by electroporation (Corbo *et al.,* 1997).

Electroporation is an easy and fast method compared to microinjection. These advantages make it possible to construct expression vectors of sg RNA and Cas9. Sg RNA requires a special promoter because sg RNA functions as small RNA. Small RNAs are short (approximately 18 to 30 nucleotides), non-coding RNA molecules that can regulate gene expression in both the cytoplasm and the nucleus via post-transcriptional gene silencing (PTGS), chromatin-dependent gene silencing (CDGS) or RNA activation (RNAa) (Zhang, 2009). For sg RNA expression, the U6 promoter of *Ciona intestinalis* was used. Once the embryos to the larval stage have been developed, genomic DNA is extracted in total and the mutations are analyzed by Cel-I analysis and sequencing of the resulted PCR products (reference method, based on the dideoxy-ribonucleotide (ddNTP) property to disrupt the DNA synthesis at the site of inclusion).

Analysis for splitting detection

Analysis for splitting detection was performed with a lysate PCR using the AmpliTaq supplied with the kit, except the assays selected for MACS cells using Pfx (platinum polymerase / Invitrogen). For AmpliTaq, the kit protocol was used with a 60°C cooling temperature. For Pfx, 30 cycles were used, 60°C annealing temperature and 68°C expansion. PCR products were purified using a QIAquick PCR purification kit (Qiagen) or a nucleoside gel purification kit (Macherey-Nagel). Denaturing, cleansing, and incubating with the detection enzyme as described in the cleansing kit. (Guschin *et al.,* 2010). Visualization was carried out with INVITROGEN SYBR Green on a 2% agarose gel at 70 V for 30 minutes.

RESULTS AND DISCUSSION

Optimize CRISPR/Cas9 components for Ciona

In order to test the Cas9 expression in *Ciona*, a Cas9-enzyme flanked by two nuclear localizing signals (nls) and a C-terminal eGFP (enhanced green fluorescent protein) tag (Chen *et. al.,* 2013, **Figure 1A**), which are highly expressed and

localized to core (**Figure 1B**). The U6 promoter (Nishiyama and Fujiwara, 2008) was used to initiate the constitutive expression of RNA polymerase III for the synthesis of single leading short RNAs (sgRNAs) (Mali *et al.,* 2013). A modified sgRNA skeleton ("F + E", **Figure 1C**) was used to circumvent this problem. In fact, by in situ hybridization, it is possible to detect transcription in the F + E spine (**Fig.1D**), but not in the original spine. This suggests that the sgRNA constructs using the original skeleton may not be effective in *Ciona* embryos

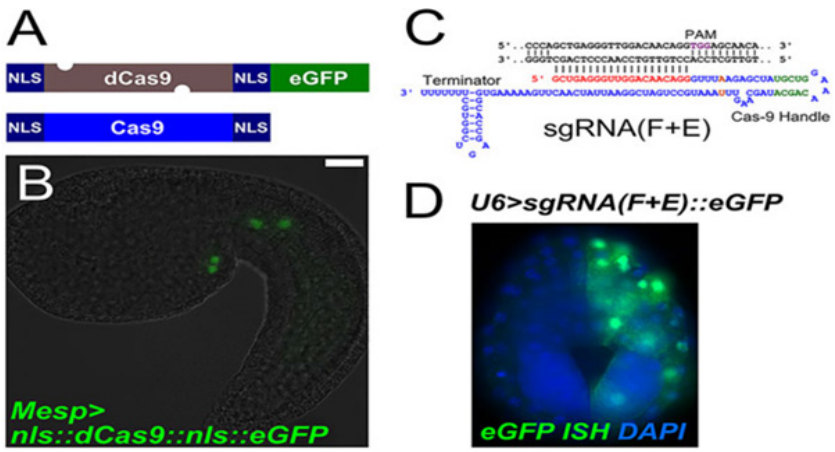

Fig. 1. CRISPR/Cas9 components in *Ciona intestinalis*

CRISPR/Cas9-induced Double Strand Breaks in Ciona Ebf-genes

Collier / Olf / EBF genes are transcriptional regulators of cell fate and differentiation in various tissues (Crozatier and Vincent, 1999). CRISPR/Cas9 was used to induce exon 9 mutations of the EBb gene (formerly known as COE, the transcription factors), the only homologue in the mammalian gene Ciona EBF1 / 2/3 / 4. This gene plays an important role in the specification of nerve motor ganglia and precursors of pharyngeal muscles (Razy-Krajka *et al.,* 2014). Ebf exon 9 encodes a portion of the IPT domain (immunoglobulin-like, plexin, transcription factor) located between the DNA-binding and helix-loop-helix (HLH) domains (**Figure 2A**).The sgRNA vectors directed to exon 9 (Ebf774 and Ebf813) and vector nls :: Cas9 :: nls expression were designed using the propagated Eef1a (EF1α) promoter (Sasakura *et al.,* 2010).

Fertilized eggs (n> 100) were harvested and electroporated with sgRNA / Cas9 plasmids. At 16-hour post-perfusion (hpf), genomic DNA derived from embryo-derived enzymes (FO-generation) was collected. Ebf exon 9 was PCR-amplified (replication) and the resulting TOPO-cloned (cloning vector) products were investigated. When the individual clones are sequenced, three of the seven sequences are listed in point 9 (**Fig. 2B**), suggesting that Cas9 specifically induces DSBs which have then been improperly repaired. Targeting with Cas9 + Ebf.774

sgRNA (not in combination with Ebf813 sgRNA) gives six additional unique Ebf mutations (**Fig.2C**).

Target mutations were generated in the 5' adjacent Fox regions (transcription factors that play an important role in regulating gene expression), suggesting that CRISPR/Cas9 may result in targeted mutagenesis of various loci in the *Ciona* genome. To confirm that CRISPR/Cas9-directed mutagenesis of the Ebf locus results in mutant Ebf transcripts, RNA is isolated by magnetic-activated cell sorting (MACS). Ebf-expressing cells from unified, dissociated embryos was electroporated with Ebf> hCD4 :: mCherry, EF1α> nls :: Cas9 :: nls and U6> Ebf.774. Partial Ebf with DNA fragments were amplified by RT-PCR (real-time RNA detection technique) and TOPO-cloned. Four of the eight consecutive branches have indices in the target sequence.

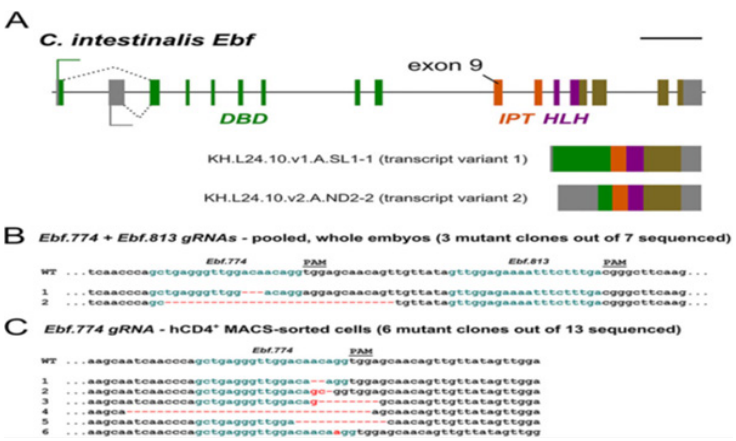

Fig. 2. CRISPR/Cas9-mediated mutagenesis of Ebf.

Cleavage assay of genomic DNA from MACS-enriched transfected cells:

Mutagenesis is effective due to the mosaic structure of electroporated plasmids in *Ciona* embryos (Zeller *et al.,* 2006). Electroporated embryos contain both transfected and nontransfected cells. To obtain more transfected cells, MACS (magnetic-activated cell sorting) is used.

On principle: Embryos dissociated as described (Christiaen *et al.,* 2009d). The OctoMACS Starter Kit (Miltenyi Biotec) is used in accordance with the manufacturer's recommendations, with modifications. Prior to stratification, the dissociated cells were resuspended in 90 µl 0.05% BSA in calcium / no artificial sea water and incubated with 10 µl of anti-hCD4 micronised antibody (Miltenyi Biotec) at 4°C for 1 hour. Afterwards the cells were distributed for pre-filtration in the column, the flow was collected as hCD4 cells. The columns were washed three times with the same buffer and the hCD4 + cells were washed with a liquid from the kit.

Cleavage analysis shows 27.1% efficiency in hCD4 + -conformed cells, while cleavable bands from CD4, more accurate-negative cells are barely visible (**Figure 3B**).

These findings show that transfection mosaicism can mask the efficacy of mutagenesis, a problem that is overcome by MACS selection of transfected cells. To verify that the cleavage efficiency can be improved by increasing the concentrations of plasmid CRISPR/Cas9 and electroporated embryos with 10 µg EF1a> hCD4 :: mCherry, 25 µg EF1a> nls :: Cas9 :: nls and 75 µg U6 > Ebf.774. This results in cleavage with an efficiency of 45.1% in unsorted cells and 66.2% in hCD4 + cells (Figure 3C). Taken together, these data suggest that the distribution and concentration of sgRNA vectors are dependent factors for effective CRISPR/Cas9-mediated mutagenesis in electroporated embryos.

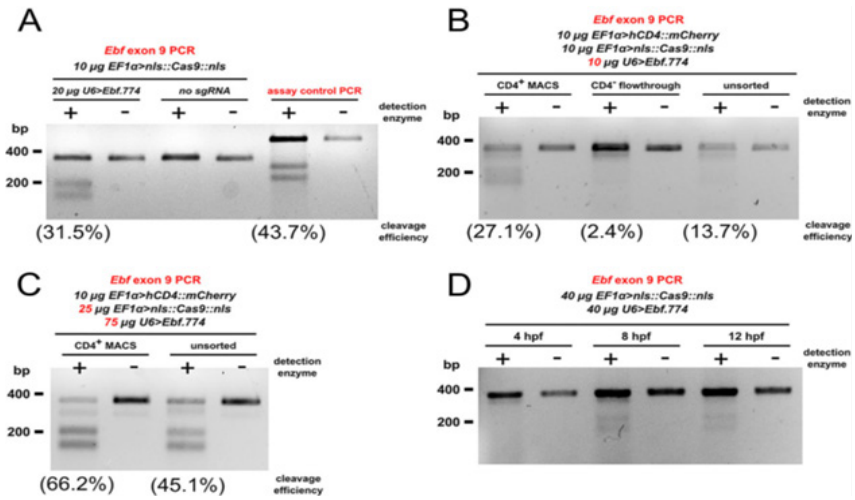

Fig. 3. Genomic cleavage assays.

CONCLUSIONS

Using the CRISRPR / Cas9 system to generate target mutations for Ebf in the specification of Isl + MNs and pharyngeal muscles is demonstrated. We believe that the effectiveness of CRISPR/Cas9, related to the ease of *Ciona* embryo transplantation, promises a quick scaling of genome editing in this string model. In this study it was shown that the CRISPR/Cas9 system could introduce mutations into the endogenous genes in the *Ciona* genome. This suggests that CRISPR/Cas9 may be another powerful tool for testing gene functions in this organism along with TALENs restriction enzymes that can cut specific DNA sequences (Treen *et al.,* 2014, Yoshida *et al.,* 2014). CRISPR/Cas9 may mutate genes that TALEN cannot (Hwang *et al.,* 2013). The reverse situation also occurs, CRISPR/

Cas9 fails to introduce mutations in genes that were successfully mutated with TALENs. CRISPR/Cas9 may be used to perform conditional gene knockouts by inducing expression of Cas9 with tissue specific promoters. The *Ciona* genome can be mutated through the CRISPR/Cas9 system. Therefore, CRISPR/Cas9-mediated mutagenesis is another powerful approach to study the function of the genes in *Ciona intestinalis*, an excellent model for elucidating the molecular mechanisms underlying the formation of the body's plan and its development. In subsequent studies with CRISPR/Cas9 it is possible to use vertebrate genes because *Ciona intestinalis* contains an almost complete set of genes inherent in ruminant animals as well.

CONFLICT OF INTERESTS: The authors declare no conflict of interests. I.A.I. have written the manuscript, M.A. and Y.K. have made the experiments and E.N. discussed and checked the text of the article and the taxonomic status of the Ciona.

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