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Communication

Outcomes comparison of SpCas9- and LbCas12a-mediated DNA editing in zebrafish embryos

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Abstract: CRISPR/Cas genome editing is a widely used research technology. Its simplest variant is gene knockout resulting from reparation errors after introduction of dsDNA breaks by Cas nuclease. We compared the outcomes of the break repair by two commonly used nucleases (SpCas9 and LbCas12a) in zebrafish embryos to reveal if application of one nuclease is advantageous in comparison to the other. To address this question, we injected ribonucleoprotein complexes of nucleases and corresponding guide RNAs in zebrafish zygotes and three days later sequenced the target gene regions. We found that LbCas12a breaks resulted in longer deletions and more rare inserts, in comparison to those generated by SpCas9, while the editing efficiencies of both nucleases were the same. On the other hand, overlapping protospacers were shown to lead to similarities in repair outcome, although they were cut by two different nucleases. Thus, our results indicate that the repair outcome depends both on the nuclease mode of action and on protospacer sequence.

Keywords: Cas9; Cas12a; Cpf1; zebrafish; gene knockout; repair outcome

1. Introduction

Gene editing with CRISPR/Cas systems has now became a hotspot of research because it has provided a simple and versatile tool for genetic studies [1]. Since decades, zebrafish (*Danio rerio*) is known as a convenient model organism, especially useful in developmental genetics [2]. However, it is being rapidly established in other fields of science such as behavioral neuroscience, oncology, and pharmacology. With a range of advantages that zebrafish poses in these fields, the choice of adequate instrument for their genetic manipulation is a question important for more rapid result obtaining [3].

Streptococcus pyogenes Cas9 (SpCas9) was the first nuclease used for gene editing and it remains the most studied one, with a dozen of modifications available for all demands [4]. Cpf1 (or LbCas12a) from *Lachnospiraceae bacterium* is gaining its popularity due to its higher accuracy and higher efficiency in homology directed editing in zebrafish. These nucleases represent two different classes of Cas proteins (II-a and V-a) and have different mechanisms of action [5]. SpCas9 produces blunt-end double-stranded DNA break very close to PAM (3 bp upstream). LbCas12a cleaves the DNA strands asymmetrically and far from PAM (18 bp downstream on the non-target strand and 23 bp upstream on the target strand), producing sticky single-stranded 5′-overhangs. This difference allows making supposition about larger deletions size after LbCas12a editing [6].

To confirm this supposition quantitatively this article aims at comparison of the properties of SpCas9 and LbCas12a in the context of generation of knockout zebrafish for behavioral research. Using Benchling service for crRNA site selection in the first exones of our target genes (*slc6a4a* and

slc6a4b), we have introduced corresponding ribonucleoprotein (RNP) complexes into the zygotes of zebrafish before the first cleavage. After embryo hatching the gene regions adjacent to the target sites were sequenced. Obtained results of gene editing were characterized for each crRNA and each nuclease that allowed us to make comparisons between them. We have found the efficiencies of both SpCas9 and LbCas12a for knockout introduction are the same. But we found higher frequency of insertions in the embryos edited with SpCas9 and increased length of deletions introduced after LbCas12a double-stranded DNA break.

2. Materials and Methods

SpCas9 was purchased from NEB as EnGen® Spy Cas9 NLS (USA). LbCas12a was extracted and purified accordingly to the previously published protocol with modifications [7]. crRNA and sgRNA were transcribed from the PCR-amplified templates using HiScribe™ T7 or Sp6 High Yield RNA Synthesis Kit (NEB, USA). *In vitro* DNA cleavage assay was performed on PCR-amplified region containing the first exone of *slc6a4a* and *slc6a4b* according to Fedorova et al. [7].

Zebrafish strain AB were maintained in the ZebTEC automated housing system (Tecniplast, Italy) in the Center of the Preclinical and Translational Research of Almazov Centre Institute of Experimental Medicine. *In vitro* fertilization was performed according to the protocol described in the Zebrafish Book [8] with modifications for sperm media from the cryopreservation protocol [9]. Obtained zygotes were microinjected with 2 nL of preformed RNP complexes with IM-300 microinjector (Narishige, Japan) and micromanipulator MK-1 (Singer Instruments, UK) according to the protocol described in [10]. Ribonucleoprotein complexes included 1,5 μmol/L of nuclease and 3 μmol/L of corresponding crRNA or sgRNA in CutSmart Buffer (NEB, USA). Embryos were raised to three days post fertilization in E3 medium according to "The Zebrafish Book" [8].

Embryos at three days post-fertilization were euthanized by freezing and their DNA was extracted with Proteinase K (Helicon, Russia) according to protocol described in [8] and purified on silica gel (Sigma, USA). Fragments containing the targets sites of the genes were PCR-amplified from 50 ng of genomic DNA with Tersus kit (Evrogen, Russia). PCR products were sequenced by fluorescent dye-terminator sequencing with Brilliant Dye kit (Nimagen, Netherlands) with analysis on ABI 3500 Genetic Analyser (Thermo, USA). The resulting sequences were analyzed with TIDE (Tracking of Indels by DEcomposition) online service [11] and for every target sequence was determined average efficiency, insertions and deletions ratios, indels ratio larger than 10 bp, and ratio of frameshift-free mutations. Average deletion length was calculated for each embryo as a weighted average of all deletions lengths. Statistical comparison was performed with Kruskal-Wallis test with post-hoc Dunn test and false discovery rate correction with R-Studio program package [12].

3. Results

3.1. crRNA and sgRNA selection and in vitro efficiency assessment

We selected the target sites in the first exones of genes *slc6a4a* and *slc6a4b* with the commonly used Benchling service (https://benchling.com/), focusing on the highest efficiency and specificity scores provided by the service [13, 14]. The list of the target sites is provided in Supplementary Table 1. For LbCas12a on-target score was not available, so we performed *in vitro* activity assessment for the selected and synthesized crRNA. We incubated preformed RNP complexes with the PCR products of the first exones of target genes and found that all of the selected crRNA variants were highly active and cut more than 90% of PCR product, except for slc6a4b-crRNA1 (Figure 1a). Besides, we synthesized marker crRNA and sgRNA to the first exone of melanocyte differentiation antigen *slc25a2* that was shown to be active in zebrafish in the work of [15] and can provide good visual control of nuclease ability to perform gene knockouts (Figure 2b). Three days after RNP complexes were injected in the zygotes of zebrafish, we obtained embryos with total or mosaic pigmentation loss. We have included this control in our subsequent experiments to verify proper nuclease and RNA storage (with conservation of their activity) and viability of the embryos in the clutch sufficient to tolerate RNP complexes microinjections.

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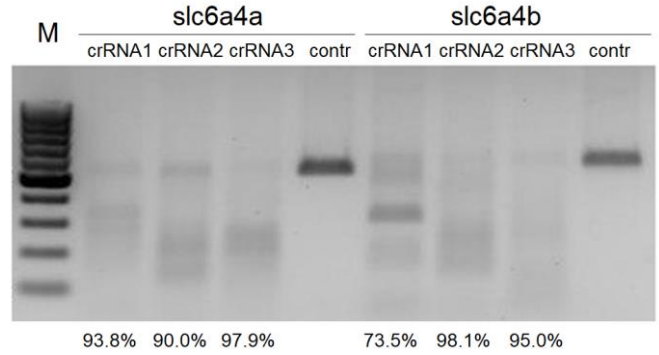
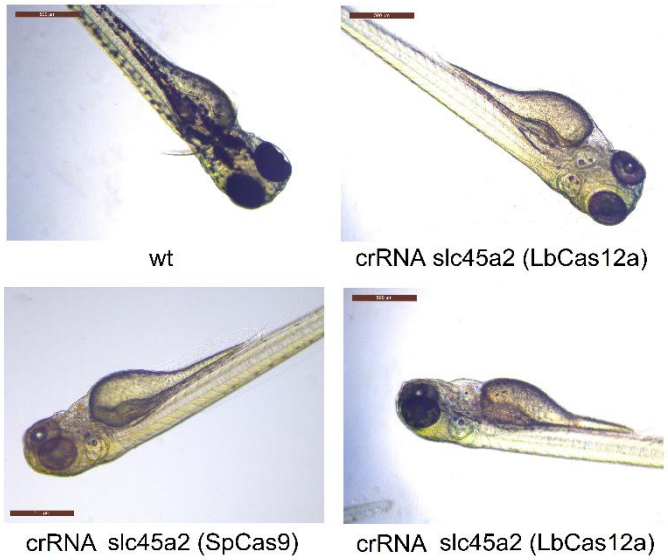


(**a**)



(**b**)

Figure 1. Activity assessment of crRNAs: (**a**) *in vitro* activity assessment of ribonucleoprotein complexes of LbCas12a with corresponding crRNAs: percentage of PCR-products that were cut by RNP complexes is given in the row under the picture; (**b**) *in vivo* visual assessment of RNP complexes activities in zebrafish embryos by melanocyte-dependent pigmentation loss.

3.2. Comparison of the resulting mutants

Selected crRNA and sgRNA were proven to be effective in the *slc6a4a* and *slc6a4b* genes, except for slc6a4b-crRNA2 that did not demonstrate *in vivo* activity in the induction of mutations, though it had rather high *in vitro* activity. Taking into consideration that the control embryos injected in parallel with slc25a2-crRNA ribonucleoprotein complex with the same aliquot of LbCas12a demonstrated obvious phenotypic disturbance in the body coloration, we can attribute this lack of activity to the individual property of this crRNA. That is why we did not take these embryos in the statistical analysis.

Sequencing of 97 mutated embryos revealed their high mosaicism in mutations generated by SpCas9 and LbCas12a. On the other hand, an essential part of these embryos did not show any wild type signal that implied they were mosaic in mutations. Thus, each embryo provided a spectrum of mutations for analysis (meanwhile, only indels less than 50 bp were available for analysis with TIDE service [11]). To characterize the restriction outcome for each type of RNP in particular and for each nuclease in general we have averaged the data about the probability of each mutation type.

We found that SpCas9 cuts result in more frequent insertions ($p = 0.00017$) than with LbCas12a (Figure 2a). Thus, we confirmed the observation that LbCas12a produces longer deletions than SpCas9 (Figures 2b and 2c), increasing both the percentage of deletions larger than 10 bp ($p < 0.00001$) and average length of deletions ($p < 0.00001$). On the other hand, we found that

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slc6a4b-sgRNA4 in complex with SpCas9 produced deletions that did not significantly differ from those produced by LbCas12a ($p = 0.46119$), but differ from the deletions produced by complexes of SpCas9 with other sgRNAs (all $p < 0.05$ with false discovery rate correction). The distinction of this sgRNA was not found for other outcome properties studied in our work that allows attributing this feature to peculiarities of surrounding sequences directing the repair to large deletions.

We found that efficiencies of both types of RNP complexes in mutating of wild type alleles are almost the same (Figure 2d), while LbCas12a produced more outcome variance, presumably depending on the protospacer sequence.

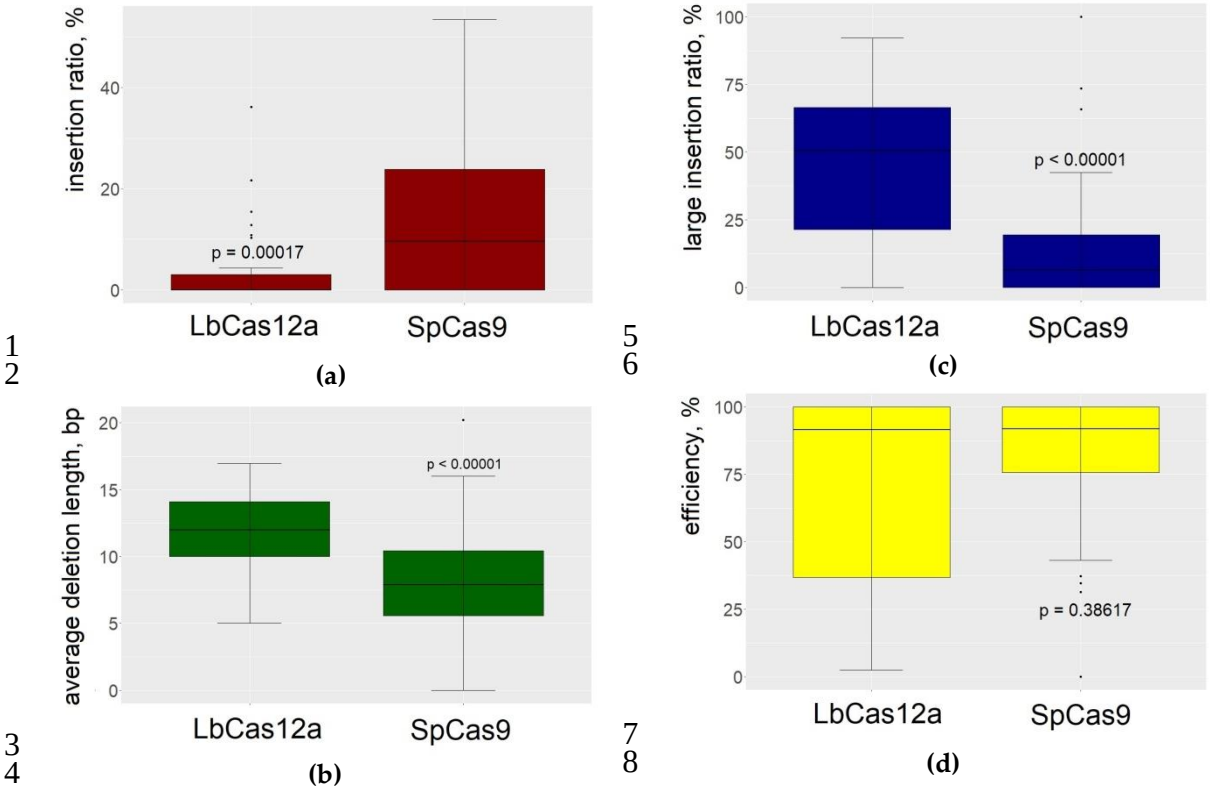

Figure 2. Comparison of LbCas12a and SpCas9 editing properties. a) SpCas9 produces higher percentage of inserts in the outcomes of restriction. b) Average length of deletion is higher in embryos mutated by LbCas12a. c) LbCas12a produces higher percentage of deletions longer than 10 bp. d) Both nucleases possess the same efficiency in editing.

4. Discussion

Here we analyzed the outcomes of SpCas9 and LbCas12a gene editing that can be used for further applications in knockout of zebrafish genes. This strategy is rather common in the research of gene function, so we selected serotonin transporter genes *slc6a4a* and *slc6a4b* regulating serotonergic signaling. Knockouts of these genes have a long history of usage for affective disorders modeling in rodents, valuable for the search of new antidepressants [16, 17].

We have applied TIDE analysis to decompose signal from Sanger sequencing and assess the spectra of indels from each embryo [11]. Low costs represent a serious advantage of the used approach in comparison to next-generation sequencing, currently considered as a gold standard. However, relatively to next-generation sequencing, TIDE underestimates the editing efficiencies and its sensitivity is limited to 2%, while being precise enough for indel sizes determination [18]. TIDE was used to estimate both efficiencies [19, 20] and features [21] of editing outcome.

Many authors have previously reported that LbCas12a produces longer deletions than SpCas9 [6, 22, 23]. This can be explained by the difference in the nuclease cleavage site: while SpCas9 cuts in the region pairing the seed region, LbCas12a cuts far from the seed region that may result in multiple rounds of recognition and subsequent cuts [5]. But lower incidence of insertions for LbCas12a that presumably similarly originates from the cut fashion of this nuclease is not mentioned

in the literature. At the same time, this difference between the two nucleases may be important for the subsequent genotyping assay design for the obtained knockout animals, especially in terms of robustness, because genotyping of experimental animals is often done in non-molecular biology labs and thus should withstand suboptimal conditions.

Our results also support an important role of protospacer sequence in the edit outcome that was already shown for human cell cultures [24]. RNP complex of slc6a4b-sgRNA4 with SpCas9 produced longer deletions than complexes of SpCas9 with other sgRNAs, which were more similar with deletions, produced by LbCas12a. This can be partly explained by the sequence overlap of this sgRNA with slc6a4b-crRNA1 that also leads to large deletions, characteristic for LbCas12a. On the other hand, sequence overlap that was found between two other pairs of protospacers (slc6a4a-crRNA1 with slc6a4a-sgRNA4 and slc6a4b-crRNA3 with slc6a4b-sgRNA6), did not result in the similarities in repair outcome. Further research is needed to distinguish the contribution of protospacer sequence from that of the nuclease cut mode in the repair outcome.

Our results support the importance of preparation of several (at least two) crRNAs for animal gene knockout production. The availability and reliability of activity predictions by online services is rapidly growing, allowing *in silico* crRNA selection. Also, ribonucleoprotein complexes allow assessment of the activity *in vitro* before the embryo injections. But crRNA selected in these preliminary tests may fail to produce knockouts with any significant efficiency *in vivo*. Our work underlines the importance of the control embryos injection with the ribonucleoprotein complex resulting in the obvious body phenotype. We used *slc45a2* (melanocyte differentiation antigen) gene, knockout of which impairs melanocyte-dependent body pigmentation [15]. This control can provide valuable information about the correctness of embryo obtaining and microinjection procedure, especially at the beginning of the facility functioning.

Supplementary Materials:

Table S1. Sequences and scores of target sites used in this work.

Group	Name	Sequence	On-target Score[1]	Off-target Score[2]
LbCas12a	slc6a4a1	TGCTGCTCTCCCCCGTACTC	NA	49.6
	slc6a4a2	AGGAGATGTACTGCGATAAC	NA	49.5
	slc6a4a3	TGATCCTTCTCCGGAACGCT	NA	49.9
	slc6a4b1	GAGGACCCGGGGCACAGAGG	NA	49.7
	slc6a4b2	CTCCATTTATCGCGGGACTC	NA	49.9
	slc6a4b3	TTTTATCGGTCATTGGATTT	NA	48.3
SpCas9	slc6a4a4	ATGATGAATCAAGAGTACGG	78.6	48.5
	slc6a4a5	TGCGGGCACTGGGACGGACA	66.1	48.6
	slc6a4a6	CAGAGTCCTAAATGTTCCAG	75.2	46.6
	slc6a4b4	TTGGAGGACCCGGGGCACAG	67.7	48.5
	slc6a4b5	CGCCGGGTACAACAGCAACC	61.6	49.5
	slc6a4b6	ATTGGATTTGCGGTAGACCT	62.7	48.3

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Abbreviations: RNP – ribonucleoprotein, TIDE - Tracking of Indels by Decomposition, SpCas9 - *Streptococcus pyogenes* Cas9, LbCas12a - *Lachnospiraceae bacterium* Cas12a, PAM – protospacer adjacent motive.

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