Response to Reviewer 1 Comments

This study shows the application of CRISPR-ScCas9, which recognize the NNG PAM, to the zebrafish system by optimizing the previously published one.

High-efficiency genome editing was induced in a target-specific manner using chemically modified guide RNA.

It is a good study showing the application so that ScCas9 can be used as one of the genome editing tools in the future.

I'd like to suggest that this paper would be published in 'Cells' after minor correction.

Point 1: In the Figure 1C, I wonder why the albino-like and few melanin phenotype are not visible in the correction induced by ScCas9 mRNA.

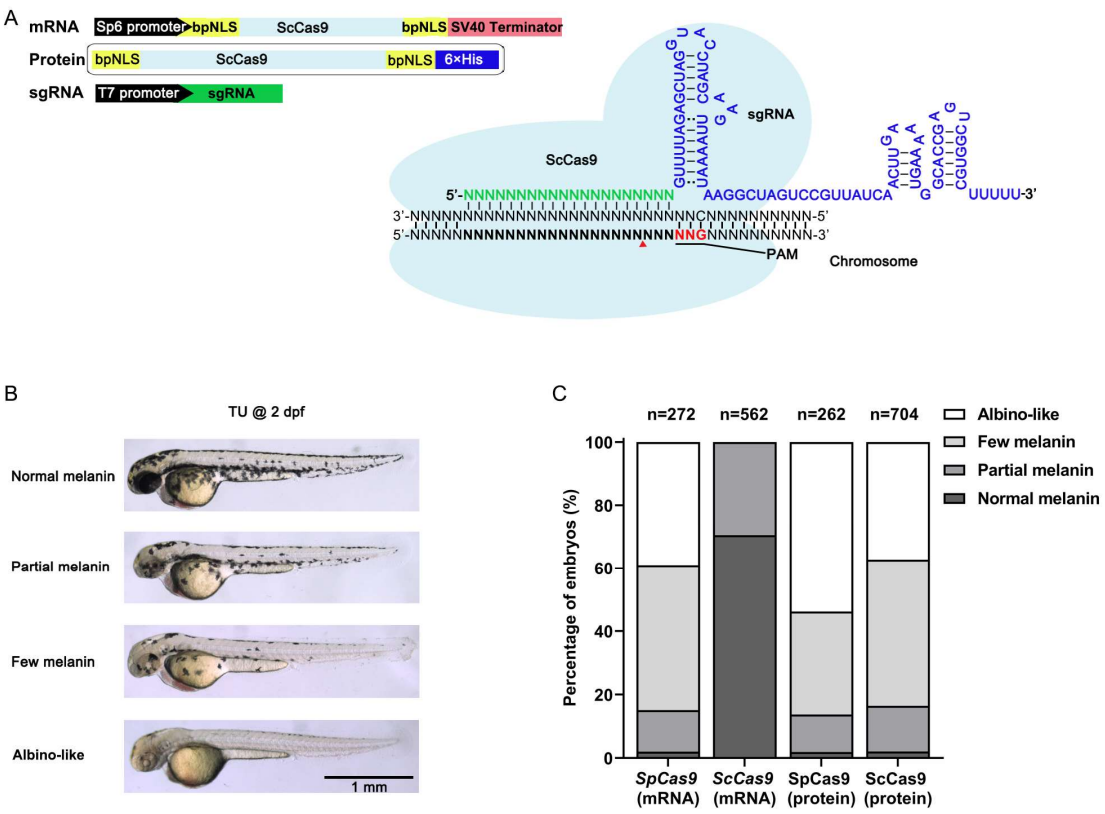
Response 1: Thanks for your questions. The low proportion of albino-like and few melanin phenotype induced by ScCas9 mRNA suggests low editing efficiency of ScCas9 mRNA *in vivo*. Previously study have shown that the chromatin-related factors might have a higher impact on eSpCas9 activity than SpCas9, which should be investigated thoroughly in future studies [1]. As an orthologue of SpCas9, ScCas9 might be affected by its own ability to target heterogeneity chromatin too. Of Couse, that the protein translational speed of ScCas9 mRNA might be an important factor. In our study, we injected the same dose of mRNA (300 pg / embryo) since the equivalent molecular weight of ScCas9 and SpCas9. We found much more malformed embryos without improved editing efficiency when increasing the dose of mRNA injection, which limits the use of ScCas9 on mRNA level. Because early zebrafish fertilized eggs cleavage very fast (cells divide once every 15 min), and SpCas9 protein can increase the indel frequency compared with SpCas9 mRNA in zebrafish (**line 142-144** in the revised manuscript), the form of ScCas9 protein directly supplied a fast mode of activity *in vivo*. Our study emphasized on the efficient gene editing method for ScCas9 in zebrafish but not for the mechanism. In fact, many other Cas9 orthologs and variants (xCas9, CjCas9) worked efficiently in cell line, but had no efficiency in zebrafish even though with RNP delivery (data not shown).

1. Jensen KT, Fløe L, Petersen TS, Huang J, Xu F, Bolund L, Luo Y, Lin L. Chromatin accessibility and guide sequence secondary structure affect CRISPR-Cas9 gene editing efficiency. *FEBS Lett* **2017**, doi: 10.1002/1873-3468.12707.

Point 2: It may confuse readers, so it would be good to show a graph organized by Cas9 expression method and phenotype, respectively.

Response 2: With your valuable advice, we have modified the phenotypic statistics chart to facilitate readers' understanding (Figure 1C).

Figure 1



Point 3: page 5 of 9, line172-173: NAG PAM targeted editing still shows low activity (12.5%, 4.2%). authors didn't do sufficient experiment to check the PAM specificity in zebrafish. So I'd like to suggest to change this sentence.

Response 3: Thanks for your suggestion. The statement has been modified in **line 177-178** in the revised manuscript.

Line 177-178 "These results indicate that ScCas9 recognizes NNG PAM sites and has a preference for NAG PAM in zebrafish."

Point 4: page 7 of 9, line234-235: authors didn't test the suitability of ScCas9 for HDR purpose. So I'd like to suggest to change this sentence.

Response 4: Thanks for your suggestion. The statement has been modified in **line 238-240** in the revised manuscript.

Line 238-240 "ScCas9 substantially increases the editing scope in zebrafish, which might potentially expand the available base-editing tools and facilitate more precise homology-directed re-pair platforms."