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Highly efficient genome editing using oocyte-specific *zcas9* transgenic zebrafish

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Since its first application to induce mutations in mammalian cells (Cong et al., 2013; Mali et al., 2013), CRISPR/Cas9 rapidly becomes a routine technique to perform genome editing in a variety of biological systems due to its facile, robust, and multiplexable features (Hwang et al., 2013; Guo et al., 2014; Wang et al., 2013). In every system, Cas9 and sgRNA should co-express to induce mutations. This is typically achieved by injection of the sgRNA mixed with Cas9 mRNA or protein into the fertilized eggs in zebrafish. As the in vivo translation of capped RNAs delay the presence of sufficient Cas9 protein, the mRNA injection produces more mosaic animals and shows relatively lower efficiency when compared to Cas9 protein injection (Burger et al., 2016). However, it is much more cost effective than using commercially available Cas9 protein, especially in the case of large-scale mutation screens. To further simplify this procedure, lower the cost and maintain an acceptable high genome editing efficiency in zebrafish, we set out to generate a transgenic line with stable and specific Cas9 expression in the egg.

zpc (zp3b) promoter (*zpc0.5*) was reported to drive robust and specific GFP expression in the oocyte (Onichtchouk et al., 2003), we thus fused it with a zebrafish codon optimized *cas9 (zcas9)* followed by an SV40 3' UTR (Fig. 1A and File S1; Liu et al., 2014), and introduced this construct to zebrafish genome by Tol2 mediated transgenesis (Urasaki et al., 2006). To screen female F0 fish harboring this transgene, the F0 female founders were outcrossed with the wild-type male, the resulting eggs were injected with an sgRNA targeting *slc45a2* (Moreno-Mateos et al., 2015), a gene essential for melanin synthesis, and assayed for their pigmentation phenotype. We found that the offspring of one founder showed a high proportion of pigmentation defect. Some of them exhibited a homozygous mutant like

albino phenotype, suggesting a robust Cas9 expression in the egg and a very high efficient gene disruption.

To examine the specificity of *zcas9* expression in the transgenic line, the offspring (F1) of this F0 founder were raised to adulthood, and the ovaries of female F1 transgenic fish were dissected and subjected to in situ hybridization (ISH) employing the probe of *zcas9*. We found that the transcripts of *zcas9* were specifically distributed in oocytes with a diameter of 40-200 μm , at around IB-II stages, while in WT embryos, no hybridization signals were detected (Fig. 1B, C). We did not detect *zcas9* expression in stage IA oocytes or in larger ones after stage II. Accordingly, by RT-PCR, we also failed to find *zcas9* transcripts in the fertilized eggs or early embryos of transgenic fish (Fig. 1D), suggesting that *zcas9* transcripts were only present in certain stages of oogenesis, and that only maternally supplied Cas9 protein remained after fertilization. We also observed an ovary specific *zcas9* expression among adult tissues, while its expression was absent in the brain, eye, gill, liver, tail fin, skin or muscle (Fig. 1D). We also injected the *slc45a2* sgRNA into the fertilized eggs from a cross between transgenic male and wild-type female, and found no pigmentation phenotype (Fig. S1). These results, when put together, suggest that we have successfully generated an oocyte specific *zcas9* transgenic zebrafish.

To establish a progeny with high genome editing efficiency, the embryos from female F1 fish were screened by injecting sgRNA targeting *slc45a2* and *bmp2b* respectively, and then assessed the efficiency by analysing the resulting phenotype. Among the six transgenic F1 females, #3 fish showed a maximum efficiency, such that some of its embryos injected with *slc45a2* sgRNA showed an albino-like phenotype in addition to high proportions of embryos with mosaic pigmentation in the retina (Fig. 1E; Fig. S2A). This high genome editing efficiency was also revealed by the severely dorsalized phenotype after injection of a sgRNA targeting *bmp2b*, a key ventralising signal during dorsoventral patterning (Fig. 1F, G; Fig. S2B-H). So we named this line originated from #3 F1 fish as Tg(*zpc:zcas9*^{high}) and compared its efficiency of genome editing with injections of *cas9* mRNA or protein.

To perform this comparison, the concentration of *slc45a2* and *bmp2b* sgRNAs was fixed to a dose of 100 pg/embryo. They were mixed separately with three different doses of Cas9 Protein (1000, 333 and 111 pg/embryo) or *zcas9* mRNA (200, 100, and 50 pg/embryo). After injection of these mixtures in 1-2 cell stage wild-type embryos, their genome editing efficiency was compared with the transgenic embryos injected with the same sgRNA dose by phenotypic analysis (Shao et al., 2017) and chromatogram quantification using the TIDE method (Etard et al., 2017). The results showed that the

mutant phenotypes induced by either sgRNA in the embryos from the Tg(*zpc:zcas9^{high}*) F1 fish is comparable to or slightly stronger than the wild-type embryos injected with the intermediate dose of either Cas9 protein or the *zcas9* mRNA (Fig. 1H, I). The quantified efficiency of *bmp2b* sgRNA at this circumference was about 38% on average, better than that of 333 pg Cas9 protein and 100 pg *zcas9* mRNA injection (Fig. 1J), both of which showed a 29% of mutation rate. This indicates that the genome editing efficiency of the embryos from a heterozygous Tg(*zpc:zcas9^{high}*) mother is high enough to perform routine knockout experiment. It would be anticipated that the efficiency in its homozygous offspring should be even higher to approach a maximum efficiency.

As Tol2 transgenesis often causes multiple copy insertion (Kawakami et al., 2000), we next performed the copy number profiling in Tg(*zpc:zcas9^{high}*) F1 transgenic fish by qPCR using primers targeting the *zpc0.5* promoter. The copy number of *zpc* fragment in the heterozygous transgenic fish is about 1.5 folds higher than that of the wild-type, suggesting single copy insertion (Fig. 1K). To identify the insertion site and to establish a reliable genotyping method (Supplementary materials and methods, Table S1), we carried out nested inverse PCR using six different restriction enzymes, and finally obtained a specific PCR product in the EcoRI group (Fig. S3). In fact, the *zpc:zcas9* cassette was inserted into the middle of the large third intron of *gfra2a* gene (Fig. 1L; File S2 for detailed sequence), which should not affect the splicing of this intron. In another aspect, the initiation of *gfra2a* expression is very late, at about Prim-15 stage, corresponding to 30 hpf or so (Fig. S4; White et al., 2017), and there is no phenotypic output of the *gfra2a* morpholino (Shepherd et al., 2004). As a result, this insertion, even if it might affect *gfra2a* expression, should not have any effect on embryogenesis, especially during early development. Accordingly, all the offspring from a cross between heterozygous Tg(*zpc:zcas9^{high}*) fish are viable, and no aberrant phenotype was observed (not shown).

In summary, we have generated an oocyte specific *cas9* transgenic line with high genome editing efficiency after fertilization. This line exhibits obvious advantages over Cas9 mRNA or protein injections. In particular, it significantly simplifies the workflow and lowers the cost of conventional gene knockout procedure in zebrafish. To target a gene, only sgRNA is synthesized, while the mRNA and commercially available Cas9 protein are dispensable to this end. As a result, this modification will further increase the capacity of sgRNA dosage, and should be of help for simultaneously targeting multiple genes. In addition, as Cas9 is stably expressed in the oocyte, this line may also provide more stable and reproducible editing efficiency compared to mRNA and protein injections. It is worth to

note that *zcas9* expression in this transgenic line exhibits little leakage and is much “cleaner” than other *cas9* transgenes driven by ubiquitous promoters (Yin et al., 2015). After fertilization, only Cas9 protein remains and the transcription of *zcas9* is silenced in the whole animal during a considerably long time. This makes genome editing in this line almost equivalent to Cas9 protein injection. The construction of this transgenic line also makes it possible to directly target maternal factors, especially those with zygotic lethal or sterile mutant phenotypes, so it would be interesting to further test the efficiency of gene knockout in the oocytes using this transgenic line.

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Fig. 1. Generation of an oocyte specific *cas9* transgenic zebrafish with high genome editing efficiency. **A:** schema showing the structure of the vector to transfer the *zpc:zcas9* cassette to the genome via Tol2 mediated transgenesis. See supplementary Data for its sequence and annotation. **B** and **C:** in situ hybridization to detect *zcas9* mRNA expression in the ovaries of wild-type (**B**) and the transgenic line (**C**). **D:** RT-PCR analysis of *zcas9* expression in the early embryos and different adult tissues of the Tg(*zpc:zcas9*) fish. β -actin is used as a loading control. **E:** representative pigmentation phenotypes at 36 hpf generated by injection of *slc45a2* sgRNA (200 pg) to the embryos from Tg(*zpc:zcas9*^{high}) heterozygous female fish (#3 fish indicated in the text). Albino-like embryos corresponding to those without any pigmentation in the retina and the body. Mosaic II indicates embryos with less than 50%

pigment coverage on the retina, while for Mosaic I, this coverage is 50%-100%. **F**: 12 hpf wild-type embryos injected with 200 pg *bmp2b* sgRNA. All embryos showed a normal development. **G**: 12 hpf injected Tg(*zpc:zCas9^{high}*) embryos. Note the typical long elliptical shape corresponding to severely dorsalized phenotypes. **H** and **I**: stacked columns showing the phenotypic comparison of Cas9 protein and mRNA injections with the transgenic line after editing the *slc45a2* (**H**) and *bmp2b* (**I**) locus. Both sgRNAs were injected at 100 pg/embryo for this experiment. The classification of dorsalized phenotype in **I** is according to Shao et al., 2017, and the representative phenotypes are presented in Fig S2. Numbers on each column indicate embryos counted in each group. **J**: quantification of the editing efficiency of Cas9 protein, mRNA and the Tg(*zpc:zCas9^{high}*) line at the *bmp2b* locus. The edited genomic region in each group was PCR amplified and Sanger sequenced. The chromatograms were subjected to quantification using the online software (<http://iai-gec-server.iai.kit.edu/uploadFiles.php>). The experiment was performed independently at least 3 times. **K**: qPCR analysis of the *zpc* copy number in Tg(*zpc:zcas9^{high}*) heterozygous fish. Three experimental repeats were performed in each group. Data in **J** and **K** are presented as mean $\pm$ standard deviation, * in **K** indicates $P < 0.05$ (paired Student's *t* test). **L**: Diagram showing the inserted site and direction of *zpc:zcas9* cassette in the third intron of *gfra2a*. Note that the length of the cassette is not in the same scale as the genome. See also supplementary data for detailed sequence. Scale bars: 100 μ m in **B** and the left most inset of **E**, 400 μ m in **E** and **F**.