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Original research

Efficient generation of zebrafish maternal-zygotic mutants through transplantation of ectopically induced and Cas9/gRNA targeted primordial germ cells

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ABSTRACT

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology has been widely utilized for knocking out genes involved in various biological processes in zebrafish. Despite this technology is efficient for generating different mutations, one of the main drawbacks is low survival rate during embryogenesis when knocking out some embryonic lethal genes. To overcome this problem, we developed a novel strategy using a combination of CRISPR/Cas9 mediated gene knockout with primordial germ cell (PGC) transplantation (PGCT) to facilitate and speed up the process of zebrafish mutant generation, particularly for embryonic lethal genes. Firstly, we optimized the procedure for CRISPR/Cas9 targeted PGCT by increasing the efficiencies of genome mutation in PGCs and induction of PGC fates in donor embryos for PGCT. Secondly, the optimized CRISPR/Cas9 targeted PGCT was utilized for generation of maternal-zygotic (MZ) mutants of *tcf7l1a* (gene essential for head development), *pou5f3* (gene essential for zygotic genome activation) and *chd* (gene essential for dorsal development) at F_1 generation with relatively high efficiency. Finally, we revealed some novel phenotypes in MZ mutants of *tcf7l1a* and *chd*, as MZ*tcf7l1a* showed elevated neural crest development while MZ*chd* had much severer ventralization than its zygotic counterparts. Therefore, this study presents an efficient and powerful method for generating MZ mutants of embryonic lethal genes in zebrafish. It is also feasible to speed up the genome editing in commercial fishes by utilizing a similar approach by surrogate production of CRISPR/Cas9 targeted germ cells.

1. Introduction

The rapid development and wide-range application of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology substantially revolutionized the genetic studies in various organisms including zebrafish (Bassett et al., 2013; Hwang et al., 2013; Tzur et al., 2013). The zebrafish has been recognized as an excellent vertebrate model organism for studies of vertebrate genetics and development, human diseases and fish physiology (Sun, 2017). The CRISPR/Cas9 mediated knockout has been well established in zebrafish (Chang et al., 2013; Hwang et al., 2013), and its application has led to generation of a large number of genetic-null mutants and unprecedented possibilities for genomic manipulation. However, using CRISPR/Cas9 to knock out essential genes that are involved in early embryogenesis is still a big challenge, because high-efficient knockout of embryonic essential genes may result in embryonic lethality in the F_0 generation. This consequently leads to the failure of germline transmission of null alleles. For instance, induction of mutagenesis of *chd*, a gene essential for the shield formation during gastrulation (Hammerschmidt et al., 1996), using conventional CRISPR/Cas9 technology causes severe ventralization and embryonic lethality (Zhang et al., 2016), which would prevent us from obtaining adult mutants for further germline screening.

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In zebrafish, large amount of RNAs and proteins are maternally deposited in the yolk, referred as maternal factors encoded by maternal genes, which are often essential for early embryonic development (Dosch et al., 2004; Wagner et al., 2004). It is often necessary to obtain maternal-zygotic (MZ) mutant in zebrafish in order to study the function of those maternal factors (Reim and Brand, 2006; Liu et al., 2018; Veil et al., 2018). The first MZ mutant of zebrafish is MZ*oep*, which shows that Oep is essential for germ layer formation, organizer development and the positioning of the anterior–posterior axis (Gritsman et al., 1999). In recent years, a large fraction of genes with maternal expression have been discovered and studied through transcriptomic approaches (Harvey et al., 2013; Lee et al., 2013; Heyn et al., 2015). However, generating MZ mutants is usually a time-consuming and labor-intensive process. It is noteworthy that, generating homozygous mutants using conventional CRISPR/Cas9 technology needs a lot of zebrafish facilities and is also time consuming, since three generations are usually required (Patton and Zon, 2001). For maternal genes, on the premise of survival of homozygous mutants, one more generation of self-cross within them has to be carried out in order to obtain MZ mutants. If homozygous mutation is lethal for early embryos, corresponding mRNAs should be considered to be overexpressed to have a rescue (Burgess et al., 2002). Otherwise, if mRNA rescued homozygous mutants could not survive to adulthood, primordial germ cells (PGCs) of homozygotes could be transferred into germ cell deprived host embryos, in order to obtain MZ mutants (Ciruna et al., 2002). With the advanced application of CRISPR/Cas9 technology in zebrafish, it is possible to directly mutate the genome of PGCs by Cas9/gRNA injection and to transplant the mutated PGCs into host embryos to produce gametes harboring mutations of lethal genes. Recently, certain PGC manipulation technologies, such as PGC-targeted overexpression (Xiong et al., 2013) and ectopic PGC induction (Ye et al., 2019), have been successfully established in zebrafish embryos. Therefore, it is possible to improve the PGC transplantation (PGCT) efficiency in zebrafish by utilizing those PGC manipulation methods.

In this study, we firstly established and optimized the procedure for gRNA targeted PGCT by increasing the mutation efficiency in PGCs and PGCT success rate. We then utilized the optimized procedure for generation of MZ mutants of *tcf7l1a* (gene essential for head development), *pou5f3* (gene essential for zygotic genome activation) and *chd* (gene essential for dorsal development) at F_1 generation with high efficiency. This technology can be applied to the large-scale generation of other embryonic lethal mutants, especially for the gene function analysis of large number of maternal factors.

2. Results

2.1. Mutants of chd and pou5f3 can rarely be obtained by conventional CRISPR/Cas9 technology

We tried to knockout zebrafish genes *chd* and *pou5f3* using conventional CRISPR/Cas9 knockout technology. As expected, after injection of *cas9-UTRsv40* mRNA and *chd* gRNA, about 96.2% (204 against 212) embryos showed typical ventralization phenotype with enlarged blood island and decreased head size (Fig. 1A and B), just mimicking its morphants or the *chordino* mutants (Schulte-Merker et al., 1997), which indicated the effectiveness and high penetrance of *chd* gRNA in zebrafish. We then analyzed the mutant types of the gRNA target sequence of *chd* in the wild-type (WT) like embryos (C1 in Fig. 1A) and the mutant like embryos (C2 in Fig. 1A). To our surprise, all the clones from the mutant like embryos showed indel (insertion or deletion) mutations of the *chd* target, while all the clones from the WT like embryos showed no mutation of the target sequence (Fig. 1C). Therefore, in order to obtain the germline mutant carriers of *chd*, we could only raise the C2 embryos. However, these embryos showed extremely low survival rate during subsequent cultivation (Fig. 1G). On the other hand, as to *pou5f3* gene, 71.7% (180/251) of the *cas9-UTRsv40* mRNA and *pou5f3* gRNA co-injected embryos showed serious developmental defects and 28.3% showed to be WT like at 30 hpf (Fig. 1D and E). Although the C2 embryos showed 100% mutation efficiency by sequencing analysis (Fig. 1F), they could not survive to adulthood. Therefore, we focused on the rest C1 embryos, which showed a mutation efficiency of about 70% (Fig. 1F). Nevertheless, most of these *pou5f3* disrupted embryos could not survive to adulthood (Fig. 1G). Although a previous study showed that injection of *cas9-UTRnanos3* mRNA could decrease the mutation efficiencies in somatic cells and led to less severe deformed embryos (Moreno-Mateos et al., 2015), we utilized a similar strategy and still could not obtain enough knockout founder fish for screening of F_1 mutant fish for *chd* and *pou5f1*, just mimicking the injection of *cas9-UTRsv40* mRNA (Fig. 1B–G). To conclude, the application of conventional CRISPR/Cas9 gene knockout technology has such obstacle and limitation to generate homozygous mutants of embryogenesis-essential (embryonic lethal) genes.

2.2. Optimization of PGC mutagenesis and PGCT

As it was difficult for us to obtain germline transmitters by using the aforementioned conventional method of CIRSPR/Cas9 by co-injection of *cas9-UTRsv40* mRNA and gRNA for embryonic lethal genes, we then tried to utilize the technology of germline replacement by transplanting the mutated PGCs to PGC-ablated embryos, and tried to optimize the efficiencies of mutagenesis in PGCs and PGCT.

Firstly, we compared the mutation efficiency of gRNA target in PGCs and the somatic cells by using a transgenic line *Tg(piwi:egfp-UTRnanos3)*^{ihb327Tg}, which specifically labels the PGCs (Ye et al., 2019). After *cas9-UTRsv40* mRNA and *tcf7l1a* gRNA were co-injected into 1-cell stage *Tg(piwi:egfp-UTRnanos3)*^{ihb327Tg} embryos, green fluorescent protein (GFP)-positive PGCs and GFP-negative somatic cells were sorted by fluorescent-activated cell sorting (FACS) at 2 dpf for further mutation analysis (Fig. 2A). To our surprise, the mutation efficiency of target sequence in PGCs was significantly lower than that in the somatic cells (Fig. 2B), indicating that the genome of germline is somehow more resistant to Cas9/gRNA induced mutagenesis. Then we co-injected *tcf7l1a* gRNA and *cas9-UTRnanos3* mRNA which could stabilize the *Cas9* expression in PGCs by the 3′UTR of *nanos3* (Koprunner et al., 2001). In contrast, PGC-specifically expressed Cas9 could significantly increase the target mutation efficiency in PGCs while decrease the mutation efficiency in somatic cells (Fig. 2C). Therefore, we utilized the *cas9-UTRnanos3* mRNA injected embryos as the donor for PGCT in subsequent study.

In theory, the efficiency of PGCT relies on the PGC number in donor embryos, therefore it is important to increase the PGC number of donor embryos in order to facilitate this method. The *buc* gene, which encodes a germplasm organizer, has been shown to be necessary and sufficient for germplasm formation and PGC induction in zebrafish (Bontems et al., 2009; Krishnakumar et al., 2018). In our recent study, we showed that overexpression of *buc* could significantly induce PGC number and even promote female development in zebrafish (Ye et al., 2019). Therefore, we speculated that whether induction of additional PGCs by injecting *buc-UTRsv40* mRNA into donor embryos could improve success rate of PGCT. As expected, *buc-UTRsv40* mRNA injection could double the PGC number in donor embryos at 3 hpf and significantly increased the PGC number at 35 hpf (Fig. 2D and E). The embryos co-injected with

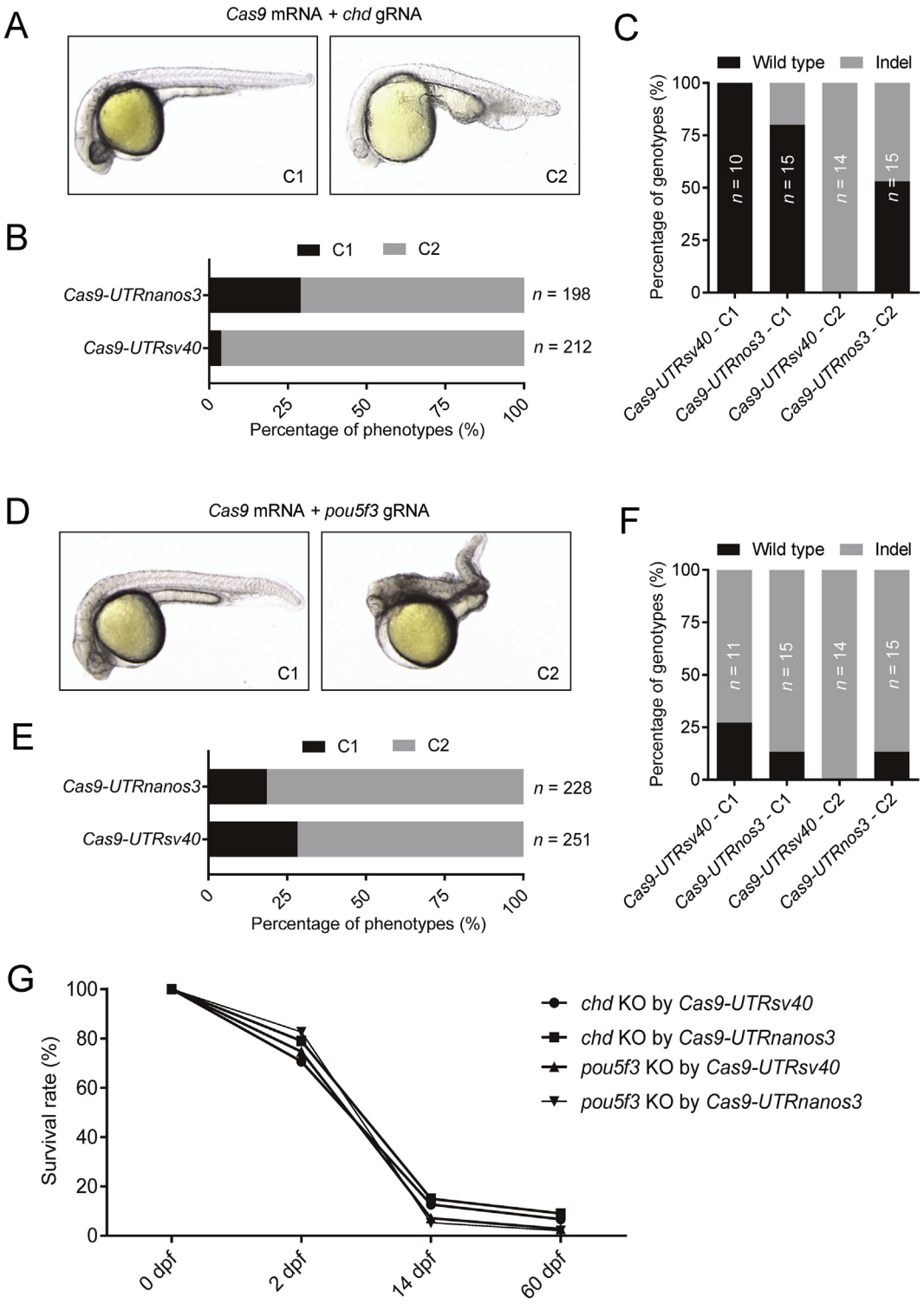

Fig. 1. Poor survival of the embryos after Cas9/gRNA injection to knock out *chd* and *pou5f3*. **A**: Different phenotypes of the embryos after injection of 400 pg *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and 80 pg *chd* gRNA. C1: wild-type (WT) like; C2: ventralization. **B**: Statistics of embryos at corresponding phenotypes after knocking out *chd* by injection of *cas9-UTRsv40* ($n = 212$) or *cas9-UTRnanos3* mRNA and *chd* gRNA ($n = 237$). *n*, number of embryos calculated. **C**: Mutation rates of embryos at corresponding phenotypes after knocking out *chd* by injection of *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and *chd* gRNA. *n*, number of clones sequenced. **D**: Different phenotypes of the embryos after co-injection of 400 pg *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and 80 pg *pou5f3* gRNA. C1: WT like; C2: dorsalization. **E**: Statistics of embryos at corresponding phenotypes after knocking out *pou5f3* by injection of *cas9-UTRsv40* ($n = 251$) or *cas9-UTRnanos3* mRNA and *pou5f3* gRNA ($n = 248$). *n*, number of embryos calculated. **F**: Mutation rates of embryos at corresponding phenotypes after knocking out *pou5f3* by injection of *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and *pou5f3* gRNA. *n*, number of clones sequenced. **G**: Statistics on survival rates of the embryos after injection of 400 pg *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and 80 pg gene specific gRNA to knock out *chd* and *pou5f3* at 0, 2, 14, and 60 dpf.

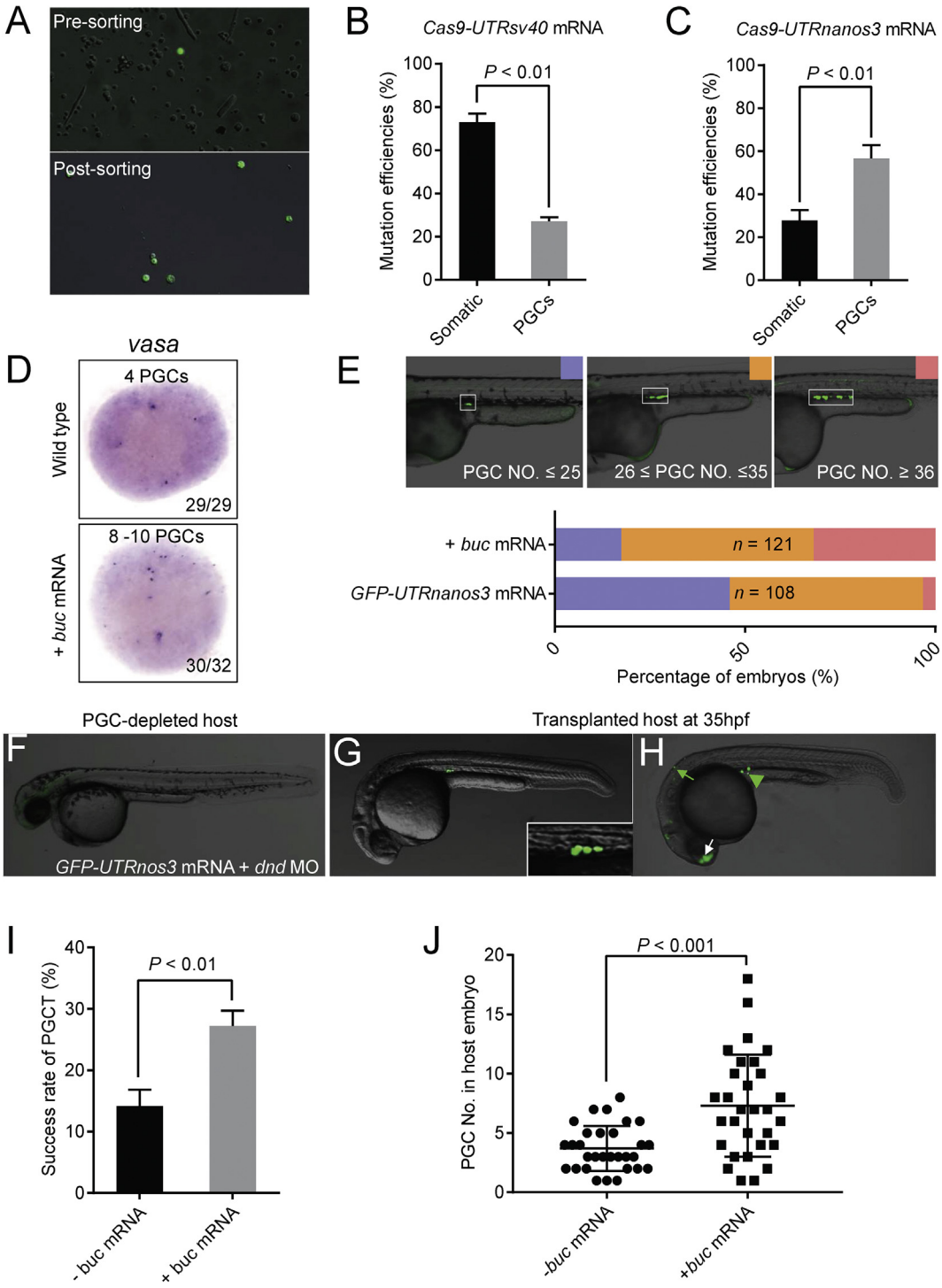

Fig. 2. Optimization of PGC-targeted mutagenesis and PGCT. **A**: Representative image of pre- and post-sorting of PGCs from a transgenic line of *Tg(piwil1:egfp-UTRnanos3)* at 2 dpf. **B** and **C**: Mutation efficiencies were calculated in somatic cells and PGCs after co-injection of *cas9-UTRsv40* mRNA and gRNA (**B**) and *cas9-UTRnanos3* mRNA and gRNA (**C**). Parallel experiments were done for three times. $P < 0.01$. **D**: Whole-mount *in situ* hybridization with *vasa* probe of the wild-type embryos and the embryos injected with 200 pg *buc-UTRsv40* mRNA at 3 hpf. The embryos were animal pole view. Note that 8–10 *vasa*-positive cells appeared in the *buc*-injected embryos, compared with 4 *vasa*-positive cells in the control embryos. **E**: *buc* mRNA induced ectopic PGCs of donor embryos. Purple, orange and pink represent larva with less (PGC number≤25), moderate (25<PGC number≤35) and many (PGC number≥36) PGCs, respectively. **F**: Representative image showing a host embryo co-injected with 200 pg *GFP-UTRnanos3* mRNA and 100 nM *dnd* morpholino ((MO), with complete loss of endogenous PGCs. **G**: Representative image showing a PGCs positive transplanted embryo screened at 35 hpf. The fluorescent PGCs from the donor embryos have been magnified at the bottom right corner. **H**: A image showing a PGCs positive transplanted embryo at 35 hpf, with mis-migrated PGCs (green arrow) and fluorescent somatic cells from donor embryo (white arrow). **I**: The success rate of PGCT, as indicated by PGC-positive transplanted embryos at 35 hpf, was significantly increased by injection of *buc* mRNA into the donor embryos. The experiment was replicated for three times. $P < 0.01$. **J**: The host embryos contained significantly higher number of PGCs compared with the control group, when using *buc*-overexpressed embryos as donors for PGCT. $P < 0.001$.

dead end (*dnd*) morpholino (MO) showed complete elimination of endogenous PGCs of the host embryos (Fig. 2F), and embryos receiving successful PGCT showed GFP-positive PGCs at the genital ridge (Fig. 2G). In some cases, the transplanted PGCs were mis-migrated into other body regions of the host embryo (Fig. 2H). We screened the PGC fluorescent embryos after PGCT and calculated the success rate of PGCT through dividing the number of PGC-positive embryos by the number of embryos manipulated. The results showed that co-injection of *buc-UTRsv40* mRNA into the donor embryos could even double the success rate of PGCT from 14.2% to 27.2% (Fig. 2I). More importantly, the host embryos contained significantly higher number of transplanted PGCs when using the *buc*-overexpressed embryos as donors when compared with control (Fig. 2J). Therefore, we utilized the *buc* overexpressed embryos as PGC donors to improve the efficiency of PGCT.

2.3. Efficient generation of MZ mutants of tcf7l1a (MZtcf7l1a)

As we optimized the efficiencies of PGC-targeted CRISPR/Cas9 gene knockout and PGCT, we then tried to generate MZ mutants of certain genes. The first gene for a test is *tcf7l1a*, whose MZ mutants showed to be headless while zygotic mutants did not show any visible defects (Kim et al., 2000). By utilizing the optimized approach of PGC-targeted mutagenesis and PGCT (Fig. 3A), we successfully obtained 18 transplanted adults, in which 4 were females and 14 were males. All the females were fertile, while only seven of the males were fertile (Table 1). By contrast, all the PGC-ablated host embryos developed into infertile males. By an outcross test, we identified the mutation efficiencies of gametes of each PGC transplanted fish, in which the female #3 and male #7 gave the highest mutation efficiencies (100% for both) among all group (Figs. 3B and S1). This indicates that target gene mutated homozygous mutants could directly be obtained just at F_1 generation.

Thereafter, the female #3 and male #7 were crossed and their offspring were phenotypically analyzed. As expected, majority (C3, 73.4%) of the offspring showed typical phenotype of headless, while the minority of them (C2, 24.2%) showed smaller eyes (Fig. 3C). We further analyzed gene expression of the mutant embryos by whole-mount *in situ hybridization analysis* (WISH) and found that *tcf7l1a* scarcely expressed in the mutant embryos throughout the early development, indicating that non-sense mediated mRNA decay occurred in the embryos (Fig. 3D). In addition, WISH showed that the expression of *emx1*, a marker of telencephalon, disappeared while *krox20*, a marker for rhombomere 3 and 5, were nearly unaffected in the mutants at early-somite stage, and the telencephalon and eyes labeled by *six3b* disappeared in the mutant embryos at 24 hpf (Fig. 3E). When compared with WT embryos, the expression of neural crest marker *foxd3* in the mutant embryos showed a slight increase, probably due to the elevated zygotic Wnt/β-catenin activity (Lewis et al., 2004). All these results undoubtedly proved that MZ mutants of *tcf7l1a* gene could be generated efficiently using combined CRISPR/Cas9 with PGCT technology.

2.4. Efficient generation of MZpou5f3 at F_1 generation

We then applied this technology to generate MZ mutants of *pou5f3*, an essential gene for early embryogenesis with both maternal and zygotic expression (Reim et al., 2004; Reim and Brand, 2006). In total, 24 F_0 adults with successful PGC transfers at the early stage were obtained following application of combined CRISPR/Cas9 with PGCT. Three adults were females and 9 out of the 21 males recovered their fertility (Table 1). Ten (1♀9♂) out of 12 (3♀9♂) fertile F_0 adults with capability producing *pou5f3* mutated gametes were screened. As shown in Fig. 4A, the mutation efficiencies of their gametes from female #1 and male #2 could reach as high as 100% (Fig. S2).

Subsequently, the female #1 was crossed with male #2 and the offspring were used for phenotypical analysis. The incrossed embryos showed gastrulation defects and severe dorsalization at 24 hpf (Fig. 4B), mimicking the previously reported mutant phenotype of MZ*pou5f3* (Reim et al., 2004; Reim and Brand, 2006). The expression analysis of several genes was conducted to confirm the phenotypes of F_0-incrossed MZ*pou5f3*. Firstly, *pou5f3* showed barely expression in mutants during early embryogenesis compared with its high expression level in WT (Fig. 4C). Secondly, compared with WT at shield stage, the expression of *chd* (labeling dorsal organizer) expanded ventrally within the germ ring and *eve1* (a ventral mesoderm marker) was significantly reduced in the mutants (Fig. 4D). Moreover, expression of *sox32* and *sox17*, the markers for endoderm development were undetectable in the mutant embryos. Notably, *myoD* was expressed in WT somites at the 6-somites stage, while in mutants its expression was fused ventrally. Lastly, *ntl* was straightly expressed in the notochord in WT embryos, but its expression was variably splited in mutant embryos which might be due to incomplete epiboly during early embryogenesis (Fig. 4B). All the results indicate that the F_0 adults could produce MZ*pou5f3* embryos with severe dorsalization as previously described (Reim and Brand, 2006). Therefore, the MZ*pou5f3* was successfully obtained at F_1 generation using combination of CRISPR/Cas9 and PGCT technology, which proved its feasibility for efficient generation of MZ mutants of zygotic essential genes with maternal and zygotic expression.

2.5. Maternal contribution of chd as revealed by MZchd phenotypical analysis

Lastly, we tried to utilize this approach to probe into the function of some embryonic essential genes with zygotic expression by generating novel MZ mutants. *chd* encodes a major antagonist of BMP signaling in early development, and both mutation analysis and MO-mediated knockdown studies revealed its essential role for dorsal development (Schulte-Merker et al., 1997; Nasevicius and Ekker, 2000). In our study, we happened to observe strong expression of *chd* in oocytes during oogenesis by fluorescent *in situ* hybridization (FISH) and reverse-transcription PCR (RT-PCR) (Fig. 5A and B), which is supported by a previous study showing slight expression of *chd* at 8-cell stage (Branam et al., 2010). Therefore, whether there is a maternal contribution of Chd activity needs to be answered by generating the MZ*chd* and comparing the MZ*chd* phenotype with its zygotic mutants (Z*chd*).

By using the optimized PGC-targeted CRISPR/Cas9 and PGCT approach, we obtained 13 fertile transplanted adults (Table 1). By direct sequencing of the target site from the genome of the test-crossed embryos, we found that 8 (2♀ 6♂) out of the 13 (5♀ 8♂) positive transplants could produce *chd* mutant gametes, while female #1 and male #1 gave the highest mutation rates (Fig. 5C). Although one transplanted adult could usually produce more than one type of mutations (Fig. S3), about 63.3% (19/30) of the mutation types resulted from microhomology-mediated end joining (MMEJ), a mechanism which facilitates the repair of DNA double-strand breaks in zebrafish early embryos (He et al., 2015; Thyme and Schier, 2016).

In the next step, the females and males of F_0 adults (♀ #1, #2 and ♂ #1) with mutated gametes were incrossed and the F_1 embryos were obtained. As expected, about 45% of the embryos showed ventralization phenotype (C2 and C3, Fig. 5D). Among the embryos showing ventralization, about 1/3 of the embryos showed severe ventralization phenotype (C3), with no forebrain and eyes and extremely expanded blood island and folded ventral tail fin, which

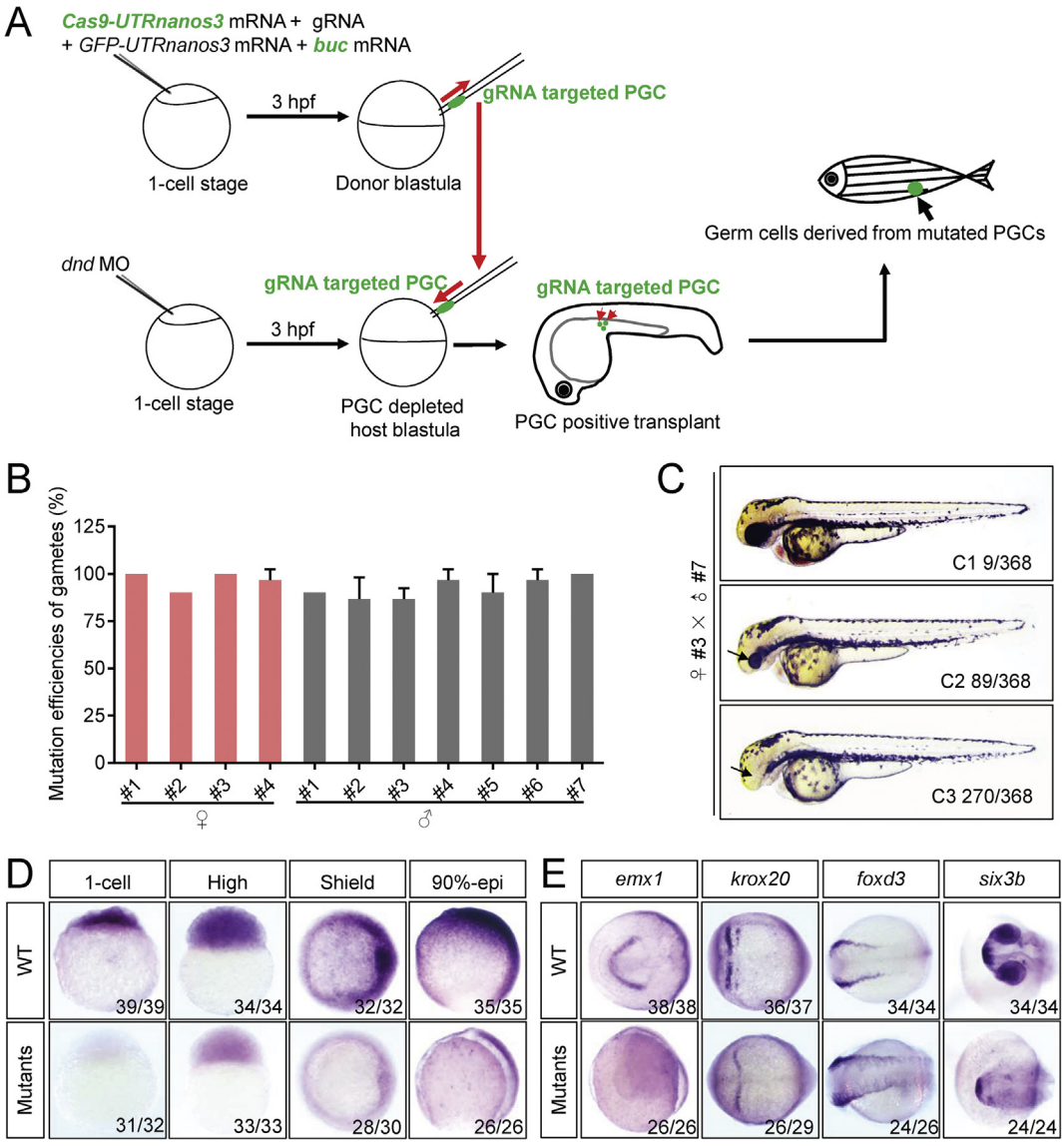

Fig. 3. Efficient generation of MZ mutants of *tcf7l1a* by combination of CRISPR/Cas9 and PGCT. **A**: Schematic workflow represents process of the optimized procedure of PGC-targeted CRISPR/Cas9 and transplantation of induced PGCs. **B**: Mutation efficiencies of gametes of each mutated positive F_0 adult fish (4♀, 7♂). **C**: The phenotypes of offspring crossed by female #3 and male #7. C1 shows the WT like phenotype, C2 shows smaller eyes, C3 shows complete loss of eyes. **D**: *tcf7l1a* was barely expressed in mutants during early embryogenesis, compared with its high expression level in WT. **E**: The marker of telencephalon *emx1* was not expressed in mutant embryos at early-somite stage; *krox20*, the marker for midbrain and hindbrain, was normally expressed in the mutants at early-somite stage; the expression of neural crest marker *foxd3* was slightly increased in mutants at early-somite stage; the expression of *six3b* at telencephalon and eyes was strongly decreased in the mutant embryos at 24 hpf.

Table 1
Positive rate of PGCT and the fertility of grown adults after PGCT.

Gene name	Test No.	Number of transplant	Number of survivors at 35 hpf	Number of successful PGCT embryos (%)	Number of survival adults	Number of fertile adults	Number of adults producing mutant embryo
tcf7l1a	1	64	30	7 (23.3)	7 (2♀5♂)	4 (2♀2♂)	4 (2♀2♂)
	2	66	37	9 (24.3)	6 (1♀5♂)	4 (1♀3♂)	4 (1♀3♂)
	3	62	31	8 (25.8)	5 (1♀4♂)	3 (1♀2♂)	3 (1♀2♂)
pou5f3	1	108	32	6 (18.7)	3 (1♀2♂)	1 (1♀0♂)	0
	2	97	45	11 (24.4)	8 (1♀7♂)	4 (1♀3♂)	3 (0♀3♂)
	3	113	63	15 (23.8)	13 (1♀12♂)	7 (1♀6♂)	7 (1♀6♂)
chd	1	72	32	9 (28.1)	7 (2♀5♂)	5 (2♀3♂)	3 (1♀2♂)
	2	56	34	10 (29.4)	6 (1♀5♂)	3 (1♀2♂)	1 (0♀1♂)
	3	67	38	10 (26.3)	8 (2♀6♂)	5 (2♀3♂)	4 (1♀3♂)

is far more severe than the ventralization phenotype of *chordino* mutant (Schulte-Merker et al., 1997). All the embryos showing C2 or C3 phenotype were randomly sampled for genetic identification and all the samples showed to be genetically homozygotes with the same or different indels at the same allele (data not shown). We carefully compared the C2 and C3 phenotypes with the zygotic mutants resulting from incross of $chd^{tt250/+}$ (Fig. 5E) or the heterozygous F_1 individuals from PGCT (data not shown), and found

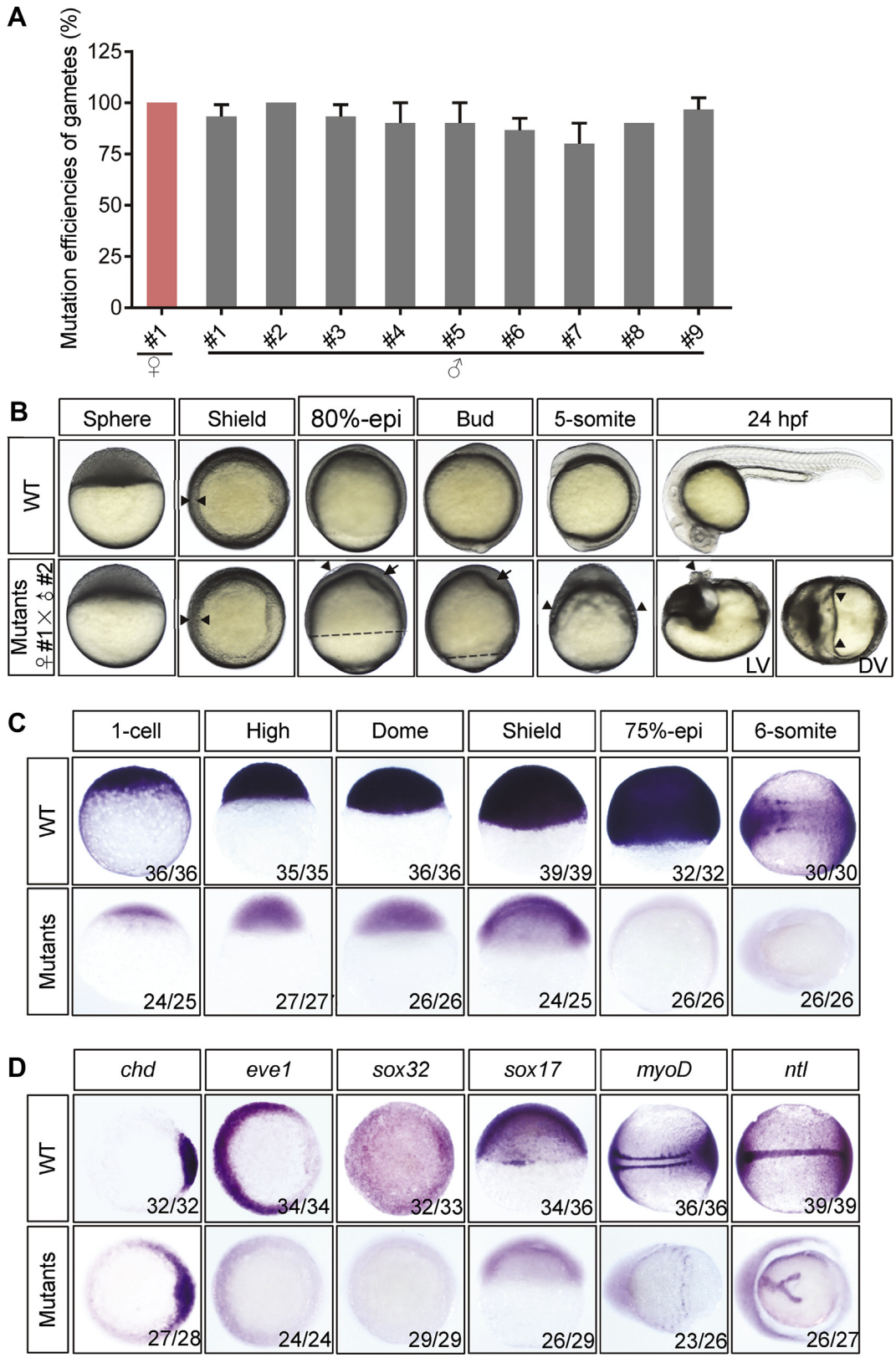

Fig. 4. Efficient generation of MZ mutants of *pou5f3* by combination of CRISPR/Cas9 and PGCT. **A**: Mutation efficiencies of gametes of each mutated positive F_0 adult fish (1♀, 9♂). **B**: The phenotypes of offspring crossed by female #1 and male #2 from sphere stage to 24 hpf. Note that the germ ring of mutant is thicker than the WT at shield stage, the epiboly is seriously affected during gastrulation, and a cluster of cells piles on the top of the dorsum (see LV, lateral view; DV, dorsal view) at 24 hpf. **C**: *pou5f3* was barely expressed in mutants, compared with its high expression in WT. **D**: The expression of *chd* was expanded ventrally within the germ ring in mutants, compared with WT; *eve1* was strongly reduced in mutants; *sox32* and *sox17*, the markers for endoderm, were undetectable in mutant embryos; the expression of *myoD* was fuses ventrally in the mutant embryos; the expression of *ntl* was variably splited in mutant embryos, in comparison with its straight expression in the notochord in WT embryos.

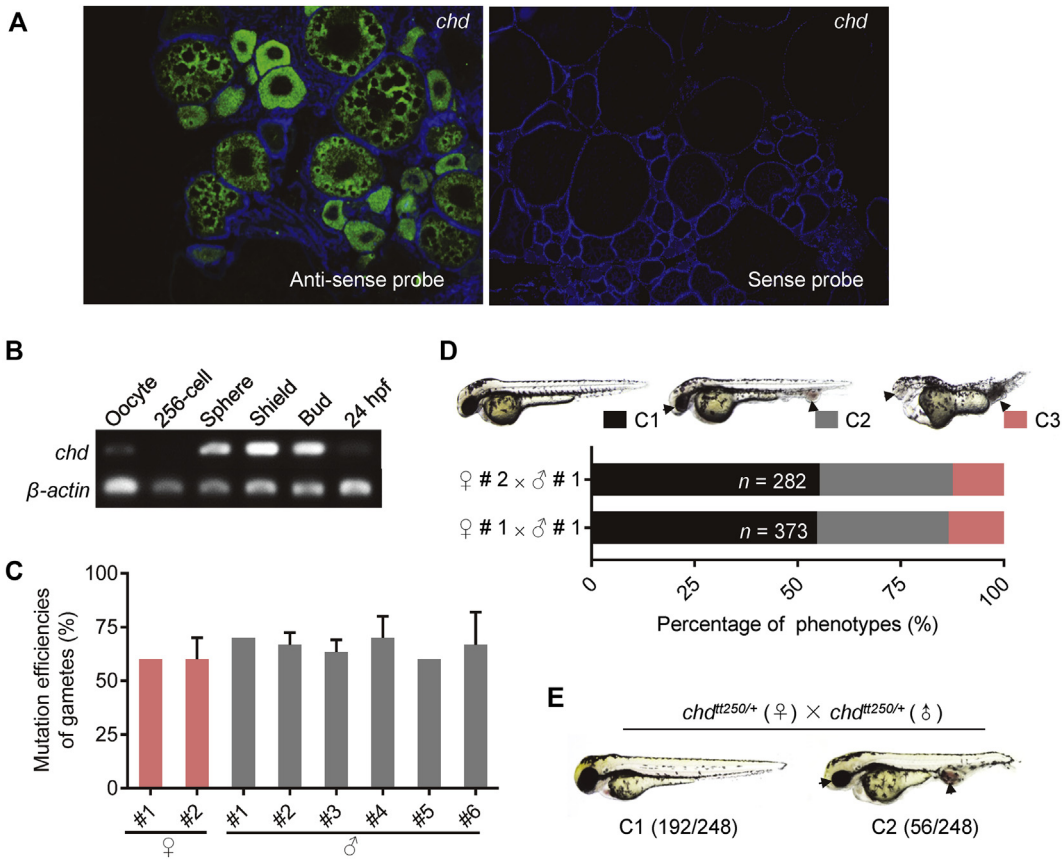

Fig. 5. Phenotypical analysis of MZ*chd* generated by combination of CRISPR/Cas9 and PGCT. **A**: The fluorescent RNA *in situ* hybridization of *chd* on cryosections of zebrafish ovaries with antisense or sense *chd* RNA probes. **B**: RT-PCR analysis of *chd* during early development, *β-actin* was used as the internal control. **C**: Mutation efficiencies of gametes of each mutated positive F_0 adult fish (2♀, 6♂). **D**: Statistics of the phenotypes of F_1 offspring incrossed by two females (#1 and #2) and the male #1. C1 shows similar phenotype of WT, C2 shows smaller eyes and enlarged blood island, C3 shows severe head defects and tail blood island enlargement. **E**: The phenotypes of F_1 offspring incrossed by heterozygotes of $chd^{tt250/+}$ mutants. C1 shows the WT like phenotype, C2 shows smaller eyes and enlarged blood island, a typical phenotype of zygotic mutant.

that C3 embryos of the MZ*chd* generated in the present study were much more severely ventralized (Fig. 5D). This strongly suggests that the maternally provided *chd* mRNA has BMP antagonistic function during zebrafish early development and the PGC-targeted mutagenesis and PGCT approach may be useful for unveiling novel functions of some classical genes.

3. Discussion

Gene targeting technology has been considered as one of the most powerful approaches to investigate gene functions. Generally, high dosage of the gene targeting vectors or RNAs will improve mutation efficiencies, but it is terribly possible to lead to developmental retardation in F_0 generation, especially when the target genes are essential for embryogenesis or organogenesis. In a previous study with zebrafish, *Cas9-UTRnanos3* mRNA injection has been reported to be an alternative to increase viability and reduce somatic mutations when knocking out some embryonic lethal genes like *dicer1* (Moreno-Mateos et al., 2015). However, in our study, the ability for *Cas9-UTRnanos3* mRNA to increase the embryonic viability seems to be limited to certain genes (Fig. 1). This is possibly due to that the translated Cas9 protein may result in efficient gene editing as early as 2 hpf, and the global expression of GFP resulting from *gfp-UTRnanos3* mRNA injection could remain till 24 hpf (Fig. S4). To overcome this conflict, in the present study, we have established a high-efficient method for generating MZ mutants of different genes, by combining the PGC-targeted CRISPR/Cas9 technology and optimized PGCT in zebrafish.

In a previous study, it has been reported that although the mutation efficiency generated by common CRISPR/Cas9 was achieved up to 50%, there was only 11% germline transmission efficiency in the progeny (Hruscha et al., 2013). This indicates that high mutation efficiency of the whole embryos generated by conventional method of CRISPR/Cas9 suffers from low mutation efficiency in germline, leading to waste of time and energy in the screening of mutants. Furthermore, we analyzed and compared the mutation efficiencies of somatic cells and PGCs resulting from ubiquitous overexpression of *Cas9* and gRNA. While for the first time, we revealed that the mutation efficiency in PGCs is much lower than that in the somatic cells. Therefore, in the conventional Cas9/gRNA injected experiment, the mutation efficiency evaluated at the whole embryo level should have been over-estimated, since the germline transmission efficiency should be much lower than the somatic mutation efficiency. In this study, high dosage of *cas9-UTRnanos3* mRNA (400 pg) and gRNAs (80 pg) were co-injected into donor embryos, aiming to create high mutation efficiencies in their PGCs. As a result, the PGC transplanted host fish could successfully produce mutated gametes with the efficiencies as high as 100%. On the other hand, by induction of additional PGCs in the donor embryos by overexpression of *buc*, we have substantially improved the efficiency of successful PGCT, which has shown to be a labor-extensive and skill-sensitive technology in previous studies (Ciruna et al., 2002; Saito et al., 2008).

In zebrafish, it is known that depletion of PGCs in early embryos leads to sterile males, and sufficient amount of PGCs is required for female development (Tzung et al., 2015). Therefore, when

conducting PGCT in zebrafish embryos, high amount of donor-derived PGCs should be transplanted into PGC-ablated host embryos, in order to obtain transplanted females. In this study, as *buc*-overexpression was used to promote PGC fate in donor embryos (Ye et al., 2019), we were able to obtain fertile females with relatively high rate in the PGC transplanted fish. In the future studies, moderate exposure of the embryos in estradiol at proper time may be an effective way to increase female numbers of the transplanted adults (Brion et al., 2004; Saito et al., 2008).

The PGCs with highly mutated target genes thus gave rise to genetically homozygous oogonia, and later maternally mutated oocytes without any contribution of maternal mRNAs of the target genes. Once mated with a homozygous male, the MZ mutants could be obtained just at F_1 generation, thus providing a novel strategy for function studies of embryonic essential genes with maternal expression. In this study, we not only obtained the MZ mutants of *tcf7l1a*, a gene only showing phenotype when it is maternal-zygotically mutated, but also generated MZ mutants of *pou5f3*, whose zygotic mutants could not survive to adulthood. Although using *cas9-UTRnanos3* mRNA could also generate MZ mutants directly at F_1 generation, the efficiency was as low as 12% (Moreno-Mateos et al., 2015), which may hinder the analysis of MZ phenotype of novel maternal genes. In our study, however, we successfully obtained 100% MZ mutants at F_1 generation for the genes *tcf7l1a* and *pou5f3*, which could be confidently used to analyze the phenotype of MZ mutants of certain novel genes. More importantly, by generating MZ mutants of *chd*, a gene essential for dorsal organizer development, we have unveiled the novel function of its maternally inherited mRNA. To our knowledge, this is the first report of combination of PGC-targeted mutagenesis method with PGCT to efficiently generate MZ mutants of zebrafish at F_1 generation. Given that genome editing of genes on chromosome level has been realized in zebrafish (Sun et al., 2020), this method may be useful for functional analysis of maternally-expressed genes in future large-scale knockout projects. On the other hand, since the mature gametes of fish species could be generated by surrogate reproduction (Lacerda et al., 2013), it is also feasible to speed up the genome editing in commercial fishes by intraspecies transplantation of CRISPR/Cas9 targeted germ cells.

4. Materials and methods

4.1. Ethics

This study was carried out in accordance with Guide for the Care and Use of Laboratory Animals at University of Chinese Academy of Sciences and Institute of Hydrobiology, Chinese Academy of Sciences.

4.2. Fish and embryos

The experimental fish used in this study were WT zebrafish of AB line, the transgenic line of *Tg(piwi:egfp-UTRnanos3)*^{ihb327Tg} (Ye et al., 2019), and the *chd*$^{tt250/+}$ mutants (Schulte-Merker et al., 1997) housed in China Zebrafish Resource Center (Wuhan, China, http://zfish.cn) and raised at 28.5 °C with a 14 h:10 h light and dark cycle. The embryos for microinjection and PGCT were harvested from natural fertilization. The stages of embryonic development were identified according to previous description (Kimmel et al., 1995).

4.3. gRNA design and synthesis

The sequence and structure information of *tcf7l1a* (ENSDARG00000038159), *pou5f3* (ENSDARG00000044774) and *chd* (ENSDARG00000006110) were obtained from zebrafish genomic database (http://www.ensembl.org/Danio_rerio/Info/Index), and the gRNA target sites for each gene were designed on the website of http://zifit.partners.org/ZiFiT/. The effective gRNA target sites are as follows: *tcf7l1a*-target: GGAGGAGGAGGTGATGACCTGGG, *pou5f3*-target: GGGTGAACTACTACACGCCATGG, and *chd*-target: GGATTAC CAGCTGCTGGTGGCGG, which are located in the N-terminal CTNNB1 binding domain coding sequence, the exon1 of the genomic sequence, and the chordin-specific (CHRD) domain coding sequence of *tcf7l1a, pou5f3* and *chd*, respectively. The underlined sequences show PAM (protospacer adjacent motif).

The gRNA templates were amplified by PCR with gene specific primers (*tcf7l1a*-gRNA-1, *pou5f3*-gRNA-1, and *chd*-gRNA-3) and a universal reverse primer gRNA-RP using plasmid pT7-gRNA as template according to a previous study (Chang et al., 2013). gRNAs were transcribed with MAXI script T7Kit (Ambion, USA). The primers used in this study are shown in Table S1.

4.4. Microinjection of mRNA and gRNA

The plasmids used for preparation of *cas9-UTRsv40* mRNA (Zhang et al., 2016), *gfp-UTRnanos3* mRNA (Xiong et al., 2013) and *buc-UTRsv40* mRNA (Ye et al., 2019) were described previously. The *nanos3* 3′-UTR was utilized to replace the *sv40* 3′-UTR in pT3:cas9-UTRsv40 to generate the PGC-targeted Cas9 expression construct, pT3:cas9-UTRnanos3. The mRNAs were transcribed using mMessage mMachine T3 UltraKit or mMessage mMachine SP6 UltraKit (Ambion). *cas9-UTRnanos3* mRNA, *cas9-UTRsv40* mRNA, *buc-UTRsv40*, *gfp-UTRnanos3* mRNA and gRNA were injected with dosages of 400 pg, 400 pg, 200 pg, 200 pg and 80 pg per embryo, respectively.

4.5. Fluorescent-activated cell sorting (FACS)

The transgenic embryos of *Tg(piwi:egfp-UTRnanos3)*^{ihb327Tg} at 1-cell stage were co-injected with either *cas9-UTRsv40* or *cas9-UTRnanos3* mRNA and gRNA. At 2 dpf, the embryos were washed three times in phosphate-buffered saline (PBS) buffer and then about 200 embryos were transferred into a 15 mL centrifuge tube (BD Falcon™ Tube) with 10 mL 0.25% trypsin. Trypsin treated embryos were passed through the syringe for two to three times to generate cell suspension, and the cell suspension was passed through a 100 μm cell strainer, centrifuged at 200 *g* for 10 min at 4 °C. The precipitated cells were washed two times with 2% fetal bovine serum (FBS)/PBS and finally resuspended in 1% FBS/PBS for sorting the GFP-positive PGCs by using a flow cytometry (FACSVerse, BD Biosciences, USA) and about 2000 GFP-positive PGCs were harvested. The GFP-negative somatic cells and GFP-positive PGCs after sorting were subject to evaluation of mutation efficiencies. Each experiment was conducted for triplicates.

4.6. Evaluation of mutation efficiency

Total DNA was isolated from the putative mutant embryos or cells, and PCR was performed with certain primers which could amplify the mutant regions. The PCR products were cloned into T-vector and subject to Sanger sequencing and sequence analysis. Except particular indication, each experiment was carried out as three independent trials.

4.7. PGC transplantation

Various types of *cas9* mRNA/gRNA co-injected donor embryos were raised till blastula stage. Meanwhile, 100 nM of *dnd* antisense morpholino oligonucleotide (5′-GCTGGGCATCCATGTC TCCGACCAT-3′) was injected into host embryos to eliminate

endogenous PGCs according to a previous report (Weidinger et al., 2003). Fluorescent donor embryos and PGC-depleted host embryos at 3 h-post-fertilization (hpf) were manipulated in 1 × Danieau's buffer under a dissecting microscope (MZX7, Olympus, Japan). Briefly, 60–100 cells at marginal region of the donor embryos were grafted into the blastula margin of PGC-depleted host embryos. At 1 hpt (hour post transplantation), the transplanted embryos were transferred to agarose plates filled with 0.3 × Danieau's buffer for further development. At 35 hpf, the PGC positive transplants were screened under an Olympus fluorescence macroscope (MVX10) and raised to adulthood.

4.8. Hybridization of transplanted adults and genotyping of the F_1 embryos

The PGC-transplanted larval fish were raised with great care and they usually became sexually matured at 2.5 months post-transplantation. Adults of transplanted fish were crossed with WT fish one by one to generate F_1 population. To evaluate the mutation rates of the gametes of the transplanted fish, 10 F_1 embryos at 1 dpf were randomly selected for PCR amplification of the gRNA target sites. The PCR products were cloned into pMD18-T vectors and subject to Sanger sequencing to analyze the mutation type and mutation efficiency. Each fish was analyzed for three independent times.

The male and female transplanted fish with the highest mutation efficiency in their gametes were selected for incross. The incrossed embryos were phenotypically analyzed with a MVX10 macroscope and used for further analysis.

4.9. Whole-mount in situ hybridization

The embryos were fixed with 4% paraformaldehyde (PFA) and digoxigenin (DIG)-labeled RNA probes were used for WISH according to a previous study (Wei et al., 2014).

4.10. Fluorescent in situ hybridization on section

8-month-old female fish were dissected and their ovaries were fixed with 4% PFA overnight at 4°C and cryo-sectioned for FISH. DIG-labeled RNA probe (sense and anti-sense) of *chd* was synthesized and the FISH was performed according to a previous study (Welten et al., 2006). The signals were developed for about 25 min using Fluor™ Tyramide reagent (Invitrogen, USA). The images were taken under the Leica SP8 confocal microscope.

4.11. Reverse-transcription PCR

Total RNA was isolated from unfertilized eggs and embryos at 256-cell stage, sphere stage, shield stage, bud stage and 24 hpf by using Trizol method. The RNA was reverse-transcribed with PrimeScript™ RT reagent Kit (Takara, Japan) and PCR was performed with primers *chd*-RT-F and *chd*-RT-R (Table S1). *β-actin* was used as the internal control.

4.12. Statistical analysis

Results were expressed as the mean ± SD. Unpaired Student's *t*-test was used to analyze the difference between two groups.

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Supplementary data

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