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Research Article

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Efficient gene editing in a medaka (*Oryzias latipes*) cell line and embryos by SpCas9/tRNA-gRNA

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Abstract: Generation of mutants with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) is commonly carried out in fish species by co-injecting a mixture of *Cas9* messenger RNA (mRNA) or protein and transcribed guide RNA (gRNA). However, the appropriate expression system to produce functional gRNAs in fish embryos and cells is rarely present. In this study, we employed a poly-transfer RNA (tRNA)-gRNA (PTG) system driven by cytomegalovirus (CMV) promoter to target the medaka (*Oryzias latipes*) endogenous gene tyrosinase (*tyr*) or paired box 6.1 (*pax6.1*) and illustrated its function in a medaka cell line and embryos. The PTG system was combined with the CRISPR/Cas9 system under high levels of promoter to successfully induce gene editing in medaka. This is a valuable step forward in potential application of the CRISPR/Cas9 system in medaka and other teleosts.

Key words: Medaka (*Oryzias latipes*); Gene editing; Poly-tRNA-gRNA; Embryos; Fish cells

1 Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) form a CRISPR/Cas system, which is an important acquired immune system for bacteria (Jinek et al., 2012; Feng et al., 2021). One type of CRISPR/Cas system, the modified CRISPR/Cas9 system, has evolved into a powerful genome-editing tool (Li et al., 2021). Cas9 protein directly cleaves the target DNA sequence under the guidance of a guide RNA (gRNA), enabling non-homologous end joining (NHEJ), homology-directed repair (HDR) (Mali et al., 2013; Ran et al., 2013), or microhomology-mediated end joining (MMEJ) (Tan JT et al., 2020). This results in insertion and deletion at the target site in the genomes of eukaryotes (Platt et al., 2014; Wefers et al., 2017), prokaryotes (Jiang et al., 2013), and viruses (Xu et al., 2015; Tang et al., 2017).

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To achieve gene knockout in particular tissues or simultaneous multiple gene knockout with the CRISPR/Cas9 system, researchers have used the specific promoter to control expression of the *Cas9* gene or used multiple RNA polymerase III promoters (such as U6 promoter, 7SK promoter, and H1 promoter) to control the expression of different gRNAs (Chen J et al., 2017; Merenda et al., 2017). However, the function and sequence of these promoters are not conserved across different species according to previous research (Liu et al., 2018). Recently, some strategies were tried to generate multiple functional gRNAs using some elements. Endonuclease CRISPR system Yersinia ribonuclease 4 (Csy4) protein was used to efficiently synthesize gRNA in yeast (Ferreira et al., 2018), plants (Čermák et al., 2017), zebrafish (Qin et al., 2015), and human cells (Nissim et al., 2014). However, in zebrafish, all injected embryos exhibited severe deformity after co-injection of *Csy4* messenger RNA (mRNA), *Cas9* mRNA, and *Csy* gRNA (Qin et al., 2015). Ribozymes can also enable efficient generation of gRNA and induce conditional gene knockout under the control of a specific RNA polymerase II-dependent promoter (He et al., 2017). Hammerhead (HH) ribozyme and hepatitis

delta virus (HDV) ribozyme were simultaneously used to cleave RNA due to their respective 5'-end and 3'-end digestion activity (Avis et al., 2012). The transfer RNA (tRNA)-linked transcript was also cleaved at the 5'-end and 3'-end of tRNA by RNaseP and RNaseZ, respectively (Forster and Altman, 1990; Schiffer et al., 2002), which made the tRNA-linked gRNA shorter than the HH+ HDV-linked gRNA. Thus, the tRNA-mediated CRISPR/Cas9 system has been used to generate multiple gene knockout lines in plants (Xie et al., 2015; Qi et al., 2016), *Drosophila* (Port and Bullock, 2016), zebrafish (Shiraki and Kawakami, 2018), and human cells (Knapp et al., 2019). Moreover, under the control of RNA polymerase II promoter, the tRNA-gRNA transcript achieved multiple gene knockout in specific mouse tissues (Xu et al., 2017). Undoubtedly, the tRNA-gRNA system is an ideal solution for multiple or conditional gene knockout.

Recently, the CRISPR/Cas9 system was used to generate a knockout fish line via injecting a gRNA and a modified *Cas9* mRNA into one-cell stage medaka (*Oryzias latipes*) embryos (Fang et al., 2018). However, gene editing methods for multiple genes or cell lines have rarely been reported in medaka. Here, we used poly-tRNA-gRNA (PTG) combined with the CRISPR/Cas9 system to induce gene editing in medaka embryos and in a fish cell line, spermatogonial cell line (SG3). Our results provid a tool for achieving gene editing in cell lines or multiple gene knockout in medaka.

2 Materials and methods

2.1 Fish and embryos

The medaka fish HdrR line was raised at Laboratory of Jimei University (Xiamen, China) and maintained in a 28 °C water circulation system with a photoperiod of 14-h light/10-h darkness (Chen TS et al., 2017). The embryos were collected from the adult fish and cultured in embryonic rearing medium until the larvae were hatched. All procedures complied with the protocol approved by the Animal Care and Use Committee of Jimei University (Xiamen, China).

2.2 Vector construction

Based on the previous study (Xie et al., 2015), we used a string of rice tRNA sequence to generate the expression vectors for this study. The pCS2-tRNA-*tyr* plasmid was constructed as follows: a string of the sequence tRNA: *Bbs*I-*Bbs*I: scaffold-tRNA: *tyr* gRNA: scaffold (Sequences S1–S4; Fang et al., 2018) was synthesized in GenScript (https://www.genscript.com.cn). We then inserted the string into pCS2+ plasmid digested by *Bam*HI and *Xho*I, and sequenced it (Fig. 1a). A DNA fragment containing medaka *pax6.1* gRNA scaffold was amplified with polymerase chain reaction (PCR) (primer: PT-F/PT-R; Table S1) and cloned into the *Bbs*I site of the pCS2-tRNA-*tyr* (GenBank accession number: MW827168) to construct pCS2-tRNA-*pax6.1* gRNA (Sequence S5)-tRNA-*tyr* gRNA (pCS2-tRNA-PT; GenBank accession number: MW827169) (Fig. 2a). To construct the pCV-zCas9 plasmid, we amplified the fragment of cytomegalovirus (CMV)-T7 (CMV-T7 F/CMV-T7 R; Table S1) and inserted it upstream of zebrafish codon-optimized Cas9 (zCas9) in pT3TS-nls-zCas9-nls (Jao et al., 2013; Fang et al., 2018). We amplified the sequence of 2A-puromycin from pX462 (Addgene: #48141) (zCas9 Afe I F/zCas9 Afe I R, zCas9 puro F/zCas9 puro R; Table S1) and inserted it downstream of zCas9 in pCV-zCas9, which formed pCV-zCas9-puro (GenBank accession number: MW827170) (Ran et al., 2013; Fang et al., 2018). The vector pCMV-tRNA-PT-zCas9-puro (GenBank accession number: MW827171) was constructed by inserting a CMV-tRNA-*pax6.1* gRNA-scaffold-tRNA-*tyr* gRNA-scaffold (Sequence S6) fragment into the CMV element of pCV-zCas9-puro plasmid (primer: all-F/all-R; Table S1, Fig. 3a). We designed the *pax6.1* gRNA using the CHOPCHOP website (https://chopchop.cbu.uib.no). The pCVpf vectors were generously given by Prof. Yunhan HONG, National University of Singapore.

2.3 Cell culture and transfection

The SG3 cell line generously given by Prof. Yunhan HONG was maintained in our lab. The cells were cultured at 28 °C in germ cell culture medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 20 mmol/L hydroxyethyl piperazineethanesulfonic acid (HEPES; pH 7.4), glutamine (2 mmol/L), Na-pyruvate (1 mmol/L), Na-selenite (2 nmol/L), non-essential amino acids (1 mmol/L), 2-mercaptoethanol (100 μmol/L), human recombinant β fibroblast growth factor (10 ng/mL), antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL), fish embryos extracted from

medaka, 15% (volume fraction) fetal bovine serum, and 1% (volume fraction) fish serum from trout (Hong et al., 2004). The cells were seeded at 7×10^5 cell/mL into six-well plates (70% confluence) before transfection. Then, cells were transfected with 2 μg of plasmid DNA (pCVpf or pCS2-tRNA-*tyr*: pCV-zCas9-puro at 1:1 (mass ratio); pCMV-tRNA-PT-zCas9-puro) using Lipofectamine™ 2000 transfection reagent (11668019; Invitrogen, USA) (Xue et al., 2018). After two days of transfection, the cells were selected with puromycin at 2 μg/mL for two weeks. The cells were observed using Ti-U inverted fluorescent microscopy (Nikon, Tokyo, Japan).

2.4 Microinjection

The *tyr* gRNA template was amplified using *tyr* gRNA F and *tyr* gRNA R (Table S1), and the *tyr* gRNA was prepared from the template with a TranscriptAid T7 High Yield Transcription kit (K0441; Thermo Scientific, USA). The pCS2-tRNA-*tyr* plasmid was digested by *Xho*I to form a template for in vitro transcription of tRNA-*tyr* gRNA. To prepare tRNA-*tyr* gRNA, we used a mMESSAGE mMACHINE™ SP6 Transcription kit (AM1340; Invitrogen). The *Cas9* mRNA was produced from the pZCas9 (Fang et al., 2018) using a mMESSAGE mMACHINE™ T7 Transcription kit (AM1344; Invitrogen). The mixture containing 100 ng/μL gRNA or 30 ng/μL plasmid, 500 ng/μL *Cas9* mRNA, and 500 μg/mL phenol red was injected into one-cell stage embryos. The injected embryos were then cultured in embryonic rearing medium at 28 °C in an incubator, and the embryos were collected 5 d post-injection (dpi) to extract genomic DNA. We imaged the embryos with a Leica M205 FA stereomicroscope (Leica, Germany).

2.5 Mutation screening

The genomic DNAs of the cells and embryos were extracted as previously described (Wang et al., 2011). About 5 dpi embryos or cells from six wells were used to extract the genomic DNA. Each sample was lysed with cell lysis buffer (10 mmol/L Tris-HCl (pH 8.0), 75 mmol/L NaCl, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 5 mg/mL sodium dodecyl sulfate (SDS)) and proteinase K (100 μg/mL) at 65 °C for 3 h. Proteins and other impurities were removed by adding NH_4Ac (7.5 mol/L), and then the DNA was precipitated with isopropanol and resuspended in TE (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0). The target fragment was amplified with PCR (Fang et al., 2018). The primers are listed in Table S1. The PCR program was set at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, then 72 °C for 10 min. The PCR products were digested by T7 Endonuclease I (EN303-01; Vazyme, China) to detect mutation and then cloned into pMD18-T (D103A; TaKaRa, Japan) for single-colony sequencing. The sequences were analyzed with the SnapGene program (https://www.snapgene.com).

3 Results

3.1 Activity of tRNA-gRNA RNA in medaka embryos

To explore whether CRISPR/Cas9 combined with the tRNA-based RNA procession system can cause gene editing in medaka, we synthesized a fragment including rice tRNA ligated by the *tyr* gRNA of medaka and inserted it into the pCS2+ plasmid to construct the pCS2-tRNA-*tyr* vector. We produced tRNA-*tyr* gRNA, *Cas9* mRNA, and *tyr* gRNA by in vitro transcription from the pCS2-tRNA-*tyr* vector, pZCas9 plasmid, and *tyr* gRNA template, respectively (Fig. 1a). Then, we co-injected tRNA-*tyr* gRNA and *Cas9* mRNA into the one-cell stage embryos, which showed mosaic loss of retinal pigment in the tRNA-*tyr* gRNA group. The injected *tyr* gRNA group, as the positive control, also exhibited mosaic loss of retinal pigment (Figs. 1b and 1c). In contrast, the normal pigment existed in the uninjected embryo group (Figs. 1b and 1c). To further verify that the loss of pigment was due to mutation of the *tyr* gene, we amplified the fragments containing the *tyr* gRNA target site and subjected them to Sanger sequencing. The result showed that the mutation sequence was indeed located at the gRNA target site (Fig. 1d), and produced at least three different types of mutants (−7 bp, −5 bp, and −5/+7 bp). Statistical data for pigment loss placed about 74% (n=32/43) of individuals in the injected tRNA-*tyr* gRNA group. By contrast, the injected *tyr* gRNA group appeared to exhibit higher mutation efficiency, with a mutation rate of 100% (n=22/22) (Fig. 1b). These results suggested that the transcribed tRNA-gRNA, similar to the gRNA, induced DNA editing in medaka embryos.

3.2 tRNA-mediated multiple gene editing in medaka embryos

To investigate the feasibility of expressing multiple functional gRNAs via CMV promoter, we constructed the pCS2-tRNA-PT vector by inserting *pax6.1* gRNA in the *Bbs*I site of pCS2-tRNA-*tyr* (Fig. 2a). In our previous study (unpublished data), *pax6.1* gRNA was validated as producing the aniridic eye in medaka, which is a canonical and visible phenotype for observation during eye development (Fig. S1). We co-injected *Cas9* mRNA with either plasmid pCS2-tRNA-*tyr* (group A) or pCS2-tRNA-PT (group B) into the one-cell stage embryos. In the group A (pCS2-tRNA-*tyr* and *zCas9* mRNA), we noted deletion of pigment (Fig. 2c). In the group B (pCS2-tRNA-PT and *zCas9* mRNA), we also found deletion of pigment and abnormal development of the eye (Fig. 2c). Subsequently, amplification of the target site and monoclonal sequencing confirmed that these phenotypes resulted from mutation of *tyr* and *pax6.1* (Figs. 2d and 2e). Additionally, statistical data on the pigment deletion showed that the mutation ratio of the injected pCS2-tRNA-*tyr* and *zCas9* mRNA group approximated 46% (n=47/102), while the injected pCS2-tRNA-PT and *zCas9* mRNA group showed higher mutation efficiency, with a mutation rate of 75% (n=45/60) (Fig. 2b). The latter result was caused by the knockout of *pax6.1*, which also led to blocked pigmentation (Fujimura et al., 2015). The mutant embryos were grown to adulthood and then intercrossed to generate albino fish (Fig. 2f). The albino fish had red eyes while the wild type still had black eyes. Thus, the tRNA-mediated gene editing enabled us to obtain fertile mutation generations. These results indicated that the tRNA-gRNA system could also produce functional gRNA under the control of CMV promoter in medaka embryos, either by RNA injection or DNA injection. Moreover, the vector pCS2-tRNA-*tyr* (*Bbs*I) provided one-step cloning to knock out any candidate genes.

3.3 tRNA-mediated gene editing in the fish cell line

Many strategies for Cas9-mediated gene editing in cells involve transfecting the vector containing U6 promoter to express gRNA stably (Yin et al., 2015). However, the U6 promoter is not conservative across the phyla (Liu et al., 2018), whereas the above results indicated that the tRNA-gRNA system can also produce functional gRNA under the control of CMV promoter. Thus, we tested the feasibility of this strategy in the fish cell line medaka SG3. We explored three different combinations of plasmids: (1) the all-in-one plasmid pCMV-tRNA-PT-zCas9-puro (Fig. 3a), (2) two separated plasmids pCV-zCas9-puro and pCS2-tRNA-*tyr*, and (3) pCVpf and pCV-zCas9-puro, as control. We transfected each of these plasmid combinations into SG3 cells (Fig. 3b). Through puromycin selection, we extracted the DNA from transfected cells to amplify the target region of *tyr*. After amplification, the products could be digested by T7 endonuclease I (Fig. 3c), which suggested that the pCMV-tRNA-PT-zCas9-puro and the mixture of pCV-zCas9-puro and pCS2-tRNA-*tyr* induced mutation of the *tyr* target site. In the subsequent sequencing, the *tyr* mutation sequence was detected. In the pCMV-tRNA-PT-zCas9-puro group, we found the +2 bp mutation type, and at least four types of mutation were also present in the pCV-zCas9-puro and pCS2-tRNA-*tyr* group (−6 bp, −20/+25 bp, −10 bp, +4 bp) (Fig. 3d). Therefore, the CMV promoter can also drive transcription of the tRNA-gRNA system in medaka SG3 cells, and produce functional gRNA to edit the target gene.

4 Discussion

Gene editing induced by the CRISPR/Cas9 system has been found in many organisms (Jiang et al., 2013; Xu et al., 2015; Fang et al., 2018; Tan YY et al., 2020). Recently, the PTG system has been employed to widen the field of application of the Cas9 system. In rice, the PTG system under the control of RNA polymerase III promoter was used to induce the mutation of multiple genes (Xie et al., 2015). Subsequently, the rice tRNA sequence was directly used to generate the mutant of genomic DNA in *Drosophila* (Port and Bullock, 2016), zebrafish (Shiraki and Kawakami, 2018), mice (Xu et al., 2017), and human cells (Dong et al., 2017). Surprisingly, no obvious differences in efficiency were found between zebrafish tRNA and rice tRNA (Shiraki and Kawakami, 2018). These findings demonstrated that the tRNA processing system is highly conserved in tested living organisms. Furthermore, the wide use of the PTG system is possible because of its non-toxicity (Xu et al., 2017) and shorter sequence structure (Mefferd et al., 2015). In this study, we also used the rice tRNA sequence to

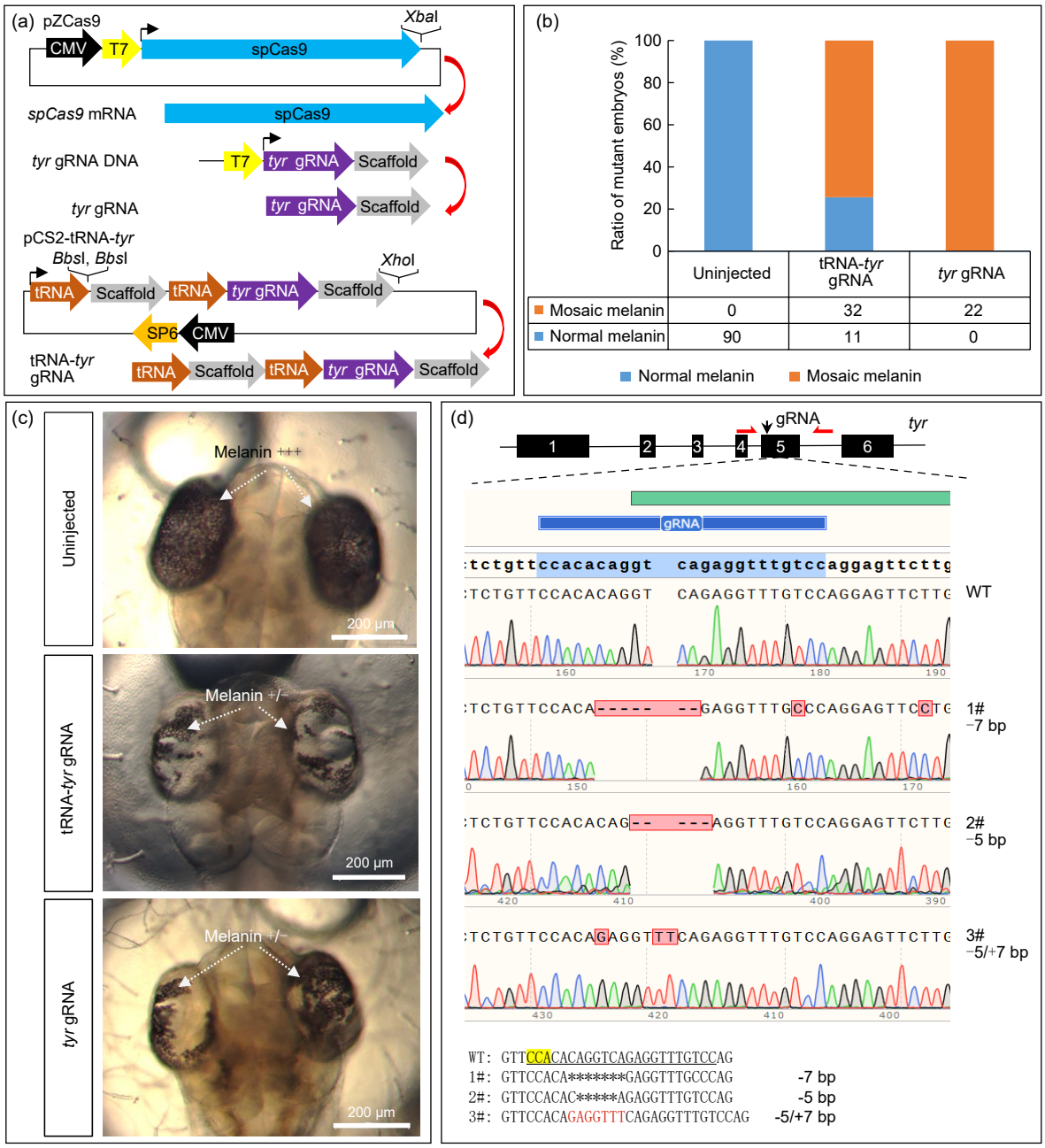

Fig. 1 tRNA-*tyr* gRNA-mediated gene editing in medaka embryos. (a) In vitro synthesis of *spCas9* mRNA, *tyr* gRNA, and tRNA-*tyr* gRNA. The elements of vectors and mRNA are marked with different colors. Black arrows represent the transcription start site, and red arrows represent the process of transcription. (b) Ratio of mutant embryos. Uninjected: wild-type embryos with no injection; tRNA-*tyr* gRNA: embryos injected with tRNA-*tyr* gRNA and *spCas9* mRNA; *tyr* gRNA: embryos injected with *tyr* gRNA and *spCas9* mRNA. (c) Diagrams displaying the phenotype of pigment cell deletion due to *tyr* gene mutation. The eye pigment cell is indicated with a white arrow. Melanin+++: normal pigmentation; Melanin+/−: mosaic pigmentation. (d) Genomic DNA structures of *tyr* and wave diagrams of single-colony sequencing. The gRNA site is indicated by a black arrow, and the red arrows indicate primers. In the wave diagrams, the gRNA is marked with blue box, and the mutation bases are marked with pink box. * represents the deleted base; red letters indicate the inserted base. PAM sites and gRNA sequences are highlighted and underlined, respectively. tRNA: transfer RNA; mRNA: messenger RNA; gRNA: guide RNA; CMV: cytomegalovirus; spCas9: *Streptococcus pyoge* Cas9; SP6: phage SP6 promoter; T7: phage T7 promoter; PAM: protospacer adjacent motif; WT: wild type.

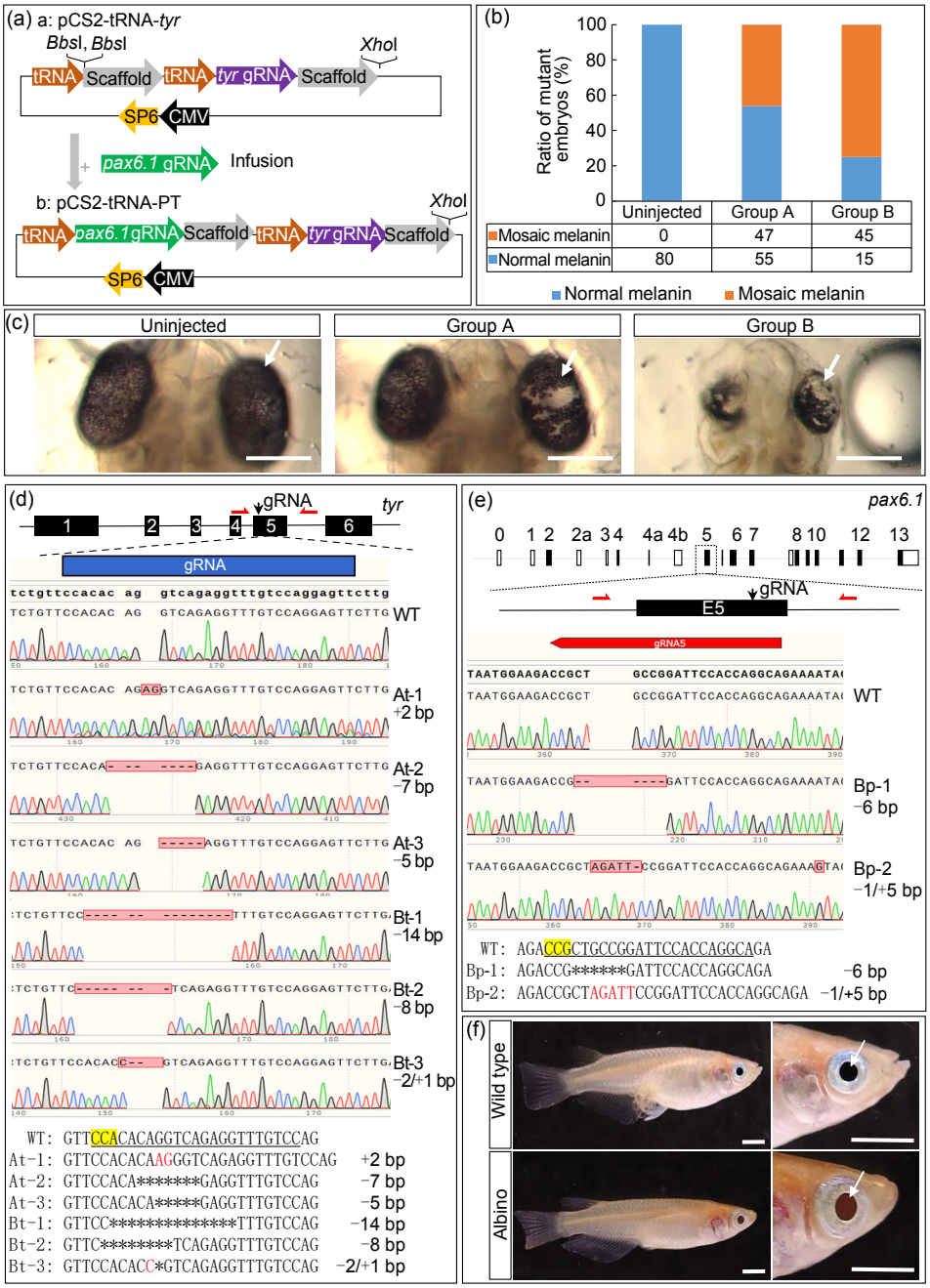

Fig. 2 tRNA-mediated multiple gene mutation in medaka embryos. (a) Construction process of pCS2-tRNA-PT. (b) Ratio of mutant embryos. Uninjected: WT embryos with no injection; Group A: embryos injected with the mixture of pCS2-tRNA-*tyr* plasmid and *spCas9* mRNA; Group B: embryos injected with the mixture of pCS2-tRNA-PT plasmid and *spCas9* mRNA. (c) Diagram showing the phenotype caused by mutation of the *tyr* gene (arrows). Scale bar=200 μm. (d) Genomic DNA structures of *tyr* and wave diagrams of single-colony sequencing. In the wave diagrams, the gRNA is marked with blue box and the mutation bases are marked with pink box. The At-1, At-2, and At-3 colonies are from embryos injected with the mixture of pCS2-tRNA-*tyr* plasmid and *spCas9* mRNA. The Bt-1, Bt-2, and Bt-3 colonies are from embryos injected with the mixture of pCS2-tRNA-PT plasmid and *spCas9* mRNA. (e) Genomic DNA structure of the *pax6.1* and wave diagrams of single-colony sequencing. In the wave diagrams, the gRNA is marked with red box and the mutation bases are marked with pink box. The Bp-1 and Bp-2 colonies are from embryos injected with the mixture of pCS2-tRNA-PT plasmid and *spCas9* mRNA. * represents the deleted base, and red letters indicate the inserted base. PAM sites and gRNA sequences are highlighted and underlined, respectively. (f) The different phenotypes of WT and albino type are indicated by white arrows. Scale bar=2 mm. tRNA: transfer RNA; gRNA: guide RNA; mRNA: messenger RNA; CMV: cytomegalovirus; PT: *pax6.1* gRNA-*tyr* gRNA; spCas9: *Streptococcus pyoge* Cas9; SP6: phage SP6 promoter; WT: wild type.

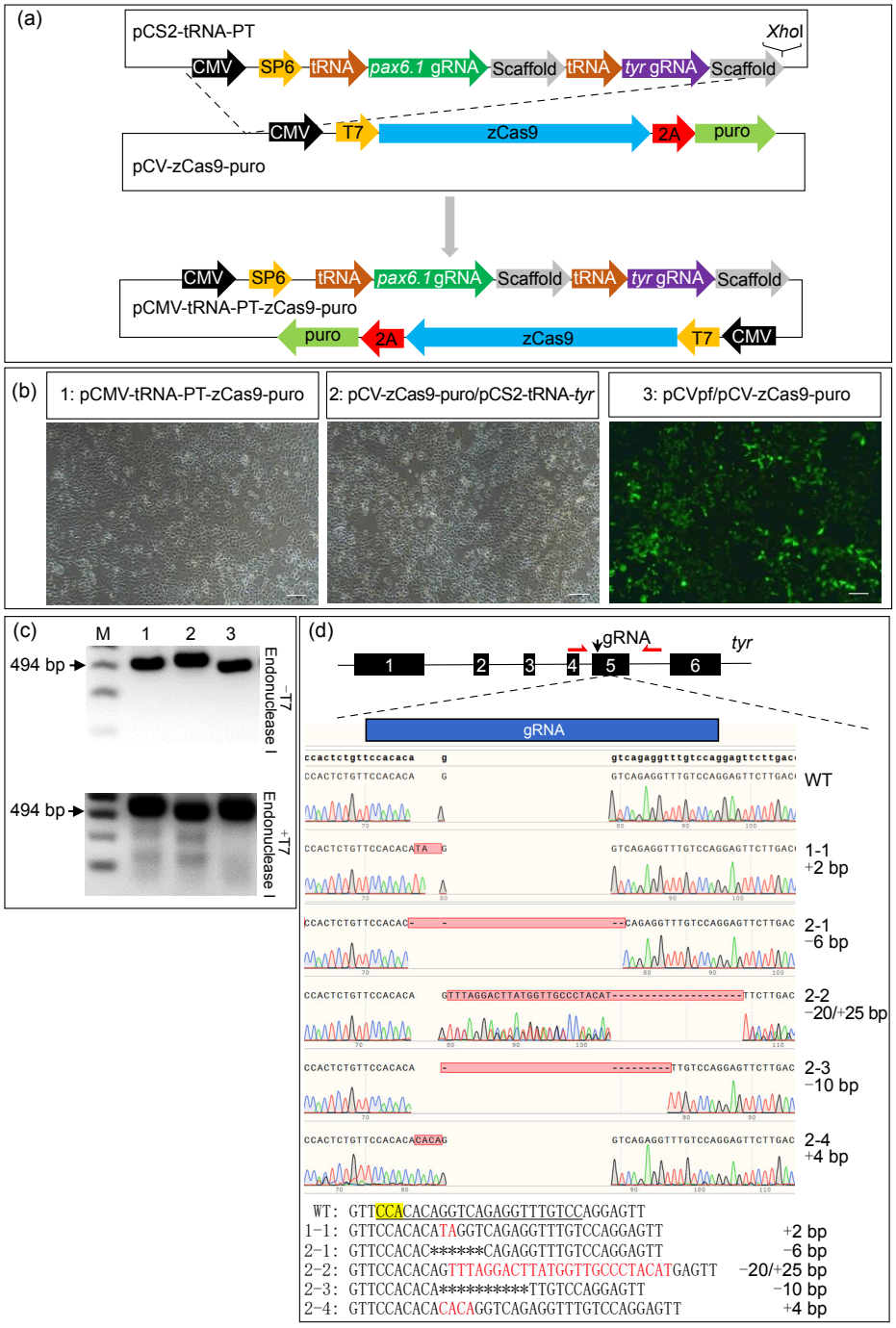

Fig. 3 tRNA-mediated gene editing in medaka SG3 cells. (a) Construction process of pCMV-tRNA-PT-zCas9-puro. (b) Vectors transfected into SG3 cell lines. 1: cells transfected with pCMV-tRNA-PT-zCas9-puro; 2: cells transfected with pCV-zCas9-puro and pCS2-tRNA-*tyr*; 3: cells transfected with pCVpf and pCV-zCas9-puro as negative control. Scale bar=100 μm. (c) PCR products of *tyr* before and after T7 endonuclease I digestion. M: DL5000 DNA marker; 1: cells transfected with pCMV-tRNA-PT-zCas9-puro; 2: cells transfected with pCV-zCas9-puro and pCS2-tRNA-*tyr*; 3: cells transfected with pCVpf and pCV-zCas9-puro as negative control. (d) Genomic DNA structure of *tyr* and wave diagrams of single-colony sequencing. In the wave diagrams, the gRNA is marked with blue box and the mutation bases are marked with pink box. 1-1: colony of cells transfected with pCMV-tRNA-PT-zCas9-puro; 2-1, 2-2, 2-3, and 2-4: colonies of cells transfected with pCV-zCas9-puro and pCS2-tRNA-*tyr*. * represents the deleted base and red letters indicate the inserted base. PAM sites and gRNA sequences are highlighted and underlined, respectively. tRNA: transfer RNA; gRNA: guide RNA; CMV: cytomegalovirus; zCas9: zebrafish codon-optimized Cas9; PT: *pax6.1-tyr*; SP6: phage SP6 promoter; T7: phage T7 promoter; puro: puromycin; 2A: self-cleaving 2A peptide; WT: wild type; PCR: polymerase chain reaction.

induce mutation of the medaka *tyr* gene in the embryos and cell line, and acquired the homozygote of *tyr* mutation by crossing and screening. We found that the PTG system did not show obvious toxicity for the embryogenesis of medaka, and the function of the tRNA processing system was also conserved between rice and medaka.

Generally, gRNA is synthesized by in vitro transcription, or by in vivo transcription from an RNA polymerase III promoter such as U6 promoter (Yin et al., 2015). gRNA synthesized by in vitro transcription has been demonstrated to induce gene editing in medaka (Fang et al., 2018), but gRNA synthesized by in vitro transcription transfects fish cells only with difficulty, and degrades quickly in culture media (Liu et al., 2018). Although human U6 can drive the transcription of gRNA to generate gene knockout in grass carp kidney cells (CIK) (Ma et al., 2018), it does not work well in medaka cells (Liu et al., 2018). Similarly, we have tried to express gRNA stably in vivo using RNA polymerase III promoters, such as medaka U6 promoter, medaka 7Sk promoter, or other U6 promoters from species like zebrafish, but these strategies were not successful in our previous experiments (data not shown). Interestingly and amazedly, the structure of gRNAs linked by tRNA can be processed into functional gRNAs under the control of CMV promoter or tissue-specific RNA polymerase II promoter in vivo (Xu et al., 2017). Further, an improved tRNA scaffold has been engineered, which enables high gRNA production from RNA polymerase II promoters without ribozymes (Knapp et al., 2019). Here, we used CMV promoter, the most popular and strongest promoter yet found, to express gRNAs spaced by tRNA in vivo, and found mutation sequences in the *tyr* gRNA target site of both embryos and cells. Moreover, the designed vector pCS2-tRNA-PT containing *Bbs*I-*Bbs*I restriction enzyme sites is also suitable for cloning any candidate gRNA in one step, which is a robust system to generate functional gRNAs. The result in fact revealed that in vivo expression of PTG under the control of CMV promoter is completely feasible in medaka. The success of this experiment has inspired us to build a transgenic/mutated cell line and fish that stably express gRNA in vivo in the future.

Although the SpCas9/tRNA-gRNA system was employed successfully in medaka, some shortcomings were also observed. The tRNA-linked transcript was cleaved at the 5'-end and 3'-end of tRNA by RNaseP and RNaseZ, respectively (Forster and Altman, 1990; Schiffer et al., 2002), which means that time is required to convert tRNA-gRNA to gRNA and form the Cas9/gRNA complex in vivo. During embryonic development, the target DNA edited by gRNA/Cas9 occurred at lower levels in embryos injected with tRNA-gRNA compared to the embryos injected with gRNA. A similar result was found in zebrafish, in which the ratio of heteroduplex bands in embryos injected with three gRNAs was significantly higher than that in embryos injected with the three tRNA-linked gRNAs (Shiraki and Kawakami, 2018).

In summary, we successfully combined the PTG system under CMV promoter with the CRISPR/Cas9 system to induce gene editing in a medaka fish model. This is the first evidence that fish cell lines can be edited by a vector system that contains the CMV promoter and tRNA-gRNA expression cassette, suggesting that the PTG system will be a valuable tool for application of the CRISPR/Cas9 system in medaka, and logically also be used in other fish embryos or cell lines.

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Author contributions

Qihua PAN designed and performed the experiment, analyzed experimental data, and wrote the original draft. Junzhi LUO, Yuewen JIANG, Zhi WANG, and Ke LU participated in part of the experiment and edited the manuscript. Tiansheng CHEN designed and supervised the experiment, analyzed experimental data, wrote and edited the manuscript. All authors have read and agreed the final version of the manuscript. The authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Qihua PAN, Junzhi LUO, Yuewen JIANG, Zhi WANG, Ke LU, and Tiansheng CHEN declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Supplementary information

Table S1; Sequences S1–S6; Fig. S1