Efficient and Heritable Gene Targeting in Tilapia by CRISPR/Cas9

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ABSTRACT Studies of gene function in non-model animals have been limited by the approaches available for eliminating gene function. The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated) system has recently become a powerful tool for targeted genome editing. Here, we report the use of the CRISPR/Cas9 system to disrupt selected genes, including *nanos2*, *nanos3*, *dmrt1*, and *foxl2*, with efficiencies as high as 95%. In addition, mutations in *dmrt1* and *foxl2* induced by CRISPR/Cas9 were efficiently transmitted through the germline to F₁. Obvious phenotypes were observed in the G0 generation after mutation of germ cell or somatic cell-specific genes. For example, loss of Nanos2 and Nanos3 in XY and XX fish resulted in germ cell-deficient gonads as demonstrated by GFP labeling and Vasa staining, respectively, while masculinization of somatic cells in both XY and XX gonads was demonstrated by Dmrt1 and Cyp11b2 immunohistochemistry and by up-regulation of serum androgen levels. Our data demonstrate that targeted, heritable gene editing can be achieved in tilapia, providing a convenient and effective approach for generating loss-of-function mutants. Furthermore, our study shows the utility of the CRISPR/Cas9 system for genetic engineering in non-model species like tilapia and potentially in many other teleost species.

RECENTLY, a simple and efficient genome editing technology, type II CRISPR/Cas9, has been developed based on the *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas9) adaptive immune system. It requires three components for effective DNA cleavage: the nuclease Cas9, a targeting CRISPR RNA (crRNA), and an additional trans-activating crRNA (tracrRNA) (Gasiunas *et al.* 2012; Jinek *et al.* 2012; Cho *et al.* 2013; Cong *et al.* 2013; Hwang *et al.* 2013; Mali *et al.* 2013). Further improvement of the system was achieved by fusing the crRNA and tracrRNA to form a single guide RNA (gRNA) that is sufficient to direct Cas9-mediated target cleavage (Hwang *et al.* 2013). Importantly, previous studies performed *in vitro* (Jinek *et al.* 2012), in bacteria (Jiang *et al.* 2013), and in human cells (Cong *et al.*

2013) have shown that Cas9-mediated cleavage can be abolished by single mismatch at the gRNA–target site interface, particularly in the last 10–12 nucleotides located in the 3' end of the 20-nt gRNA targeting region. Compared to the other two engineered nuclease genome-editing technologies, zinc-finger nucleases (ZFNs) (Urnov *et al.* 2005; Doyon *et al.* 2008) and transcription activator-like effector nucleases (TALENs) (Huang *et al.* 2011; Sander *et al.* 2011; Tesson *et al.* 2011), the CRISPR/Cas9 system is substantially less expensive and much easier to program for editing new target sites. This new approach has been widely used for genome engineering in model animals, including *Caenorhabditis elegans* (Dickinson *et al.* 2013; Friedland *et al.* 2013; Tzur *et al.* 2013), *Drosophila* (Bassett *et al.* 2013; Ren *et al.* 2013; Yu *et al.* 2013), zebrafish (Chang *et al.* 2013; Hruscha *et al.* 2013; Hwang *et al.* 2013), rat (W. Li *et al.* 2013) and mouse (Wang *et al.* 2013; Yang *et al.* 2013). The editing efficiencies of CRISPR/Cas9 in these species are similar to or surpass those obtained by ZFNs and TALENs. However, to date there are no reports showing the application of CRISPR/Cas9 in any non-model animals. As genome sequences become available for many more economically important, non-model species, development of an efficient and precise method becomes urgent.

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The Nile tilapia (*Oreochromis niloticus*), a gonochoristic teleost with a stable XX/XY sex determination system, has become one of the most important species in global aquaculture. It is also an important laboratory model for understanding the developmental genetic basis of sex determination. The availability of monosex populations, together with the whole-genome sequence of Nile tilapia, has made it much easier to study the genes involved in sex determination (Soler *et al.* 2010; M. H. Li *et al.* 2013). To date, numerous genes with conserved function in gonadal sex differentiation in vertebrates have been examined, but most of our knowledge comes from studying their expression patterns because no approaches were available for altering gene function. Here, we report development of the CRISPR/Cas9 system for genome editing in Nile tilapia. The simplicity, efficiency, and power of the CRISPR/Cas9 genome-editing system described in this study will allow mutations in a chosen gene to be generated within a short time, greatly facilitating the study of gene function in tilapia.

Materials and Methods

Fish

Nile tilapias, *O. niloticus*, were kept in recirculating freshwater tanks at 26° before use. All-XX and all-XY progenies were obtained by crossing the sex-reversed XX pseudomale and YY supermale with the normal female (XX), respectively. Animal experiments were conducted in accordance with the regulations of the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University.

gRNA design and transcription

The gRNA target sites were selected from sequences corresponding to GGN₁₈NGG on the sense or antisense strand of DNA (Chang *et al.* 2013). Candidate target sequences were compared to the entire tilapia genome using the Basic Local Alignment Search Tool (BLAST) to avoid cleavage of off-target sites. Any candidate sequences with perfectly matched off-target alignments [*i.e.*, the final 12 nt of the target and NGG protospacer adjacent motif (PAM) sequence] were discarded (Cong *et al.* 2013). For gRNA *in vitro* transcription, the DNA templates were obtained from the pMD19-T gRNA scaffold vector (kindly provided by J. W. Xiong, Peking University, Beijing, China) by polymerase chain reaction (PCR) amplification (Chang *et al.* 2013). The forward primer contained the T7 polymerase binding site, the 20-bp gRNA target sequence, and a partial sequence of gRNA scaffold. The reverse primer was located at the 3' end of the gRNA scaffold. *In vitro* transcription was performed with the Megascript T7 Kit (Ambion) for 4 hr at 37° using 300 ng purified DNA (PCR products) as template. The transcribed gRNA was purified and quantified using a NanoDrop-2000 (Thermo Scientific), diluted to 50 and 150 ng/μl in RNase-free water and stored at -80° until use.

Cas9 messenger RNA *in vitro* transcription

The Cas9 nuclease expression vector pcDNA3.1 (+) (Invitrogen) was used for *in vitro* transcription of the Cas9 messenger RNA (mRNA) as previously described (Chang *et al.* 2013). Plasmid templates were prepared using a plasmid midi kit, linearized with *Xba*I, and purified by ethanol precipitation. Cas9 mRNA was produced by *in vitro* transcription of 1 μg DNA using a T7 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions. The resulting mRNA was purified using the MegaClear Kit (Ambion), suspended in RNase-free water and quantified using a NanoDrop-2000.

Microinjection, genomic DNA extraction, and mutation detection assay

To determine the optimal quantity of gRNA and Cas9 mRNA, varying concentrations of both gRNA and Cas9 mRNA were microinjected into all XX or XY tilapia embryos at the one-cell stage (*nanos2* and *dmrt1* in XY embryos, *nanos3* and *foxl2* in XX embryos). The injected embryos were incubated at 26°, and survival rates were calculated at 10 days after hatching (dah). Twenty injected embryos were collected 72 hr after injection. The genomic DNA extracted from these pooled embryos was quantified using a NanoDrop-2000 and then used as template for PCR. DNA fragments spanning the target site for each gene were amplified using gene-specific primers (Table 1). The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen). A restriction enzyme cutting site (*Pci*I, *Bam*HI, *Cac*8I, and *Hpy*99I for *nanos2*, *nanos3*, *dmrt1*, and *foxl2*, respectively) adjacent to the NGG PAM sequence was selected to analyze the putative mutants by digestion of the amplified fragment. After restriction enzyme digestion (RED), the fragments were separated by gel electrophoresis. The uncleaved bands were recovered and subcloned and screened by PCR. The positive clones were sequenced and then aligned with the wild-type sequences to determine whether they were mutated. In addition, the percentage of uncleaved band was measured by quantifying the band intensity with Quantity One Software (Bio-Rad) (Henriques *et al.* 2012). The indel frequency was calculated by dividing uncleaved band intensity to the total band intensity from a single digestion experiment.

To screen the G0 fish, a piece of tail fin was clipped from each individual, and genomic DNA was extracted as described above. Target genomic loci were amplified using the primers listed in Table 1. Mutations were assessed by RED. For each target site, up to eight G0 animals were screened. The indel mutation frequency within each individual was also estimated by quantifying the band intensity of the restriction enzyme digestion.

Detection of heritable mutations

To investigate whether CRISPR/Cas9-mediated mutations were also induced in the germline and transmitted to subsequent

Table 1 Sequences of primers used in the present study

Primer	Sequence (5'–3')	Purpose
<i>nanos2</i> -Cas9-F	GGTCTTAAGAGGTCCTAAGG	Positive gene knockout fish screening
<i>nanos2</i> -Cas9-R	GGAAGTGTGGACCTTACTCCAG	
<i>nanos3</i> -Cas9-F	GGATCCAGTGGATGGTGTGGC	
<i>nanos3</i> -Cas9-R	GGCGTACACGGAGCTGTATGCG	
<i>dmrt1</i> -Cas9-F	GGTGATATCAACAGTTTATCTG	RT-PCR and <i>in situ</i> hybridization
<i>dmrt1</i> -Cas9-R	CCTGTGACAGCAGAGGTGGC	
<i>foxl2</i> -Cas9-F	GCGAGAGAAAGGGGAATTACTG	
<i>foxl2</i> -Cas9-R	GATGAGGGGGCTGACAGCCCCCT	
<i>nanos2</i> -ISH-F	CTGCTTTAACATGTGGCAGGAC	
<i>nanos2</i> -ISH-R	CAGAAAACCTTCCCCTCGTCTGA	
<i>nanos3</i> -ISH-F	GGCCTCGGAGCAGAGAGTGCGC	
<i>nanos3</i> -ISH-R	GTCTTATTGCTCCTTGCCACCTG	
M13+	CGCCAGGGTTTTCCAGTCACG	Sequencing and clone screening
M13–	AGCGGATAACAATTTACACAG	

generations, the *dmrt1* and *foxl2* mutant fish with the highest indel frequency were used as G0 founders. They were raised to sexual maturity and mated with wild-type tilapia. F₁ larvae were collected at 10 dah and genotyped by PCR amplification and subsequent *Cac8I* and *Hpy99I* digestion. The uncleaved band was purified, subcloned into the pMD-19T vector, and sequenced to confirm the mutation.

Preparation of enhanced GFP-vasa 3' UTR mRNA and germ-cell labeling

The T7 polymerase binding site and three restriction cutting sites, *XhoI*, *BglII*, and *NotI*, were introduced at the 5' and 3' ends of the enhanced GFP (eGFP) ORF by PCR using pTOL2 (Stratagene) plasmids as template with forward (5'-TAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAGC-3'; underlining represents the T7 polymerase binding site) and reverse (5'-CTCGAGAGATCTGCGGC CGGATCTAGAGGATCATAATCAG-3'; underlining represents *XhoI*, *BglII*, and *NotI* sites) primers. The amplified PCR products were cloned into the pMD-19T vector to create the eGFP pMD-19T constructs. The Nile tilapia vasa 3' UTR (280 bp) was amplified by PCR using its complementary DNA clone as template with a forward primer designed after the termination codon (5'-GCGGCCGCGAGCAGCGCAGTCA CACAGCAATG-3'; underlining represents the *NotI* site) and reverse primer flanking the poly(A) tail (5'-AGAT CTGGCCGAGGCGGCCGACATG-3'; underlining represents the *BglII* site). The amplified PCR products were cloned into the eGFP pMD-19T construct after digestion with *NotI* and *BglII*. The eGFP-vasa-3' UTR plasmid was linearized using *XhoI* (Takara) and used for *in vitro* transcription using a T7 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's instructions. RNA was purified and dissolved in RNase-free water at a final concentration of 200 ng/μl. A total of 100 pg of RNA solution was microinjected into the animal pole of one-cell-stage embryos after fertilization. For each fish, 300 eggs were microinjected, and at least 30 randomly selected embryos were used for fluorescent observation.

Germ-cell labeling with GFP and Nanos2 and Nanos3 mutation by CRISPR/Cas9

eGFP-vasa 3' UTR mRNA, *nanos2* or *nanos3* gRNA, and Cas9 mRNA were co-injected into the XY or XX one-cell-stage fertilized eggs. Control injection used only eGFP-vasa 3' UTR mRNA. The absence of fluorescent germ cells in the gonads was confirmed at 72 hr postfertilization by fluorescence microstereoscopy. Embryo with no GFP observed was raised for 2 or 3 months. In addition, mutant animals were further assessed by RED and Sanger sequencing (SS). Gonads of 60 or 90 dah fish from the *nanos2* or *nanos3* targeted group and the control group were dissected and fixed in Bouin's solution for 24 hr. They were subsequently dehydrated, embedded in paraffin, and then serially sectioned to a 5-μm thickness. The sections were stained with hematoxylin–eosin or with immunohistochemistry (IHC) counterstained with hematoxylin and visualized to confirm the ablation of germ cells.

Immunohistochemistry

Expression of Vasa, Cyp19a1a, Cyp11b2, and Dmrt1 was analyzed in mutant gonads by IHC, which was performed as described previously (M. H. Li *et al.* 2013).

Measurement of steroid hormones

Serum E2 (estradiol-17β) and 11-ketotestosterone (11-KT; the native androgen in most teleosts) levels were measured using the Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI). Sample purification and assays were performed according to the manufacturer's instructions.

Results

Efficient and heritable site-directed disruption of tilapia genes by CRISPR/Cas9

nanos2, *nanos3*, *foxl2*, and *dmrt1* were selected as targets to demonstrate the feasibility of CRISPR/Cas9-mediated mutagenesis in tilapia. First, gRNAs containing restriction enzyme

Figure 1 Efficient disruption of tilapia genes by CRISPR/Cas9. *nanos3* (A), *nanos2* (B), *foxl2* (C), and *dmrt1* (D) were selected as targets to demonstrate the feasibility of CRISPR/Cas9-mediated mutagenesis. gRNA was designed in the coding sequence of target containing a restriction enzyme cutting site (underlined). *In vitro*-synthesized 500 ng/ μ l of Cas9 mRNA and 50 ng/ μ l of gRNA were co-injected into one-cell-stage embryos. At 72 hr after injection, 20 embryos were randomly selected and pooled to extract their genomic DNA for PCR amplification, and the indels were confirmed with two assays, restriction enzyme digestion and Sanger sequencing. The Cas9 and gRNA were added as indicated. For each gene, two cleavage bands were detected in the control group, while an intact DNA fragment (indicated by white arrowheads) was observed in embryos injected with both Cas9 mRNA and target gRNA. The percentage of uncleaved band was measured by quantifying band intensity. The indel frequency was obtained from a single digestion experiment. Sanger sequencing results from the uncleaved bands are listed. Substitutions are marked in lowercase letters, deletions and insertions by dashes and blue letters. The protospacer adjacent motif (PAM) is highlighted in green. Numbers to the right of the sequences indicate the loss or gain of bases for each allele, with the number of bases inserted (+) or deleted (–) indicated in parentheses. WT, wild type.

sites were designed based on the coding sequences of these genes. Then, *in vitro*-synthesized Cas9 mRNA and gRNA were microinjected into fertilized one-cell eggs. At 72 hr after injection, 20 embryos were randomly selected and pooled to extract their genomic DNA for PCR amplification, and the insertion and deletion (indels) were confirmed by RED and SS. Complete digestion with a selected restriction enzyme produced two fragments in the control group while an intact DNA fragment was observed in embryos injected with both Cas9 mRNA and target gRNA. In-frame and frameshift deletions induced at the target site were confirmed by SS. Finally, the mutation frequency of the target gene was calculated by quantifying band intensity in one

RED. The indel frequencies of these genes in pools of 20 embryos reached 38% (*nanos2*), 49% (*nanos3*), 42% (*foxl2*), and 22% (*dmrt1*), respectively (Figure 1).

To determine the optimal quantity of gRNA and Cas9 mRNA to be used for gene editing, combinations of various concentrations of gRNA and Cas9 mRNA for genome editing were microinjected into fertilized one-cell eggs. All four combinations resulted in indels. With the decrease in mRNA concentration, the survival rate following injection increased from 7 to 33% in *nanos2*, while the proportion of indel mutation rate decreased from 52 to 13% (Table 2). The efficiency of mutation was Cas9 mRNA concentration dependent. The optimal mutation rate for *nanos2* was obtained

Table 2 Mutagenesis is Cas9 mRNA concentration dependent

Gene	gRNA/Cas9 concentration (ng/ μ l)	No. of injected embryos	No. survived	Survival rate (%)	Mutation rate (%)
<i>nanos2</i>	50/100	300	100	33	13
<i>nanos2</i>	50/300	300	66	22	24
<i>nanos2</i>	50/500	300	38	12.60	51
<i>nanos2</i>	150/800	300	21	7.00	52
<i>nanos3</i>	50/100	300	81	27	8
<i>nanos3</i>	50/300	300	65	21	19
<i>nanos3</i>	50/500	300	22	7	38
<i>nanos3</i>	150/800	300	15	5	36

Various concentrations of gRNA and Cas9 mRNA were used to induce target gene mutation. Indel frequency was estimated by quantifying the band intensity of the restriction enzyme digestion of pooled genomic DNA from up to 20 embryos. Survival rate of embryos was calculated at 14 days after injection.

with 50 ng/ μ l gRNA and 500 ng/ μ l Cas9 mRNA, while the concentration also resulted in the highest toxicity as shown by the percentage of embryos that died after injection (Table 2). The same results were obtained in *nanos3* (Table 2).

To investigate whether CRISPR/Cas9-mediated mutations can be transmitted to subsequent generations, G0 founders were screened by RED and SS (Figure 2). The *dmrt1* and *foxl2* mutant fish with a high mutation rate (>85%) were raised to sexual maturity and mated with

wild-type tilapia. Mutations were transmitted to their F₁ progeny at a rate of 22.2% (4 of 18 for *dmrt1*) and 58.3% (10 of 24 for *foxl2*), respectively. The F₁ *foxl2* larvae carried deletion mutations including in-frame and frameshift deletions as their G0 founders. In contrast, the F₁ *dmrt1* larvae carried only 3- or 21-bp in-frame deletions, the same as found in the sperm used for fertilization but different from the G0 founders that carried both in-frame and frameshift deletions (Figure 2).

Figure 2 CRISPR/Cas9-induced mutations are transmitted efficiently through the germline to the F₁. *dmrt1* (A) and *foxl2* (B) mutant fish were screened as founders by restriction enzyme digestion. The mutation rates of *dmrt1* and *foxl2* induced by CRISPR/Cas9 were >85% as quantified the band intensity. DNA sequencing confirmed that the uncleaved band, indicated by white arrowheads, had various mutant sequences. Deletions are indicated by dashes. The numbers at the right side show the number of deleted (–) base pairs. The *dmrt1* and *foxl2* mutant fish was raised to sexual maturity and mated with wild-type tilapia. F₁ larvae were collected 10 dah and genotyped by PCR amplification and subsequent *Cac81* and *Hpy99I* digestion using genomic DNA extracted from each F₁ larva. The percentage of wild-type and CRISPR/Cas9-disrupted alleles in F₁ tilapias was derived from the number of mutated fish among the fish screened. The transmission rates were 22.2% (4 of 18 for *dmrt1*) and 58.3% (10 of 24 for *foxl2*), respectively. WT, wild type; n, the number of F₁ fish examined. The mutation sequences in the F₁ tilapias are listed. The F₁ *foxl2* larvae carried deletion mutations including in-frame and frameshift deletions. In contrast, the F₁ *dmrt1* larvae carried only 3- or 21-bp in-frame deletions.

Table 3 Mutation rates of four tilapia genes induced by CRISPR/Cas9

Gene	No. of G0 analyzed	No. of mutants	Frequency (%)	Indel mutation frequency (%)							
				#1	#2	#3	#4	#5	#6	#7	#8
<i>nanos2</i>	26	8	31	51	67	43	66	32	87	95	92
<i>nanos3</i>	33	8	24	44	71	77	69	58	91	86	89
<i>dmrt1</i>	18	8	44	31	48	90	85	72	67	81	84
<i>foxl2</i>	16	8	50	29	52	61	75	45	95	90	86

For each gene, G0 fish were screened until exactly eight mutants were found. The indel mutation frequency within each individual was estimated by quantifying the band intensity of the restriction enzyme digestion.

Screening of the gRNA- and Cas9 mRNA-injected fish (G0) showed average mutation rates of 31% (8 of 26) for *nanos2*, 24% (8 of 33) for *nanos3*, 44% (8 of 18) for *dmrt1*, and 50% (8 of 16) for *foxl2* (Table 3). The mutation rates were estimated to be in the range of 18–95% by quantifying the band intensity of restriction enzyme digests for each of the four genes. The maximum mutation efficiency reached was 95% in *nanos2* and *foxl2*.

Phenotypes of gene mutation induced by CRISPR/Cas9 in tilapia

In agreement with the gonadal phenotype of *Dmrt1* and *Foxl2* deficiency induced by TALENs (M. H. Li *et al.* 2013), *foxl2* mutations induced by Cas9/gRNA lead to downregulation of aromatase expression and sex reversal. *Dmrt1* deficiency resulted in up-regulation of aromatase expression in the testis (data not shown).

In the present study, *nanos2* and *nanos3* were found to be expressed in male and female germ cells, respectively, by tissue distribution, ontogeny, and *in situ* hybridization analyses (Supporting Information, Figure S1, File S1). eGFP-*vasa* 3' UTR RNA was transcribed *in vitro* to observe the effects of *nanos2* and *nanos3* mutation in germ cells. In the control group, GFP-labeled germ cells were located along the axis on both sides of the embryo 72 hr after injection (Figure 3, A and C). In contrast, no GFP was observed after co-injection of eGFP-*vasa* 3' UTR mRNA, *nanos3* gRNA, and Cas9 mRNA in XX embryos (Figure 3B). The embryos with no GFP were raised to 2 months old. Gonads of the *nanos3* mutant XX G0 fish displayed a single tube-like structure with no germ cells observed in histological sections (Figure 3, E–H). This result was further confirmed by IHC with *Vasa*, a germ-cell marker (Figure 3, E and M). Among the G0 *nanos3* mutant XX tilapia examined ($n = 10$), 40% ($n = 4/10$) of individuals did not possess germ cells in the gonads. The germ-cell-less *nanos3* mutant XX gonads experienced female-to-male sex reversal. IHC of these gonads identified expression of *Dmrt1* (a Sertoli cell marker) (Figure 3, G and O) and *Cyp11b2* (a Leydig cell marker, the key enzyme responsible for the production of the androgen 11-KT) (Figure 3, H and P). However, like the control testis (Figure 3F) but unlike the control ovary (Figure 3N), the *nanos3* mutant XX gonads displayed no *Cyp19a1a* (aromatase, the key enzyme responsible for the production of the estrogen estradiol-17 β) expression. Con-

sistent with the *Cyp19a1a* and *Cyp11b2* IHC results, *nanos3* mutant XX fish showed lower serum E2 and higher 11-KT compared with the XX control (Figure 4). On the other hand, co-injection of eGFP-*vasa* 3' UTR, *nanos2* gRNA, and Cas9 mRNA also led to germ-cell ablation in the XY testis, which was further demonstrated by GFP (Figure 3D) and anti-*Vasa* IHC (Figure 3, I–L). The gonads of *nanos2*-deficient XY fish also showed a single tube-like structure, and displayed no sex reversal as revealed by IHC for *Dmrt1* and *Cyp11b2* expression in the Sertoli cells and Leydig cells (Figure 3, K and L). Among the G0 *nanos2* mutant XY tilapia examined ($n = 16$), 18% ($n = 3/16$) of individuals did not possess germ cells in the gonads.

Discussion

Reverse genetics approaches have been important in demonstrating gene functions, genetic engineering, and understanding complex biological processes. In the present study, we successfully established the CRISPR/Cas9 technique to create targeted mutations with high efficiency in tilapia. Targeted mutagenesis was successfully obtained in four genes (*nanos2*, *nanos3*, *dmrt1*, and *foxl2*), demonstrating the broad applicability of this technology in tilapia genome editing. To our knowledge, this is the first report on targeted disruption of endogenous genes in tilapia as well as in non-model teleosts using CRISPR/Cas9. In addition, gRNA is the only component that needs customization for each target, thus greatly simplifying the design and lowering the cost of gene targeting. This allows the production of a desired mutation within a short time, thereby permitting future high-throughput analyses of gene function.

Successful germline transmission is essential for establishment of knockout lines. In this study, *foxl2* and *dmrt1* mutations induced by CRISPR/Cas9 were efficiently transmitted through the germline to F₁ in tilapia, which indicated that CRISPR/Cas9-induced gene disruption in tilapia is heritable. The F₁ *foxl2* larvae carried deletion mutations including in-frame and frameshift deletions like their G0 founders. In contrast, the F₁ *dmrt1* larvae carried only 3- or 21-bp in-frame deletions, the same as those found in the sperm used for fertilization, but different from the G0 founders that carried both in-frame and frameshift deletions. It has been reported that loss of *Dmrt1* in mice embryos disrupts germ-cell development, especially in terms of mitotic reactivation,

Figure 3 Mutation of *nanos2* and *nanos3* by CRISPR/Cas9 resulted in germ-cell-deficient gonads. *In vitro*-synthesized eGFP-*vasa* 3' UTR mRNA was injected into fertilized eggs to label germ cells. GFP-labeled germ cells were located in the gonadal primordium (box') in the normal XX and XY embryos at 72 hr postfertilization (A and C) while no GFP-labeled germ cells were observed in embryos co-injected with *nanos3* (B) or *nanos2* (D) gRNA, Cas9, and eGFP-*vasa* 3' UTR mRNA at the same stage. (A', B', C', and D') Magnification of the boxed areas in A, B, C, and D, respectively. (E–L) By histology, both gonads from *nanos3* (60 dah) and *nanos2* (90 dah) mutant fish displayed a single tube-like structure with no germ cells, different from control XX ovary (N) and XY testis (M, O, P), which contained germ cells at different developmental stages. The absence of germ cells in mutant gonads was further confirmed by immunohistochemistry with anti-Vasa, a germ-cell marker, which was observed in control XY testis (M), but not detected in *nanos3* (E) or *nanos2* (I) mutant gonads. Cyp19a1a was expressed in control XX ovary (N), but not expressed in the germ-cell-deficient XX (F) and XY (J) gonads. Dmrt1, which was expressed in Sertoli

cells of control XY testis (O), was detected in both germ-cell-deficient XX (G) and XY (K) gonads. Similarly, Cyp11b2, which was detected in Leydig cells of control XY testis (P), was also detected in germ-cell-deficient XX (H) and XY gonads (L). Bar in E and I–L, 15 μ m; in F–H and M–P, 10 μ m.

meiosis initiation, and germ-cell survival (Kim *et al.* 2007; Matson *et al.* 2010). Therefore, frameshift deletions in *Dmrt1* in tilapia germ cells probably affect their development, meiosis, and maturation in tilapia. The mechanism underlying this phenomenon needs further investigation. Additionally, this may explain the fact that the transmission rate of the *dmrt1* mutation (22.2%) was much lower than that of *foxl2* (58.3%), even though the mutation rate of G0 flounder of both *dmrt1* and *foxl2* was nearly the same.

Based on our observations, the maximum efficiency of mutation induced by CRISPR/Cas9 was up to 95%, suggesting that both alleles were disrupted in most of the cells. As reported in *Drosophila* (Bassett *et al.* 2013) and zebrafish (Jao *et al.* 2013), the high frequency of induced mutation resulted in phenotypes in G0 founders. Just because there is an indel at a genetic locus does not necessarily lead to a loss of function. Indeed, some of the mutations are likely in-frame, which might not reduce gene function at all. However, most of *nanos2* and *nanos3* mutations induced by CRISPR/Cas9 were frameshift indels, and these mutations generated obvious phenotypes. Previously, the *dmrt1* and *foxl2* loci had been successfully mutated by TALENs and produced obvious phenotypes (M. H. Li *et al.* 2013). In this report, mutation of *dmrt1* and *foxl2* induced by Cas9/gRNA lead to the same phenotypes as mutation of the two genes

induced by TALEN, indicating that the CRISPR/Cas9 system can serve as a more rapid alternative strategy for loss-of-function studies.

In this study, *nanos2* and *nanos3*, which are specifically expressed germ cells of the testis and ovary, respectively, were mutated by Cas9/gRNA. Germ cells were lost in the gonads after *nanos2* and *nanos3* mutation, as demonstrated by GFP labeling and Vasa staining. In line with the results obtained from medaka and zebrafish (Slanchev *et al.* 2005; Kurokawa *et al.* 2007), but contrary to those from goldfish and loach (Fujimoto *et al.* 2010; Goto *et al.* 2012), our study showed that germ-cell-deficient XX tilapia displayed female-to-male sex reversal after *nanos3* mutation. In contrast, Cyp19a1a, an ovarian-specific gene, was not detected in *nanos3* mutant XX gonads. On the other hand, germ-cell deficiency in XY tilapia testis did not affect the sex differentiation in somatic cells, which is consistent with the results from the four fishes mentioned above (Slanchev *et al.* 2005; Kurokawa *et al.* 2007; Fujimoto *et al.* 2010; Goto *et al.* 2012). Together, these results demonstrate that the effects of germ-cell ablation gonadal fate are species-specific.

Previous reports indicated that mutations induced by CRISPR/Cas9 showed high specificity with few or no off-target events (Bassett *et al.* 2013; Jao *et al.* 2013; Ren *et al.* 2013; Wang *et al.* 2013). Therefore, in the present study, no

Figure 4 Impact of *nanos3* deficiency on tilapia serum E2 and 11-KT levels. Knockout of *nanos3* in the XX fish resulted in elevated 11-KT and decreased E2, compared with the control fish. Results are presented as the means $\pm$ SD. Bars bearing different letters differ ($P < 0.05$) by one-way ANOVA. Sample numbers are shown.

experiment was performed to determine such events. Instead, to avoid any possible off-target events, CRISPR/Cas9 target sites were strictly selected and analyzed within the tilapia genome using a BLAST search. Sequences that perfectly matched the final 12 nt of the target and NGG PAM sequence were strictly discarded (Cong *et al.* 2013). However, off-target effects are very complicated in Cas9/CRISPR systems. Many off-target cutting sites are not highly homologous to the target sequences (Fu *et al.* 2013). Therefore, off-target events may not be completely excluded by genome BLAST approach.

In summary, we demonstrated successful targeted mutagenesis in non-model animal tilapia using CRISPR/Cas9. Mutations in *foxl2* and *dmrt1* induced by CRISPR/Cas9 were efficiently transmitted through the germline to the F₁ generation. In addition, obvious phenotypes were observed in the G0 generation after mutation of germ-cell- or somatic-cell-specific genes. Our study goes beyond model animals and shows the utility of the CRISPR/Cas9 as an efficient tool in generating genetically engineered tilapia, and potentially other aquacultured fish, with high efficiency. Taken together, our data demonstrate that targeted, heritable gene editing can be achieved in tilapia, providing a convenient and effective approach for generating loss-of-function mutants.

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Literature Cited

- Bassett, A. R., C. Tibbit, C. P. Ponting, and J. L. Liu, 2013 Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell Rep.* 4: 220–228.
- Chang, N., C. Sun, L. Gao, D. Zhu, X. Xu *et al.*, 2013 Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. *Cell Res.* 23: 465–472.
- Cho, S. W., S. Kim, J. M. Kim, and J. S. Kim, 2013 Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31: 230–232.
- Cong, L., F. A. Ran, D. Cox, S. Lin, R. Barretto *et al.*, 2013 Multiplex genome engineering using CRISPR/Cas systems. *Science* 339: 819–823.
- Dickinson, D. J., J. D. Ward, D. J. Reiner, and B. Goldstein, 2013 Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat. Methods* 10: 1028–1034.
- Doyon, Y., J. M. McCammon, J. C. Miller, F. Faraji, C. Ngo *et al.*, 2008 Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.* 26: 702–708.
- Friedland, A. E., Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church *et al.*, 2013 Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nat. Methods* 10: 741–743.
- Fu, Y., J. A. Foden, C. Khayter, M. L. Maeder, D. Reyon *et al.*, 2013 High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31: 822–826.
- Fujimoto, T., T. Nishimura, R. Goto-Kazeto, Y. Kawakami, E. Yamaha *et al.*, 2010 Sexual dimorphism of gonadal structure and gene expression in germ cell-deficient loach, a teleost fish. *Proc. Natl. Acad. Sci. USA* 107: 17211–17216.
- Gasiunas, G., R. Barrangou, P. Horvath, and V. Siksnys, 2012 Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA* 109: E2579–E2586.
- Goto, R., T. Saito, T. Takeda, T. Fujimoto, M. Takagi *et al.*, 2012 Germ cells are not the primary factor for sexual fate determination in goldfish. *Dev. Biol.* 370: 98–109.
- Henriques, A., F. Carvalho, R. Pombinho, O. Reis, S. Sousa *et al.*, 2012 PCR-based screening of targeted mutants for the fast and simultaneous identification of bacterial virulence factors. *Biotechniques*. DOI: .10.2144/000113906
- Hruscha, A., P. Krawitz, A. Reichenberg, V. Heinrich, J. Hecht *et al.*, 2013 Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development* 140: 4982–4987.
- Huang, P., A. Xiao, M. Zhou, Z. Zhu, S. Lin *et al.*, 2011 Heritable gene targeting in zebrafish using customized TALENs. *Nat. Biotechnol.* 29: 699–700.
- Hwang, W. Y., Y. Fu, D. Reyon, M. L. Maeder, S. Q. Tsai *et al.*, 2013 Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.* 31: 227–229.
- Jao, L. E., S. R. Wentz, and W. Chen, 2013 Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc. Natl. Acad. Sci. USA* 110: 13904–13909.
- Jiang, W., D. Bikard, D. Cox, F. Zhang, and L. A. Marraffini, 2013 RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat. Biotechnol.* 31: 233–239.
- Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna *et al.*, 2012 A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337: 816–821.
- Kim, S., V. J. Bardwell, and D. Zarkower, 2007 Cell type-autonomous and non-autonomous requirements for *Dmrt1* in postnatal testis differentiation. *Dev. Biol.* 307: 314–327.
- Kurokawa, H., D. Saito, S. Nakamura, Y. Katoh-Fukui, K. Ohta *et al.*, 2007 Germ cells are essential for sexual dimorphism in the medaka gonad. *Proc. Natl. Acad. Sci. USA* 104: 16958–16963.

- Li, M. H., H. H. Yang, M. R. Li, Y. L. Sun, X. L. Jiang *et al.*, 2013 Antagonistic roles of Dmrt1 and Foxl2 in sex differentiation via estrogen production in tilapia as demonstrated by TALENs. *Endocrinology* 154: 4814–4825.
- Li, W., F. Teng, T. Li, and Q. Zhou, 2013 Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat. Biotechnol.* 31: 684–686.
- Mali, P., L. Yang, K. M. Esvelt, J. Aach, M. Guell *et al.*, 2013 RNA-guided human genome engineering via Cas9. *Science* 339: 823–826.
- Matson, C. K., M. W. Murphy, M. D. Griswold, S. Yoshida, V. J. Bardwell *et al.*, 2010 The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis vs. meiosis decision in male germ cells. *Dev. Cell* 19: 612–624.
- Ren, X., J. Sun, B. E. Housden, Y. Hu, C. Roesel *et al.*, 2013 Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. *Proc. Natl. Acad. Sci. USA* 110: 19012–19017.
- Sander, J., D. L. Cade, C. Khayter, D. Reyon, R. T. Peterson *et al.*, 2011 Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nat. Biotechnol.* 29: 697–708.
- Slanchev, K., J. Stebler, G. de la Cueva-Méndez, and E. Raz, 2005 Development without germ cells: the role of the germ line in zebrafish sex differentiation. *Proc. Natl. Acad. Sci. USA* 102: 4074–4079.
- Soler, L., M. A. Conte, T. Katagiri, A. E. Howe, B. Y. Lee *et al.*, 2010 Comparative physical maps derived from BAC end sequences of tilapia (*Oreochromis niloticus*). *BMC Genomics* 11: 636.
- Tesson, L., C. Usal, S. Ménoret, E. Leung, B. J. Niles *et al.*, 2011 Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.* 29: 695–706.
- Tzur, Y. B., A. E. Friedland, S. Nadarajan, G. M. Church, J. A. Calarco *et al.*, 2013 Heritable custom genomic modifications in *Caenorhabditis elegans* via a CRISPR-Cas9 system. *Genetics* 95: 1181–1185.
- Urnov, F. D., J. C. Miller, Y. L. Lee, C. M. Beausejour, J. M. Rock *et al.*, 2005 Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435: 646–651.
- Wang, H., H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng *et al.*, 2013 One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153: 910–918.
- Yang, H., H. Wang, C. S. Shivalila, A. W. Cheng, L. Shi *et al.*, 2013 One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154: 1370–1379.
- Yu, Z., M. Ren, Z. Wang, B. Zhang, Y. S. Rong *et al.*, 2013 Highly efficient genome modifications mediated by CRISPR/Cas9 in *Drosophila*. *Genetics* 195: 289–291.

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GENETICS

Supporting Information

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Efficient and Heritable Gene Targeting in Tilapia by CRISPR/Cas9

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Figure S1 Specific expression of *nanos2* and *nanos3* in germ cells of XY and XX gonad, respectively. Tissue distribution and early ontogenic expression of *nanos2* (A) and *nanos3* (F) in tilapia was investigated by RT-PCR. *nanos2* was specifically expressed in the testis but not in the ovary. In addition, *nanos2* was also expressed in XY embryos from 36 to 72 hours post fertilization (hpf). β -actin was used as an internal control. P, positive control; N, negative control. *In situ* hybridization analysis showed that *nanos2* was specifically expressed in male germ cells in testis (B, C), but not in ovary (D). *nanos3* was specifically expressed in the ovary but not in the testis, and also highly expressed XX embryos from 36 to 72 hpf (F). *In situ* hybridization analysis showed that *nanos3* was specifically expressed in oocytes in ovary (G, H), but not in testis (I). E, J, *nanos2* and *nanos3* sense probe, respectively. O, ovary; T, testis. Scale bar, B, G, 10 μ m; C, E, 100 μ m; D, I, 50 μ m; H, J, 15 μ m.

Supplemental method

***In situ* hybridization**

Gonads from 30, 60 and 180 dah tilapia were dissected and fixed in 4% paraformaldehyde in 0.1M phosphate buffer at 4°C overnight. After fixation, gonads were embedded in paraffin. Cross-sections were cut at 5µm. Probes of sense and antisense digoxigenin (DIG)-labeled RNA strands were transcribed *in vitro* from linearized plasmid containing partial ORF (open reading frame) of *nanos2* and *nanos3*, using an RNA labeling kit (Roche, Germany). *In situ* hybridization was performed as follows: sections were deparaffinized, hydrated and treated with proteinase K (10 mg/ml) and then hybridized with the sense or antisense DIG-labeled RNA probe at 60°C for 18–24 hrs. The hybridization signals were then detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche, Germany) and NBT as the chromogen.

RT-PCR

Total RNAs (2.0 µg) were isolated from testis and ovary tissues of adults (180 dah), and embryos from 36 to 72 hours post fertilization. Thereafter, total RNAs were treated with DNase I to eliminate the genomic DNA contamination. Then first strand cDNAs were synthesized and RT-PCR was carried out to check the expression of tilapia *nanos2* and *nanos3*. The templates for positive and negative controls were set with plasmid DNA containing *nanos2* or *nanos3* and deionized water, respectively. A 342-bp fragment of b-actin was amplified as internal control to test the quality of the cDNAs used in the PCR. The PCR products were subjected to agarose gel (1.2%) electrophoresis.