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Research Article

Generation of Ectodysplasin A *(eda)*-targeted Knockout Zebrafish Via the CRISPR/Cas9 System

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Abstract

Ectodysplasin A (EDA) plays a vital role in the development of skin appendages, especially in fish scales. Zebrafish model with the mutation of *eda* was found using CRISPR/Cas9 system. CRISPR/Cas9 nucleases targeting to two loci in exon 4 of *eda*, were constructed and injected into zebrafish embryos, respectively. CRISPR-Cas9 mediated mutation frequency toward eda exon 4 was approximately 16%, which was relatively low compared with that of other genes in zebrafish. Five *eda* mutant types were obtained in F0 generation including a deletion of 5 bp, 6 bp, 8 bp and 87 bp, and an insertion of 11 bp around the targeted site respectively, and all of which happened just in one allele. But the scales of all F0 founders were normal compared with their wild counterparts. In the F1 generation, five scale loss mutants with few scales covered were achieved that were all caused by bi-allelic 11-bp insertion in *eda*. The insertion results in frameshift mutation of *eda* and leads to loss of expression and function inactivation of EDA as determined by western blotting. This provides a good model for elucidating the function of EDA in the development of fish scales.

Keywords: *Gene knockout, eda, Zebrafish mutant, Scale loss*

CRISPR/Cas9 Sistemi Kullanılarak Ectodysplasin A *(eda)* İfade Etmeyen Zebra Balığı Üretimi

Öz

Ektodisplasin A (EDA) deri uzantılarının gelişiminde, özellikle balık pullarında, hayati bir rol oynar. CRISPR/Cas9 sistemi kullanılarak *eda* mutasyonlu zebra balığı modeli bulundu. *Eda*'nın ekson 4'ünde iki lokusu hedefleyen CRISPR/Cas9 nükleazları oluşturuldu ve zebra balığı embriyolarına enjekte edildi. *Eda* ekson 4'e yönelik CRISPR-Cas9 aracılı mutasyon sıklığı yaklaşık %16 idi; bu, zebra balığındaki diğer genlerinkine kıyasla nispeten düşüktü. F0 jenerasyonunda, hedeflenen bölge etrafında sırasıyla 5 bp, 6 bp, 8 bp ve 87 bp'lik bir silme ve 11 bp'lik bir ekleme dahil olmak üzere beş *eda* mutant türü elde edildi ve bunların hepsi sadece bir allelde gerçekleşti. Ancak tüm F0 üyelerinin pulları yabani akranlarına kıyasla normaldi. F1 jenerasyonunda, tamamı *eda*'ya bi-allelik 11-bp ilavesinden kaynaklanan az sayıda pulla çevrili beş adet pulsuz mutant elde edildi. Ekleme, *eda*'nın çerçeve kayması mutasyonuna neden olur ve Western Blot tekniği ile belirlendiği üzere EDA'nın ekspresyonunda azalma ve fonksiyon inaktivasyonuna yol açar. Bu çalışma, balık pullarının gelişiminde EDA'nın işlevini açıklamak için iyi bir model oluşturmaktadır.

Anahtar sözcükler: *Gen nakavt, eda, Zebra balığı mutantı, Pul kaybı*

INTRODUCTION

In the last decade, the emergence of three major genome-engineering technologies have provided efficient support for site-specific modification of genes in various species [1]. The core principle of engineered zinc-finger nuclease (ZFN), transcription activator like effector nuclease (TALEN), and clustered regularly interspaced short palindromic

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repeats (CRISPR)/CRISPR-associated Cas9 is recognizing and introducing double-strand breaks (DSBs) in targeted region of the genome precisely. In comparison, CRISPR/Cas9 systems make this process much more feasible and efficient.

Ectodysplasin A (EDA) belongs to TNF family which involved in the development of various structures derived from the ectoderm, including hair, teeth, sweat glands, feathers, armor plates, and scales. The EDA protein contains four transmembrane region (TM) domains, a furin consensus cleavage site, a collagen like domain (CL), and a tumor necrosis factor domain (TNF) [2]. In humans, X-linked hypohidrotic ecto-dermal dysplasia (XLHED) is a rare, inherited disorder that has been observed globally [3]. The phenotypic characteristics associated with this disorder include sparse hair, abnormal or missing teeth, and inability to sweat as a result of absent sweat glands. Several different inheritance patterns have been observed in relation to XLHED. The majority of these cases were caused by mutations in the *eda*1 gene [4]. In mice, Srivastava et al.[5] found that the tabby phenotype (characteristic hair defects, tooth abnormalities, and eccrine sweat gland morphology) was caused by a mutation in *eda*. *eda* mutants in above studies associated the development defects of skin attachment and accumulation with the TNF regions. In zebrafish, Harris et al.[6] screened a similar scale loss mutant caused by a mutation location in the TNF domain of EDA, demonstrating that TNF domain played a vital role in the development of skin appendages. Nevertheless, several in-frame deletions have been observed in HED patients and Schizothoracinae fishes [7-10], indicating that the collagen-like domain also harbored other important functions which are still unknown.

Therefore, in order to determine the function of other domains in the EDA protein except TNF, we employed CRISPR/Cas9 systems to induce mutation in the *eda* of zebrafish and achieved the scale loss mutants. It appears that it will provide a good model for researching the genetic mechanisms of skin appendage development, drug screening, and gene function identification.

MATERIAL and METHODS

Targeted Sites Design

In order to determine the role of EDA CL domain in fish scale development, we designed a CRISPR/Cas9 system to knockout *eda* in the zebrafish genome. According to the sequence alignment results, the CL domain is coded by exon 4 in zebrafish EDA. Therefore, two target sites were selected *(Fig. 1)* at the *eda* exon 4 locus based on online ZIFIT software *(http://zifit.partners.org/ZiFiT/)*. Accordingly two small guide RNAs targeting to the screened sites were designed namely gRNA-1(TTAGGCAAGAAAGGGCCCCCTGG) and gRNA -2(AGCAACGCCATGGGTCCCTCTGG)

Plasmid Construction and RNA Synthesis

According to Hwang et al.[11], the plasmids required to practice CRISPR-Cas/RNA-Guided Nuclease (RGN) technology in zebrafish include Cas9 expression vector and guide RNA expression vector. The Cas9 expression plasmid MLM3613 and the guide RNA expression vector were a gift from Keith Joung (Addgene plasmid # 42251 and # 42250; http://n2t.net/addgene: 42251, 42250; RRID: Addgene_42251 and 42250). The methods used to facilitate plasmid construction and RNA synthesis are as follows [11]. In brief, the guide RNAs gRNA -1 and gRNA -2 (comprising a 20-bp target sequence complementary to the genomic target adjacent to a protospacer adjacent motif (PAM) with a sequence of NGG) toward the selected targeting sites were cloned into the pDR274 vector cut by *Bsa*I. The obtained plasmid were named as pDR274-eda1 and pDR274-eda2 which encoding sgRNA-1 and sgRNA-2, respectively. Cas9 mRNA was transcribed using *Pme*I-digested pMLM3613 as template by mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies). sgRNA-1 and sgRNA-2 were transcribed using *Dra*I-digested pDR274-*eda*1 and pDR274-*eda*2 as templates by the MAXIscript T7 kit (Life Technologies).

Experimental Animals

According to the standard of zebrafish breeding, adult zebrafish were bred at 28°C in circulating water on a 14 h light cycle followed by a 10 h dark cycle and fed twice daily. The injected eggs were incubated at 28.5°C in an embryo medium, to the larvae out of the membrane and the yolk sac disappear, then the larvae were fed with yolk as opening bait twice-daily. All animal experiments were approved by the Animal Care and Use Committees of the Northwest Institute of Plateau Biology, Chinese Academy of Sciences and the Agriculture Department of Qinghai Province, China.

Microinjection and Activity Determination

The mixtures containing 50 pg of sgRNA-1 and 250 pg of Cas9 mRNA, or 50 pg sgRNA-2 and 250 pg Cas9 mRNA, were injected directly into 70 single-cell-stage embryos as described by Hwang et al.[11], respectively. Aind the embryos were derived from the Tübingen wild-type cross. 24 h after injection, we collected randomly 20 embryos which were lysed as template of PCR to detect whether mutations were introduced near the PAM site, the remaining embryos were kept until 3 months of age. The remaining 50 fish were sedated in 0.016% solution of Tricaine (Sigma) until the startle response ceased and buoyancy equilibrium was lost, after which fin clips were obtained using a clean eye scissors. Post-op recovery tanks contained dilute methylene blue as anti-infection treatment. Genomic DNA was extracted using Genome extraction kit (B518221, Sangon Biotech). A pair of primers (F: TTGTTTTGCTTCTCATCAGTTG, R: TTTGCTCTGCTGCTTCACTC) around the PAM sites were

designed and used to detect the mutation events. The length of products using the primers and with wild zebrafish genomic DNA as template is 357 bp. The following procedure was used to amplify the targeted sequences: pre-denaturation was performed at 94°C for 5 min; then 32 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec; and finally extended at 72°C for 7 min using Veriti Thermal Cycler PCR system (Applied Biosystems, USA). Appropriate PCR products with length between the range of 250 bp and 450 bp were sequenced directly, and those showing multi-peaks set close to the PAM site were cloned into pMD18-T, then sent to further sequence and analysis. The zebrafish with the sequence differed from that of their wild type (such as substitution or transversion of single nucleotide, and insertion or deletion of short nucleotides) around the PAM sites were named as F0 founder.

F1 Mutant Detection and Observation

The day before breeding, using partitions to separate the breeding male and female F0 founders at 10-hour dark cycle. The next day removed partitions and let the parents fish mated randomly. Resulting embryos were collected and grown to 45 days of age, and then PCR was performed to identify gene-targeted modifying events. The F1 mutants were further stained via Alizarin red to observe the patterns of scale development as described by Harris et al.[6] with microscope (OLYMPUS SZ61).

Western Blotting

The whole bodies of the two samples (one EDA mutant and one wild-type fish) were frozen in liquid nitrogen and homogenized with electric homogenizer adding lysing RIPA buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM EDTA), respectively. After shaking for 1 h, the supernatant fluid containing the whole protein were obtained through centrifugation at 4°C (13000 rpm for 5 min). 20 μL of total protein were separated by 10% SDS-PAGE at 100V for 1.5-2 h, and then transfered to nitrocellulose membranes at 100V for 1-1.5 h. Membranes were blocked in 5% milk in TBST (TBS with 0.05% Tween) overnight at 4°C. The primary antibodies, rabbit polyclonal antibodies against human EDA (ab84311, Abcam) and against zebrafish β-actin (ab16039, Abcam), were diluted 1:5000 with antibody solution, and incubated with the blocked membrane in blocking buffer overnight at 4°C. Washing the membrane at least three times with TBST, 5 min each, and then incubating with the secondary antibody of Goat Anti-Rabbit HRP (ab205718, Abcam, 1:5000) in blocking buffer at room temperature for 1 h. Images were acquired using darkroom development techniques for chemiluminescence and ChemiDoc XRS+ (BIO-RAD).

RESULTS

Targeted Site Design and Activity Detection

In this work, we carried out a targeted disruption of the *eda* exon 4 loci in zebrafish using CRISPR-Cas9 system *(Fig. 1)* to further verify the function of EDA CL domain in fish scale development. Two designed small guide RNA, namely sgRNA-1 and sgRNA-2 were injected into 70 one-cell stage embryos, respectively. According to the sequencing results of target sites, eight individuals of F0 generation showed multi-peaks set close to the PAM sequence *(Fig. 2)* in the 50 adults after injection of sgRNA-1; CRISPR-Cas9-mediated mutation frequency within the *eda* exon 4 was approximately 16%. Nevertheless, multi-peaks sets were observed in the 70 embryos by injecting sgRNA-2. It was estimated that there was no activity of sgRNA-2 *in vivo*.

Mutation Detection and Typing

According to *Fig. 2,* deletion and insertion mutation types were introduced near the PAM site in founder 0 (F0). The screened five *eda* mutation types including a deletion of 6 bp (F0 1#, 2#), a deletion of 5 bp (F0 3#, 4#), a deletion of 87 bp (F0 5#), an insertion of 11 bp (F0 6#, 7#), and a

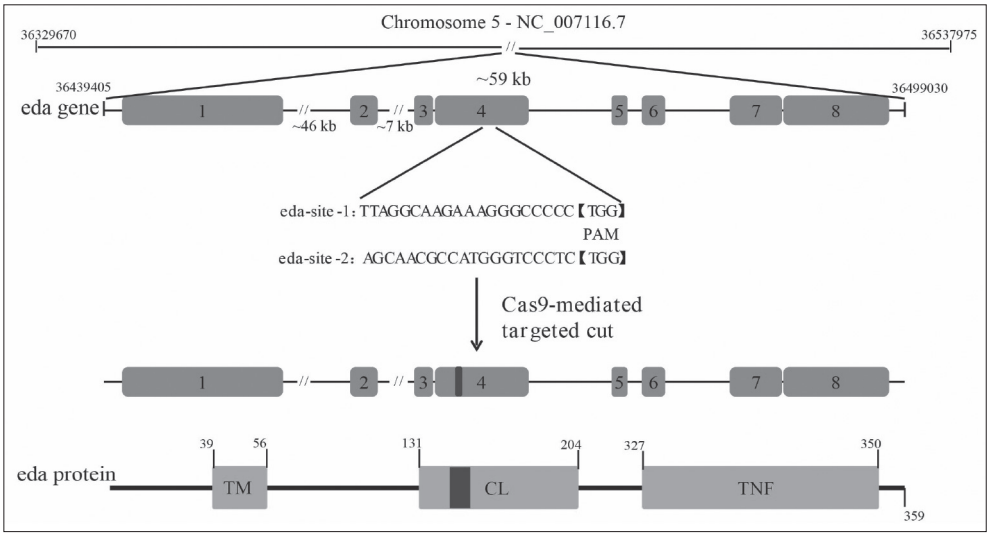

Fig 1. Engineered CRISPR-Cas systems and associated target Eda gene loci used in this study. The Eda gene has eight exons (gray box) in zebrafish. We designed two target sites including Eda-site-1 and Eda-site-2 at exon 4 on chromosome 5. The TGG of the target sequence is the protospacer adjacent motif (PAM). When sgRNA-1 and sgRNA-2 were injected into the single-cell stage embryos, the mutation (black bar) will be induced at the exon 4 locus. EDA protein are mainly transmembrane (TM), collagen-like (CL) and tumor necrosis factor (TNF) domains, and the CL domain are edited by exon 4

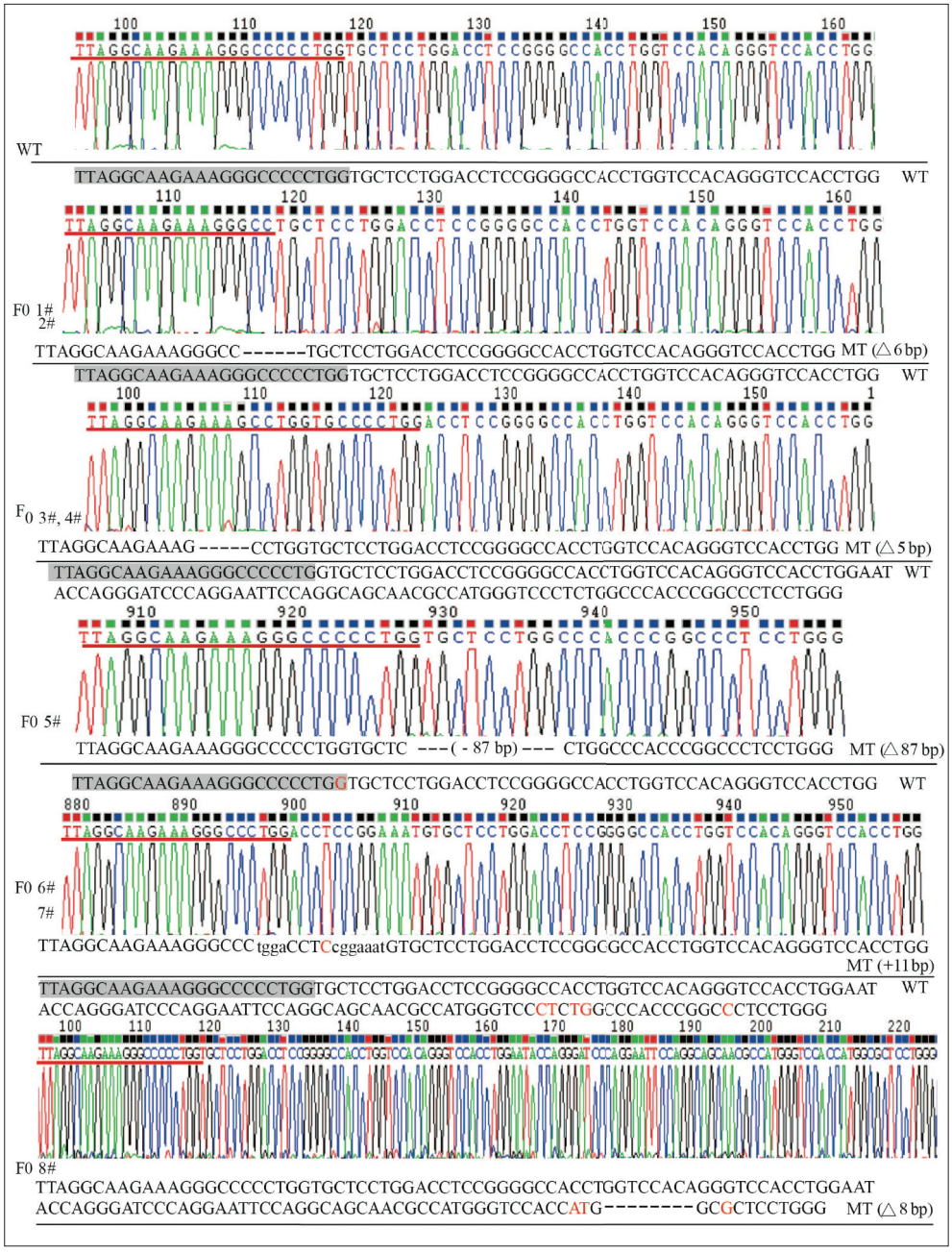

Fig 2. The PCR products detected in the founder 0 (F0) mutated zebrafish induced by injection of Cas9 enzyme together with sgRNA. Double-peaks and the sequences corresponding mutants were detected near the PAM site in the wild type (WT), 1#, 2#, 3#, 4#, 5#, 6#, 7#, and 8# mutants, and the results suggested that deletion and insertion mutations were induced close to the PAM site. The target sequence including PAM sites are marked in light gray and red line. Abbreviations: deletion sequences are indicated by "-----", insertion sequence is lowercase letters; Wild-type (WT), mutation-type (MT), deletion/insertion (△/+)

deletion of 8 bp (F0 8#). Among them, the deletion of 5 bp, a deletion of 6 bp, and the insertion of 11 bp were detected in two individuals, respectively. An ORF analysis indicated that the deletion of 5 bp, 8 bp and the deletion of 87 bp were in-frame mutations, while the deletion of 6 bp and the insertion of 11 bp were out-frame mutations. The scale phenotype of all F0 mutation types were normal by microscopic observation (OLYMPUS SZ61).

Scale-loss Mutants

In this research, the two male F0 founders (6# and 8#), were mated randomly with the six female F0 founders (1#, 2#, 3#, 4#, 5#, and 7#). Then we obtained 53 embryos and eventually 26 adult zebrafish after feeding for three months, the survival rate of 49% is half that of the wild-type zebrafish (90%). This might be due to inbreeding or the mutations leading to decrease in their survival ability, but it served to remind us to maintain stringent conditions in the mutant breeding program. The 26 living zebrafish were detected by microscopic observation. Finally, only five of the mutants had no scales coating the skin, and the PCR detection results showed that these mutants were all with bi-allelic insertion of 11 bp nucleotides. We further analyzed scale development using Alizarin red staining method. As shown in *Fig. 3*, scales coated the whole body of the wild type *(Fig. 3-A,B),* while the F1 mutant *(Fig. 3-C,D)* contained just few scales on the body. Meanwhile, the *eda* expression pattern were assayed by Western blotting in both the mutant and wild zebrafish *(Fig. 3-E),* with β-actin as the internal reference *(Fig. 3-F)*. We could see obvious

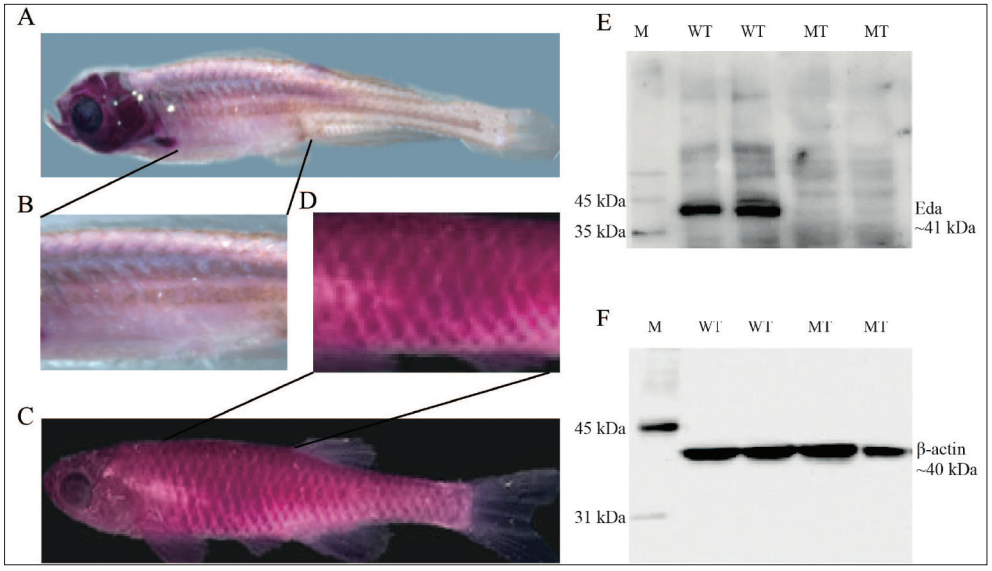

Fig 3. The Alizarin red staining and Eda expression assay of wild-type (WT) and mutation-type (MT) zebrafish. **A, B:** Individual containing an insertion 11 bp allelic mutation contains abnormal scaling when compared to the WT individual **(C, D)**. **E, F:** Western blotting of lysates from WT and the mutant carrying bi-allelic frameshift mutation with anti-Eda antibody and β-actin, respectively. M is the Unstained Protein Marker (NO. C600525 and NO. C600526, Sangon Biotech)

results as illustrated in *Fig. 3-E* that there is no targeted product in the mutants (MT) compared with that of the wild type (WT) zebrafish.

DISCUSSION

To date, a number of genes have been edited by the CRISPR-Cas9 system in zebrafish, and the mutation frequencies have been recorded up to 75% [12] and 86% [13]. By comparison, the mutation frequency of *eda* exon 4 was only 16% which was relatively low in the present work. And no mutations were detected in the embryos injected with sgRNA-2. It has proved that mutation frequencies depend on target loci in the zebrafish genome [11], which may be caused by the specific nucleotide of the targeting sequence in *eda*. Gene mutation include base substitution, deletion and insertion mutations, deletion and insertion mutations are the major mutations by the the CRISPR-Cas9 system introduced in zebrafish, especially deletion mutation [12-15]. In the paper, we obtained eight mutants, seven of them were deletion mutations, one of them is insertion mutation, however base substitutions were also occured with deletion and insertion mutations (shown in F0 6#, 7# and 8# of *Fig. 2)*. In addition, a large relatively fragment of 87 bp deletion (F0 5#) was detected in this study, which was less common in other similar studies.

The above mutations, whichever are all likely to change the structure of protein. Five *eda* mutation types were detected in the embryos injected with sgRNA-1, the 6 bp deletion and the 11 bp insertion mutations resulted in the reading frame shift mutation of EDA protein. The frameshift mutations in the target gene are expected to affect the function of the EDA protein. However, the scale phenotype of all F0 mutation types were normal. Makino et al.[16] research suggested that all of the bi-allelic out-of-frame mutations expressed the target gene products are due to illegitimate translation. This means that the individuals with homozygous mutations of genes will produce a pronounced phenotype. In consequence, F1 generations are necessary to achieve bi-allelic mutants.

It is well known that homozygous mutant in F0 generations is hard to achieve; however, phenotyping could be done in F1 generation by inbreeding two founder fish [17], significantly reducing animal husbandry and time. In the study, five mutants were obtained without scales coating the skin, the results sugguested that bi-allelic 11-bp insertions in *eda* by PCR and sequecing detection. Western blotting were executed to further verify the function of EDA protein, the results showed that there was no expression product of EDA protein in scale-less fish compared with wild-type fish (shown in *Fig. 3)*. We speculated that a frameshift mutation was caused by bi-allelic 11-bp insertions, and then leads to function inactivation of the CL and TNF domains. Similar experimental verifications have been performed in mouse and stickleback models [16], all of which suggested that changing the EDA signal pathways resulted in altered plate development in sticklebacks. Colosimo et al.[18] injected single-celled embryos from low-plated parents with full-length mouse EDA-A1 cDNA. The numbers of armor plates from the transgenic fish were then compared to the wild type fish, confirming that Eda signaling triggers lateral plate formation. Our results confirmed further that EDA was a crucial gene in scale development. Mutation in the *eda* leading to an out-frame mutation at the exon 4 locus affected scale development in zebrafish. The mutants will provide a good model for unveiling the role of EDA in fish scale development and elucidating the CL domain function. Before that, we need to consider the off-target effects of the CRISPR/Cas9 system as well as ZFN [19].

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Competing Interests

The authors declare that they have no competing interests.

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