Gene Editing of Luteinizing Hormone, Follicle-stimulating Hormone and Gonadotropin-releasing Hormone Genes to Sterilize Channel Catfish, *Ictalurus punctatus,* using Zinc Finger Nuclease, Transcription Activator-like Effector Nuclease and Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Technologies

by

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Abstract

Channel catfish (*Ictalurus punctatus*) is the leading freshwater aquaculture species in the US, but the catfish industry has been in crisis during the past few years. Genetically enhanced fish can greatly contribute to production efficiency and profit, but could pose environmental and ecological risk upon escapement. Targeted gene editing technologies, zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 were applied to induce mutagenesis of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and catfish type gonadotropin-releasing hormone (cfGnRH) genes, and to sterilize channel catfish.

Three sets of ZFNs targeting LH gene were electroporated into one-cell embryos, and different concentrations were tested. The overall mutation rate was 19.7% and the best treatment was ZFN set 1 at a concentration of 25 μg/ml. Channel catfish carrying the mutated LH gene were sterile, as confirmed by mating experiments. The plasmids were eventually degraded without integration as they were not detectable in mutated individuals. Carp pituitary extract failed to induce spawning and restoration of fertility indicating the need for developing other hormone therapies to achieve reversal of sterility upon demand.

TALENs were electroporated into one-cell embryos targeting LH, FSH and cfGnRH genes. Mutations were produced and confirmed with Cel-I assay and DNA sequencing. The mutation

rates of LH, FSH and cfGnRH genes were 44.7%, 63.2% and 52.9% respectively. Embryo hatch rates and fry survival rates were not different among either TALEN groups ($p=0.387$, $p=0.182$) or among the TALEN and control groups ($p=0.340$, $p=0.107$), indicating low cell toxicity of TALENs.

CRISPR/Cas9 plasmids were electroporated into embryos to mutate LH and cfGnRH genes, and CRISPR/Cas9 RNAs were microinjected to target cfGnRH gene. Both methods successfully produced mutations. The mutation rates for the electroporation were 37.5% and 38.5%, and 100% for microinjection. However, the egg hatch rate was only 9.0% using microinjection and significant lower than that for electroporation (23.0% and 21.0%, $p=0.0004$). Low cell toxicity was also confirmed.

These results and future research will help in understanding the roles of reproductive genes, sterilization of teleost fish and is a step towards control of domestic, hybrid, exotic, invasive and transgenic fish.

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I Introduction

Catfish farming, primarily channel catfish (*Ictalurus punctatus*) and its hybrid, channel catfish ♀ X blue catfish (*Ictalurus furcatus*) ♂ (Chatakonidi, personal communication), is the largest aquaculture industry in the US in terms of both weight and value, which accounting for more than 70% of all US aquaculture production and more than 40% of all sales (NASS, 2012; NASS, 2013). However, the catfish industry is in crisis. Catfish production has contracted since a high mark in 2003 (Harvey, 2006; Hanson and Sites, 2012, 2015) and catfish production acres keep decreasing (NASS, 2013). The industry is currently struggling to keep pace with the increasing cost of inputs, particularly feed cost driven by the demand and high price of soybeans and corn, production inefficiencies, and competition with inexpensive imported frozen fish, which now accounts for more than two-thirds of all US sales of frozen catfish fillets (Hanson and Sites, 2012, 2015). Food safety will become a large issue if much of the aquatic food needs to be imported from other countries. In the last 2-3 years, all of these issues have stabilized (Hanson and Sites, 2015), but the industry has opportunity to grow and recapture its lost market share with improved production and production efficiency.

Exploiting fish genetics can greatly contribute to production efficiency and enhancing aquaculture production. Genetic enhancement of farmed fish has advanced to the point that it is

now impacting aquaculture worldwide (Dunham et al., 2000; Dunham et al., 2001). Gene transfer holds great promise for genetic improvement of farmed fish and catfish.

Transgenic technology is a powerful technology which has high potential to increase both production and profit of catfish industry. Transgenic fish have been developed that have improved growth, color, disease resistance, tolerance of heavy metals, survival in cold and body composition, and that can produce pharmaceutical proteins (Dunham and Liu, 2006; Dunham, 2009). The application of transgenic techniques to fish that has received the most attention is growth enhancement. Transgenic fish such as Atlantic salmon (*Salmo salar*) (Du et al., 1992), coho salmon (*Oncorhynchus kisutch*) (Devlin et al., 1995) and mud loach (*Misgurnus mizolepis*) (Nam et al., 2001) have been produced, for which growth enhancement is dramatic, with fish growing to be more than ten-fold faster, and as high as 35-fold faster in mud loach, than control growth that can probably never be achieved by traditional breeding. Transfer of growth hormone (GH) genes also enhances performance in channel catfish. Five generations of mass selection has increased growth in channel catfish by 65% (Dunham and Liu, 2003). By inserting the GH gene, an additional 41% increase in growth is obtained (Dunham and Liu, 2003). GH gene transfer in catfish has numerous other positive effects including increasing percent protein, lowering fat percentage and improving flavor and texture.

Disease is the most critical problem of the catfish industry, which leads to the largest economic loss (NAHMS, 2011). The most effective mechanism for increasing disease resistance in catfish is transgenesis via transfer of cecropin genes found originally in the moth (*Hyalophora cecropia*) (Steiner et al., 2009), which gave two to four-fold increase in bacterial disease resistance

and was much better than what was obtained through selection (Dunham et al., 2002). There is evidence that cecropin might also have anti-viral and anti-fungal properties (Sarmasik et al., 2002).

Type 3 antifreeze protein from ocean pout (*Macrozoarces americanus*) had been introduced into goldfish (*Carassius auratus*) with an attendant increase in cold tolerance (Wang et al., 1995). Transgenic zebrafish (*Danio rerio*) (Yoshizaki et al., 2005) and common carp (*Cyprinus carpio*) (Cheng et al., 2014) containing the β-actin-salmon desaturase gene had been reported to have enhanced levels of the omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).

Concern and controversy exists regarding the possible ecological impacts of domestic fish as well as interspecific hybrids, exotic species and transgenic fish. Physical containment and physicochemical containment are options to confine various types of fish (Wong and Van Eenennaam, 2008). However, these options work in very limited cases, have inherent disadvantages, and usually are not practical. Chemical and mechanical sterilization are additional approaches, but the disadvantages are that the effects are temporary and are not feasible on a commercial scale in fish (Dunham, 1990; Dunham, 1995). Monosexing may be an approach for some fish. The sex ratio of Nile tilapia and loach were altered when fry are cultured in different temperature during their sex differentiation period, and hormonal sex control can lead to populations having the genotype of a single sex (Nomura et al., 1998; Dunham et al., 2001). This approach will only provide confinement if the fish are utilized in locations where conspecifics are not present.

Virtually all ecological issues become moot if genetically sterile aquatic organisms and brood

stock are used. Several genetic mechanisms can result in sterility, but unfortunately all of these systems have significant shortcomings, making them unacceptable to address these ecological problems, especially on a commercial scale.

Induction of triploidy is a popular sterilization option, but it still requires fertile diploid brood stock, so risk is not eliminated. Additionally, in some cases triploidy can decrease performance in fish (Lilyestrom et al., 1999; Dunham, 2011) and is not feasible in catfish and many other species of fish industry on a commercial scale.

Transgenic sterilization is a promising option to confine various biotechnological forms of fish including transgenics (Templeton, 2005; Thresher et al., 2009). Several potential transgenic sterilization strategies exist each having advantages and disadvantages.

Targets for transgenic sterilization would primarily include those responsible for gamete maturation and release. Gonadal maturation in teleost fish is primarily regulated by the hypothalamic-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is known and named for its role as the final common signaling molecule used by the brain to regulate reproduction in all vertebrates (Fernald and White, 1999). The GnRH decapeptide is synthesized by neurosecretory cells in the hypothalamus and secreted into portal vessels, to be transported to the pituitary gland and simulated the synthesis and release of hypophysial gonadotropin (including LH and FSH) which then simulates the secretion of steroid hormone from the gonads (Amano et al., 2002). These steroid hormones, in turn, feed back to the brain and the pituitary to complete the axis and to regulate the reproductive cycle (Redding et al., 1993; Zohar et al., 2010).

To date, comparative endocrinological studies have revealed 14 different types of GnRH in

vertebrates (Kah et al., 2007; Okubo and Nagahama, 2008). All these peptides were originally named after the first species in which they have been discovered. Chicken GnRH-II (cGnRH-II) is the GnRH that presents throughout the vertebrates (King and Millar, 1992; Sherwood et al., 1993; White et al., 1998), which has the demonstrated function of inhibiting amphibian K^+ channels sympathetic ganglia and the potential functions in neural development and sexual arousal (Millar, 2003). In teleost fish, two or three forms of GnRH exist in the brain (Nabissi et al., 2000; Amano et al., 2002; Dubois et al., 2002; Somoza et al., 2002). The *Siluriformes* order to which channel catfish belongs has two forms of GnRH, the first one is cGnRH-II and the second one named catfish type GnRH (cfGnRH) (Zandbergen et al., 1995; Amano et al., 1997), of which cfGnRH is the hypophysiotropic GnRH form and considered to be the gonadotropin releaser, which plays a key role in sexual maturation in catfish (Bogerd et al., 1994; Zandbergen et al., 1995).

The glycoprotein hormone (GpH) family consists of follicle-stimulating hormone (FSH), luteinizing hormone (LH), chorionic gonadotropin (CG), thyroid-stimulating hormone (TSH), and thyrostimulin (Hsu et al., 2002). These glycoproteins all share the same α subunit in a given species in a functional heterodimer consist of one α subunit and one β subunit, where the different β subunit confers distinct functions to each of them (Pierce and Parsons, 1981; Liu et al., 2001).

In teleost, two types of gonadotropin were first identified in chum salmon (*Oncorhynchus keta*) (Suzuki et al., 1988) and are now generally accepted as homologues, FSH and LH. These two pituitary gonadotropins play central roles in regulating gametogenesis and the production of gonadal hormones required for the development of sexual behavior and secondary sex characters.

In salmonid fish, FSH is primarily involved in spermatogenesis and vitellogenesis, where LH stimulates the maturation of oocytes, ovulation and spermiation (Swanson et al., 2003). Both LH and FSH stimulate the maturation-inducing steroid testicular 11-ketotestosterone (11-KT) and 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P) in males (Planas and Swanson, 1995; Kagawa et al., 1998), whereas in females they stimulate production of ovarian estradiol-17β (E2) and 17,20β-P (Suzuki et al., 1988; Kagawa et al., 2003).

Although FSH and LH are similar in structure, they are synthesized in two different cell types (Nozaki et al., 1990; Naito et al., 1993) and at different stages of the reproductive cycle (Naito et al., 1991). FSH is expressed in the glandular cords of the proximal pars distalis (PPD), in close association with somatotrophs; LH is expressed in the central parts of the glandular cords of the PPD. FSH is expressed first in ontogeny and involved in early reproductive events such as steroidogenesis, vitellogenesis and spermatogenesis, whereas LH is expressed in later stages of reproductive cycle and regulates spermiation and ovulation (Naito et al., 1991; Weil et al., 1995; Hassin et al., 2000; Kajimura et al., 2001).

The introduction of a recombinant vector containing gonadotropin-releasing hormone (GnRH) antisense sequence can result in transgenic fish expressing GnRH antisense mRNA, which disturbs the normal function of GnRH and leads to sterile fish (Uzbekova et al., 2000; Hu et al., 2007). The fertility of the fish could be restored by therapy with exogenous artificial GnRH. The disadvantage is that GnRH is needed at different time points of fish development. They may need to be captured multiple times to administer the therapy. This would be feasible, but highly inconvenient and costly on large commercial farms.

Hammerhead ribozymes have been used to knockdown gene expression in zebrafish (*Danio rerio*) (Xie et al., 1997). Ribozymes can be designed to affect the selective cutting of a particular specific messenger RNA (Sokol et al., 1998). However, this technology has not proved invariably successful when used in other species.

Gonad-specific transgenic excision utilizes site-specific recombinases, such as Cre and FLP, to exact excision, insertion, or inversion events in DNA at specific recognition sequences (Rodríguez et al., 2000; Wong and Van Eenennaam, 2008). This technique has been successfully demonstrated in zebrafish (*Danio rerio*) (Dong and Stuart, 2004; Le et al., 2007). The disadvantage is that it requires fertile transgenic brook stock and does not totally eliminate the risk of escape.

Repressible Tet-off and modified Tet-off based systems have been developed to knockdown genes essential for embryonic development or reproduction. Constructs are fused into fish genome so that a specific promoter is coupled to a repressible element that in turn drives expression of a blocker gene, antisense RNA, dsRNA, sense RNA or ribozyme to an early key developmental gene (Thresher et al., 2009; Dunham, 2011; Thresher et al., 2014). The transgenic fish will be sterile or even die during their early development stage if they won't receive the treatment of the repressor chemicals (Thresher et al., 2009). But this system is relatively complex and still under evaluation.

Complementary DNA overexpression and short hairpin RNA interference (shRNAi) had been applied to decrease expression of primordial germ cell (PGC) marker genes *nanos*, *dead end* and *vasa* in common carp (*Cyprinus carpio*) (Su et al., 2014) and channel catfish (*Ictalurus punctatus*) (Su et al., 2015). Sexual maturity had been successfully reduced or repressed in both cases. This was an environmental safe method that exhibited good potential for repressible transgenic

sterilization allowing. But this method still needs further optimization to realize complete sterilization.

In the past decade, new approaches enabling researchers to accurately manipulate genes via gene editing have been developed. This technology could be used to knock out reproductive function. The core technology is based on the use of engineered nucleases composed of specific DNA recognition and binding domains and non-specific DNA cleavage modules (Gaj et al., 2013). These methods can produce DNA double-strand breaks (DSB) at the targeted sequences and stimulate DNA repair mechanisms by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Wyman and Kanaar, 2006), which induce efficient alteration of genes by nucleotide deletion and/or insertion and modify normal gene functions. Zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) are developed as the popular targeted gene editing technologies.

Zinc finger nucleases (ZFNs) are a class of engineered proteins that create a highly targeted DSB within the genome and enable the manipulation of the genome with unprecedented ease and precision (Urnov et al., 2005). ZFNs consist of two domains, a zinc finger DNA binding domain comprised of a chain of zinc finger proteins and a DNA-cleaving domain comprised of catalytic nuclease domain of *Fok*I (Santiago et al., 2008). The zinc finger domains usually consist of 3-6 zinc finger proteins that each can specifically recognize and bind to 9-18 base pair target DNA. The nuclease domain comes from a restriction endonuclease *Fok*I and more importantly is it has been reengineered to function as an obligate heterodimer to cleave DNA (Miller et al., 2007). The

relatively easier modular design offers a greater number of combinatorial possibilities that could be designed to target a number of genes in the genome.

Transcription activator-like effector nucleases (TALENs) are proteins engineered by fusion of a *Fok*I endonuclease domain with a transcription activator-like (TAL) effectors DNA binding domain. TAL effectors are specific DNA binding proteins produced by plant pathogenic bacteria *Xanthamonas* to modulate host gene expression (Aigner et al., 2010) and composed with highly conserved 33-35 amino acid repeats. The amino acids in positions 12 and 13, referred to as "repeat variable di-residue" (RVD), vary and specify DNA-binding properties (Boch et al., 2009; Moscou and Bogdanove, 2009). Thus TALENs can be designed by the combination of TAL effectors to recognize much of the genome. Whereas the nuclease domain is similar to that of ZFNs with the restriction endonuclease *Fok*I and works obligately as heterodimer (Ansai et al., 2013).

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements so that protects bacteria and archaea from invading viruses and plasmids (Bhaya et al., 2011). Three major types of CRISPR (types I-III) have been categorized on the basis of locus organization and conservation (Makarova et al., 2011), in which the type II CRISPR system is one of the best characterized consisting the nuclease Cas9, the crRNA array that encodes the guide RNAs and a required auxiliary trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units (Gasiunas et al., 2012; Jinek et al., 2012). Each crRNA unit then contains a 20-nt guide sequence and a partial direct repeat, where the former direct Cas9 to a 20-bp DNA target. The crRNA and tracrRNA can be fused together to create a

chimeric, single-guide RNA (sgRNA) (Ran et al., 2013). Cas9 can thus be re-directed toward almost any target of interest in the genome.

Our long-term goal of this study is to realize reversible genetic sterilization in channel catfish, to reproductively confine and prevent the impact of transgenic or domestic species on the natural environment and to protect the ecosystem. Our specific objectives include sterilizing channel catfish using ZFN, TALEN and CRSPR/Cas9 technologies by inactivating LH, FSH and cfGnRH genes either with electroporation or microinjection, evaluating their efficiencies and potential plasmids integration, as well as the hormone therapy of fertility restoration. This research could serve as a model for other aquaculture species including any domestic genotype, interspecific hybrid, transgenic or exotic to minimize impacts on the natural environment, protect genetic biodiversity and ecosystems, increasing environmental friendliness of aquaculture and transgenic fish.

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II Gene Editing of Luteinizing Hormone Gene to Sterilize Channel Catfish, *Ictalurus punctatus*, using a Modified Zinc Finger Nuclease Technology

Abstract

Channel catfish (*Ictalurus punctatus*) is the most important freshwater aquaculture species in the US. Genetically enhanced fish that are sterile could both profit the catfish industry and overcome potential environmental and ecological risk. As the first step to generate sterile channel catfish, three sets of zinc finger nuclease (ZFN) plasmids targeting the luteinizing hormone (LH) gene were designed and electroporated into one-cell embryos; different concentrations were introduced and the Cel-Ⅰ assay was conducted to detect mutations. Channel catfish carrying the mutated LH gene were sterile, as confirmed by DNA sequencing and mating experiments. The overall mutation rate was 19.7% for 66 channel catfish, and the best treatment was ZFN set 1 at the concentration 25 μg/ml. The introduction of the ZFN plasmids may have reduced mosaicism as mutated individuals were gene edited in every tissue evaluated. Apparently, the plasmids were eventually degraded without integration as they were not detectable in mutated individuals using PCR. Carp pituitary extract failed to induce spawning and restoration of fertility indicating the need for developing other hormone therapies to achieve reversal of sterility upon demand. This is the first sterilization achieved using ZFNs and plasmids electroporation in an aquaculture species,

and the first successful gene editing of channel catfish. Our results will help in understanding the roles of LH gene, sterilization of teleost fish and is a step towards control of domestic, hybrid, exotic, invasive and transgenic fish.

1 Introduction

Technology for targeted gene editing is advancing rapidly. Zinc finger nuclease (ZFN) was a major breakthrough, which allowed targeted gene manipulation. ZFN technology is a novel gene editing tool developed in recent years, which are a class of engineered proteins that could create a highly targeted DNA double-strand break (DSB) within the genome and enable the manipulation of the genome with unprecedented ease and precision (Urnov et al., 2005).

ZFN consists of two domains, a zinc finger DNA binding domain comprised of a chain of zinc finger proteins that could recognize and combine to the target sequences, and a DNA-cleaving domain comprised of catalytic endonuclease *Fok*I which creates DNA strand break. When a pair of ZFNs binds to their target in the correct orientation, *Fok*I monomers can dimerize at the target site and introduce a DNA DSB. Then, cells will initiate the DNA repair processes by one of two highly conserved processes, homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Urnov et al., 2010; Stoddard, 2011). The NHEJ is an error prone process that usually leads to mutations (nonsense, deletion, insertion or frame shift) at the target site and the gene product usually loses its function.

ZFN-mediated mutations have been reported in a number of animals, including fruit fly (*Drosophila melanogaster*) (Bibikova et al., 2002; Beumer et al., 2008), sea urchin (*Hemicentrotus*

pulcherrimus) (Ochiat et al., 2010), frog (*Xenopus tropicalis*) (Young et al., 2011; Nakajima et al., 2012), zebrafish (*Danio rerio*) (Doyon et al., 2008; Meng et al., 2008; Foley et al., 2009), rat (*Rattus norvegicus*) (Geurts et al., 2009; Mashimo et al., 2010; Cui et al., 2011) and rabbit (Flisikowska et al., 2011). In all these studies, ZFNs could be successfully applied to introduce the target gene mutations, lead to the loss of gene function and the corresponding phenotype modification. In addition, these gene mutations could also be inherited by their offspring and a mutated family could be built.

Channel catfish (*Ictalurus punctatus*) and its hybrid, channel catfish ♀ X blue catfish (*Ictalurus furcatus*) ♂ (Chatakonidi, personal communication), are the leading freshwater aquaculture organism in the US and catfish farming is the largest aquaculture industry in both weight and value, accounting for more than 70% of all US aquaculture production and more than 40% of all sales (Zhang et al., 2014b; NASS, 2013). However, the catfish industry is in crisis, struggling with the rising cost of inputs, production inefficiencies and competing with inexpensive imported frozen fish. Exploiting fish genetics can greatly contribute to production efficiency and enhancement. Genetic enhancement of farmed fish has advanced to the point that it is now impacting aquaculture worldwide (Dunham et al., 2000; Dunham, 2011). Gene editing is a powerful technology which has high potential to increase production of aquaculture and profit to catfish industry. A critical problem in aquaculture is the potential escapement of domestic, hybrid, exotic and transgenic fish with potential adverse effects on the natural environment. Virtually all ecological issues become moot if genetically sterile brood stocks are used.

Luteinizing hormone (LH) is one of the gonadotropins that belongs to the glycoprotein

hormone family (Hsu et al., 2002). Glycoproteins are functional heterodimers, consisting of one α subunit and one β subunit. Within a given fish species all α subunits are identical while the different β subunit confers the physiological specificity of the hormone (Liu et al., 2001). LH plays central roles in regulating gametogenesis and the production of gonadal hormones. LH stimulates secretion of the maturation-inducing steroid testicular 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in males (Planas and Swanson, 1995; Kagawa et al., 1998), while working in females by producing ovarian estradiol-17β (E2) and 17,20β-P (Suzuki et al., 1988; Kagawa et al., 2003). LH is expressed in later stages of reproductive cycle and regulates spermiation and ovulation (Rosenfeld et al., 2007; Levavi-Sivan et al., 2010).

In the present study, we delivered the ZFN plasmids, targeting the LH gene, into fertilized eggs with electroporation process and our study aims to create a gene edited sterile channel catfish by ZFN mediated targeting mutagenesis. Usually, ZFN mRNA is delivered by microinjection, thus, the electroporation of ZFNs plasmids offers a novel and potentially more effective delivery mechanism not previously evaluated in fish. The potential disadvantage is integration of the plasmids, which would result in a transgenic organism obviating one of the advantages of gene editing. Evaluation of potential integration was another objective of the current study.

2 Material and Methods

2.1 Construction of zinc finger nuclease

The design and assembly of ZFNs plasmids, “CompoZr Custom ZFN” plasmids, were provided by Sigma-Aldrich Company (St. Louis, MO). All the plasmids are driven by the

cytomegalovirus (CMV) promoter and the T7 promoter, followed with the zinc finger domain, *Fok*I domain and the kanamycin resistant element (Fig.1).

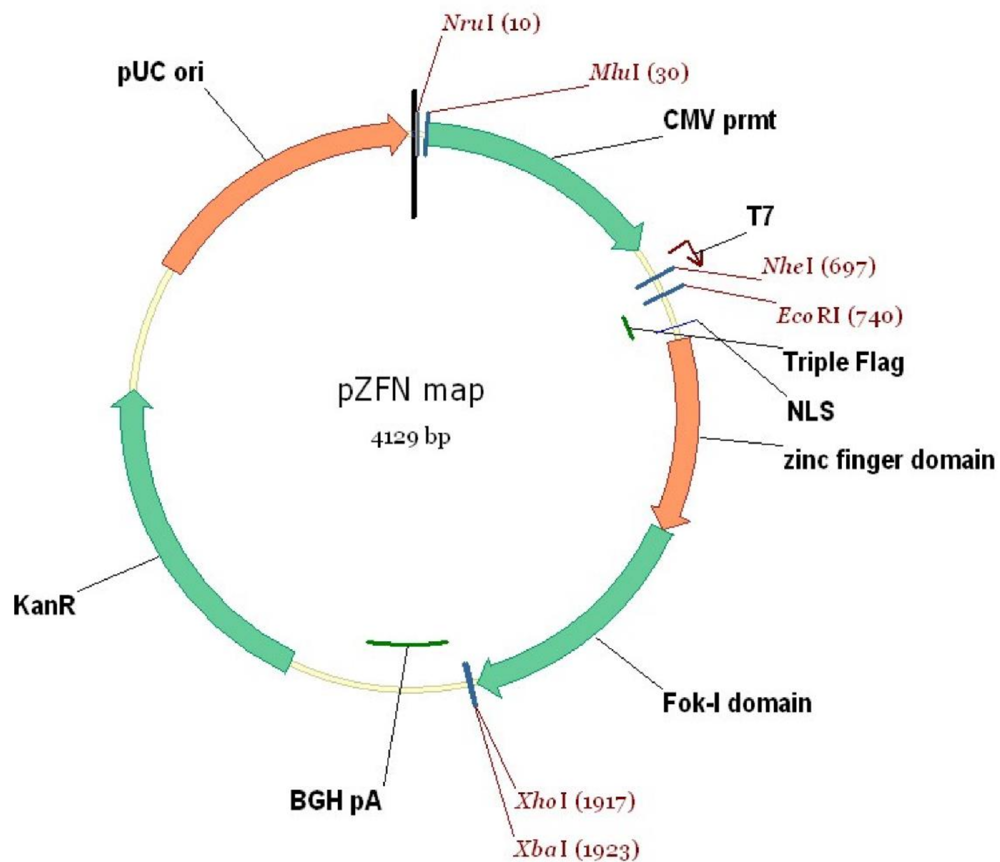

Fig.1 Schematic representation of zinc finger nuclease (ZFN) plasmid structure targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene.

The channel catfish (*I. punctatus*) LH gene β subunit (AF112192) (Liu et al., 2001) was chosen as the target gene and three targeting sites were selected (Fig.2) for gene editing and

sterilization of channel catfish. Three sets of ZFN plasmids targeting each site were prepared and MEL-Ⅰ assay was performed by the company to check the ZFNs activity as previously described (Urnov et al., 2005; Doyon et al., 2008).

1) ZFN set1

CACAGAAACAGTCTCATTAACAGGTTCGCAGTGTGGCA

2) ZFN set2

CTCATTAACAGGTTCGCAGTGTGGCAGAATGTAGCT

3) ZFN set3

CTCATTAACAGGTTCGCAGTGTGGCAGAATGTAGCTTTGAGCG

Fig.2 Three sets of zinc finger nucleases (ZFN) targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene. ZFN binding sites are underlined and cutting site is in grey.

Three sets of ZFNs were designed to disrupt the *I. punctatus* LH gene, and the targeting sites were all located within the ORF of channel catfish LH gene β subunit (Fig.3).

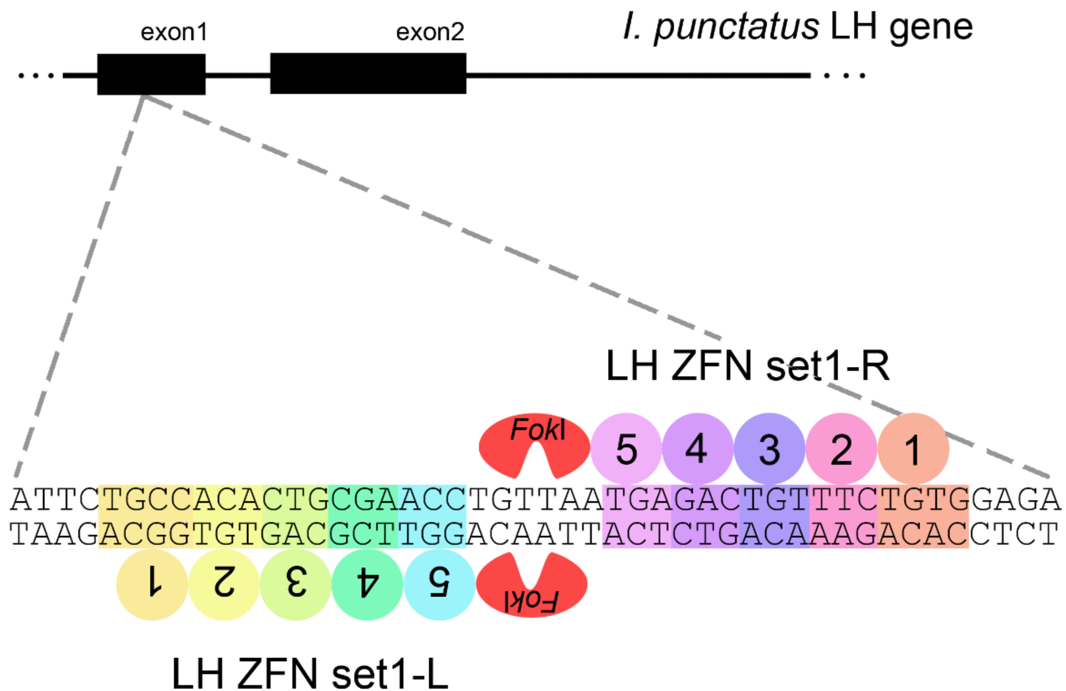

Fig.3 The location of the zinc finger nuclease (ZFN) target site in the *Ictalurus punctatus* luteinizing hormone (LH) gene. Exons and introns of LH gene are indicated by black boxes and lines, respectively. Different numbers and colors indicate different zinc finger proteins in the DNA binding domain of ZFN set1-L and R.

2.2 Plasmid DNA preparation

ZFN plasmids were transformed into One Shot Top 10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY) following the manufacturer's instruction and cultured in LB broth. 100 μl transformation mix was used to spread on the LB agar plate with 50 μg/ml kanamycin. A single colony was picked up from each plate and cultured in 400 ml LB broth with 50 μg/ml kanamycin. Plasmids were then extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville,

Holliston, MA) and their quantity and quality were inspected by gel electrophoresis and spectrophotometry.

The ZFN plasmids of each set were prepared separately for the purpose of double electroporation (Su, 2012; Dunham and Winn, 2014). Equal amount of both left and right ZFNs were mixed together and diluted with 2 ml saline (0.9% NaCl) to the final concentration of 10 μg/ml, 25 μg/ml and 50 μg/ml for the first electroporation of sperm. The purpose of the saline was to dehydrate the sperm once they were introduced to the solution; when rehydrated, transformation rates of the embryos can be improved (Kang et al., 1999; Collares et al., 2010). Meanwhile, the same concentration of ZFNs was diluted with 5 ml TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0), which was then used for a second electroporation of embryos.

2.3 Brood stock spawning

Sexually mature channel catfish were harvested from the Fisheries Genetics Unit, E.W. Shell Research Center (Auburn University, Auburn, AL) and artificially spawned in aquaria with flowing water at 26 to 27℃. Kansas random female channel catfish were implanted with luteinizing hormone releasing hormone analog (LHRHa) at 90 μg/kg body weight to facilitate ovulation. Eggs were stripped from two ovulating females into metal pie pans coated with grease (Crisco, Orrville, OH). Two male channel catfish (Kansas random and AR) (Dunham and Smitherman, 1984) were euthanized and the testes were macerated into saline (0.9 % NaCl) to release sperm and produce a sperm homogenate.

2.4 Fertilization, electroporation and incubation

Two drops of sperm solution were added to the ZFNs plasmids saline solution, mixed and incubated at room temperature for 5min. Then the mixture was poured into a 10ml petri dish and filled with fresh water. This solution was then electroporated with a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters set at 6 kV, 2^7 pulses, 0.8s burst, 4 cycles, 160 μs (Powers et al., 1991).

Two hundred eggs were fertilized with these sperm and incubated in fresh water for 60min. Then fertilized eggs were transferred into a 10ml petri dish and ZFNs plasmids TE solution was poured into it. After 10min of incubation, the embryos were electroporated again as described above. Control groups were treated similarly with double electroporation using saline and TE buffer without ZFNs plasmids.

Embryos were moved into separate 10L tubs filled with Holtfreter's solution (Bart and Dunham, 1996) containing 10ppm doxycycline and reared at 27℃ until hatch. Dead embryos were picked out and water was changed daily. Then channel catfish fry were transferred into a recirculating system.

2.5 Sample collection and DNA extraction

The pelvic fin and barbel of 6-month-old fingerlings were sampled for DNA analysis. Fingerling were also euthanized and samples from barbel, brain, muscle, intestine and pelvic fin were collected, to study the mosaicism of the mutations. All samples were stored at -20℃.

To extract DNA, samples were digested with 100 μg/ml proteinase K followed by protein

precipitation and DNA ethanol precipitation as described by Kurita et al. (2004). DNA quantity and quality were determined with gel electrophoresis and spectrophotometry.

2.6 Mutation analysis

Channel catfish LH gene β subunit specific primers (Table 1) were designed and Roche Expand High Fidelity^{Plus} PCR System (Roche, Indianapolis, IN) was used to amply these DNA samples. The PCR amplification procedure was as follows: initial denaturation for 2min at 94℃; followed by 35 cycles of 94℃ for 30s, 60℃ for 30s and 72℃ for 1min; and a final elongation for 10min at 72℃. PCR products were examined by gel electrophoresis.

Table 1 Primer sequences used for the amplification of luteinizing hormone (LH) β subunit in channel catfish (*Ictalurus punctatus*).

Primer	Sequence (5'-3')	Product size (bp)	Description
LH-F	AGGATGTCAGTGCCAGCTTC	572	LH gene amplification and mutation analysis
LH-R	CTTGGAGTAAATGGACTCGTTG		

LH gene mutations were detected through the Cel-Ⅰ mutation detection assay with SURVEYOR Mutation Detection Kit (Integrated DNA Technologies, Coralville, IA) as described by Miller et al. (2007). This approach takes advantage of the NHEJ error prone DNA repairing process. After the denaturing and re-annealing treatment of the PCR products, the wild type and modified amplicons could anneal to create the heteroduplex structure, which will be cleaved by

the CEL-Ⅰ mismatch endonuclease. Thus, three bands will be seen after electrophoresis, which indicates the existence of mutation. In contrast, only one band on the gel represents no mutation occurs. Briefly, PCR products were denature and re-annealed as follows: 94℃ 10min; 94℃ to 85℃ -2℃/s; 85℃ to 25℃ -0.1℃/s; cooling to 4℃; then 1 μl Enhancer S and 1 μl Nuclease S were added into 5 μl of the products above and incubated at 42℃ for 30min; The digested PCR products were resolved on 2% UltraPure Agrose-1000 high resolution agarose gel (Invitrogen, Grand Island, NY).

2.7 TA clone and sequencing

To identify the exact modification of LH gene, the DNA samples were amplified with Roche Expand High Fidelity^{Plus} PCR system as described above. After purification with IsoPure DNA Purification Kit (Denville, Holliston, MA), PCR products were inserted into the vector of TOPO TA Cloning Kit for Sequencing (Invitrogen, Grand Island, NY) and transformed into the One Shot TOP10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY). The positive competent cells harboring the putative mutated DNA fragments of each individual were selected with LB agar plate containing 100 μg/ml ampicillin.

At least 10 colonies, carrying mutated LH DNA from each individual, were picked up and amplified with LB broth. The plasmids were extracted using Zyppy™ Plasmid Miniprep Kit (ZYMO Research, Irvine, CA) and sequenced by Lucigen Corporation (Middleton, WI).

When the sequencing results came back, the quality of the sequencing was detected by examining the .ab1 file using the software FinchTV (version 1.4.0). Sequences of each results were

then aligned with the wild type channel catfish LH gene β subunit using the online multiple sequence alignment tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.8 Plasmid integration inspection

To determine the presence of plasmids integrated into the channel catfish genome or persisting in the cytoplasm, three pairs of primers (Table 2), amplifying the vector backbone, ZFN domain and CMV promoter region, respectively, were designed to detect ZFN plasmids in LH mutated channel catfish. The PCR procedure was the same as above and products were inspected with electrophoresis.

Table 2 Primer sequences used for the detection of zinc finger nuclease (ZFN) plasmids integration in channel catfish (*Ictalurus punctatus*).

Primer	Sequence (5'-3')	Product size (bp)	Description
I1-F I1-R	GTGTACGGCTACAGGGGAAA TTGGGGTTGAGGTGCTTATC	203	ZFN plasmids integration detection
I2-F I2-R	CGTGACCGAGTTCAAGTTCC AAGTTGATCTCGCCGTTGTT	204	ZFN plasmids integration detection
I3-F I3-R	TACAAAGACCATGACGGTGA TGCAGATTCGACACTGGAAG	148	ZFN plasmids integration detection

2.9 Reproductive evaluation of LH mutants

When LH mutated channel catfish were two years old, four males and four females were paired in individual aquarium to mate. Meanwhile, four pairs of control catfish were also paired in individual aquaria. When three years old, 11 pairs of putative LH mutated channel catfish were paired in aquaria. In each case they were given 14 days to spawn naturally and then were injected

with a priming dose of 2 mg/kg of carp pituitary extract (CPE) followed by a resolving dose of 6 mg/kg CPE 12 hours later. Fifteen control pairs were utilized at the same time. When the three-year old fish did not respond to the CPE injections after 4 days, they were given two more injections of CPE at 2 mg/kg every 3 days.

2.10 Statistical analysis

Mutation rates, hatch rate and survival rates from different ZFN sets and different concentration groups were analyzed utilizing Pearson Chi-square Test and Fisher's Exact Test (McDonald, 2014). All analysis were performed with statistical software R (version 3.1.3).

3 Results

3.1 ZFN activity examination

All these three sets of ZFNs displayed high activities in the yeast MEL-Ⅰ assay (Doyon et al., 2008) (Fig.4), among which ZFNs set1 had the highest activity.

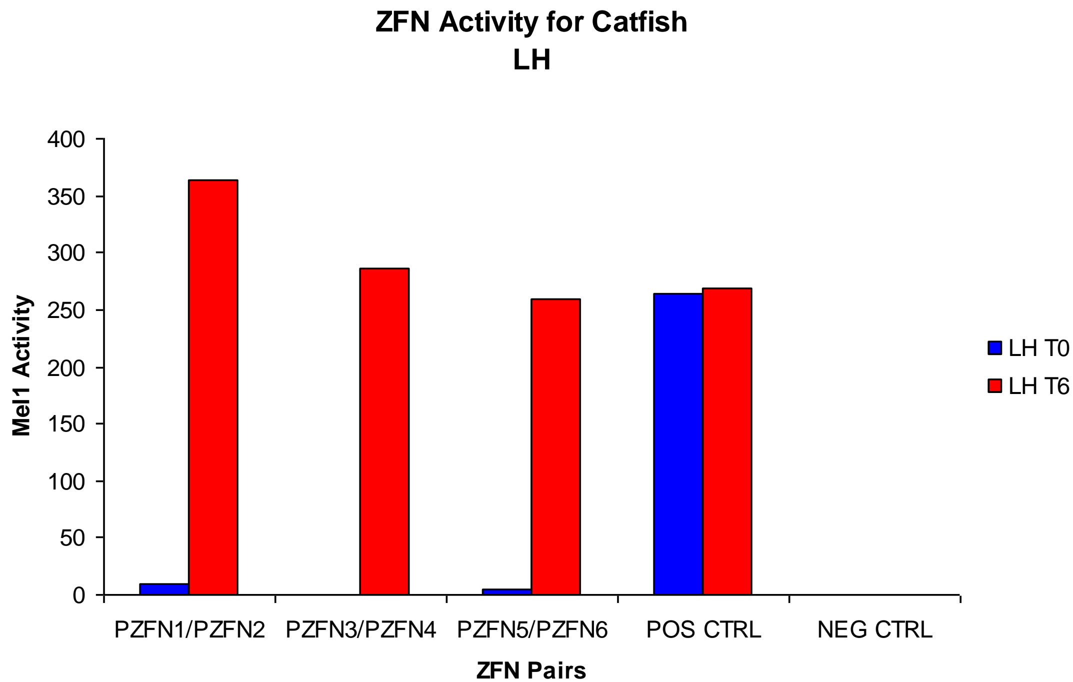

Fig.4 Zinc finger nuclease (ZFN) activity as measured by the yeast MEL-1 reporter assay. ZFN cleavage activity is measured before induction (0h, blue bars) and after induction of ZFN expression (6h, red bars). MEL-I levels positively correlate with ZFN ability to create double strand breaks at the desired target site.

3.2 Hatch rate and survival rate

Two hundred eggs were double electroporated for each ZFN treatment group and control group. 47 of them hatched from ZFN set1 group (hatch rate 23.5%), 38 hatched in ZFN set2 group (19%) and 42 hatched from ZFN set3 group (hatch rate 21%). After 6-month growing, 32 out of 47 (68.1%) fry survived from ZFN set1 group, 12 of 38 (31.6%) and 22 of 42 (52.4%) survived in ZFN set2 and set3 group, respectively (Table 3). Statistical analysis was performed and indicated the embryo hatch rate was not different from each group (p=0.544), but the fry survival rate was significantly different (p=0.004).

In contrast, the control group has a hatch rate of 48% (96 of 200) and survival rate of 82.3% (79 of 96), both of which were significant higher than treatment groups (*p*<0.001 and *p*=0.033).

Table 3 Comparison of the embryo hatch rate and fry survival rate for different sets of zinc finger nuclease (ZFN) treatments in channel catfish (*Ictalurus punctatus*).

Constructs	Eggs	Fry hatched	Hatch rate (%)*	Fry survival	Survival rate (%)†
ZFN-set1	200	47	23.5	32	68.1
ZFN-set2	200	38	19.0	12	31.6
ZFN-set3	200	42	21.0	22	52.4
Control	200	96	48.0	79	82.3

*No difference among treatment groups (*p*=0.544); significant difference between treatment and control groups (p<0.0001).

†Significant difference among treatment groups (*p*=0.004) and between treatment and control groups (*p*=0.033).

3.3 Mutation detection

Pelvic fin and barbel samples were collected from each of the survived 66 fingerlings and DNA is extracted. After PCR amplification and Cel-I assay, the products were detected with high resolution gel electrophoresis. Three bands appearing on the gel indicated the individual containing mutated LH gene (Fig.5).

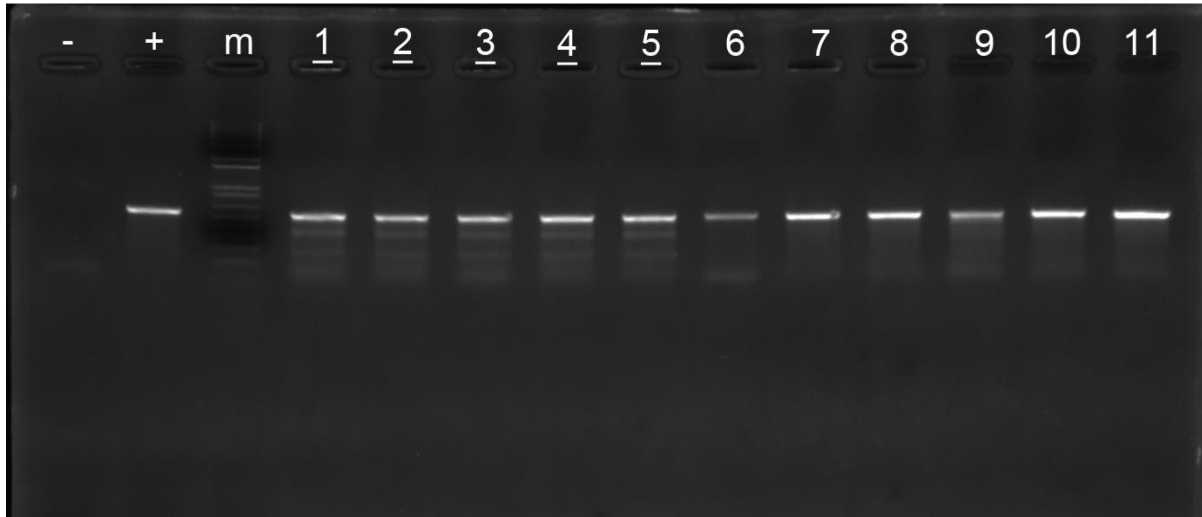

Fig.5 Identification of edited luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*) using Cel-Ⅰ mutation detection assay. “-” indicates the negative control without template; “+” indicates the control with wild-type channel catfish DNA as template; “m” indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Three bands indicate mutations occurred and the individual number of mutated channel catfish are underlined.

The Cel-Ⅰ assay indicated that the ZFN sets varied in effectiveness (Fisher’s Exact Test, p=0.100). ZFN set1 treatment group had 31.3% (10 of 32) of individuals containing mutated LH gene (Table 4), for which 37.5% (3 of 8) occurred at the concentration 10 μg/ml, 38.9% (7 of 18) at 25 μg/ml and 0 (0 of 6) at 50 μg/ml. In contrast, zero (0 of 12) mutation was detected for ZFNs set 2 and 13.6% (3 of 22) for ZFNs set 3, including 25.0% (3 of 12) at concentration 25 μg/ml and zero at others. The overall mutation rate was 19.7% for a these 66 channel catfish, which rises to 25.0% if the non-working concentration 50 μg/ml is excluded.

Table 4 Comparison of luteinizing hormone (LH) gene mutation rate for different sets of zinc finger nucleases (ZFN) electroporated at different concentrations in channel catfish (*Ictalurus punctatus*).

ZFN sets	Concentration (μg/ml)	N fry	N mutated fry	Mutation rate (%)*
ZFN set1	10	8	3	37.5
	25	18	7	38.9
	50	6	0	0
ZFN set2	10	3	0	0
	25	5	0	0
	50	4	0	0
ZFN set3	10	6	0	0
	25	12	3	25.0
	50	4	0	0
Overall		66	13	19.7

*Mutation rate was significantly different among treatments (Fisher's Exact Test, p=0.100) (50 μg/ml was excluded from analysis).

Two mutated fingerlings, confirmed with the analysis of pelvic fin and barbel DNA, from the ZFNs set1 group were sacrificed and DNA was extracted from the brain, muscle and intestine to detect mosaicism of the LH gene mutation. Same procedure was conducted as above and the Cel-Ⅰ assay results of all these tissues showed three bands on the gel (Fig.6), indicative of all these tissues tested containing mutated LH gene.

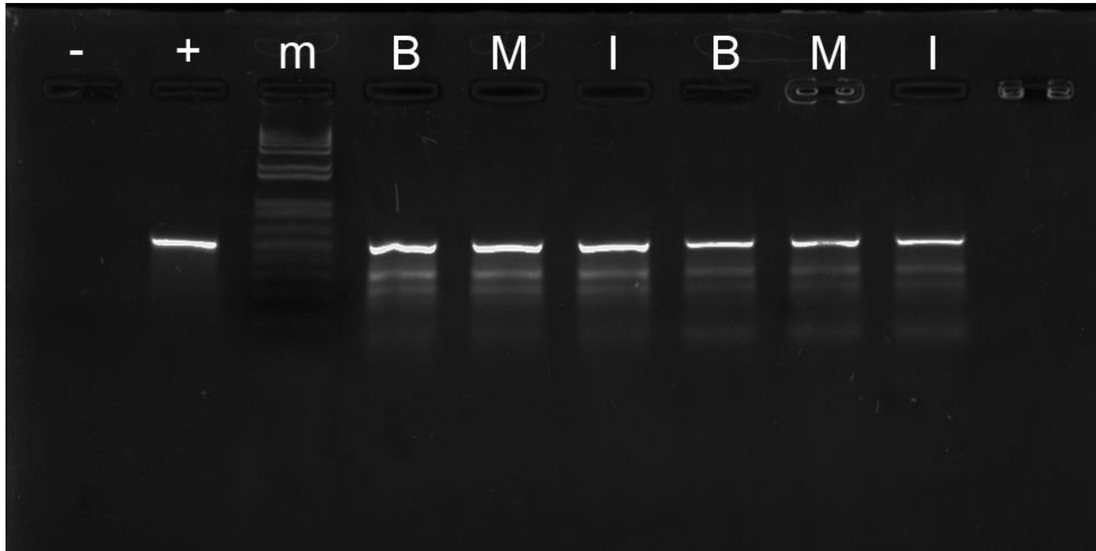

Fig.6 Identification of edited luteinizing hormone (LH) gene in different tissues from two mutated channel catfish using Cel-Ⅰ mutation detection assay. “-” indicates the negative control without template; “+” indicates the control with wild-type channel catfish DNA as template; “m” indicates 1 kb DNA ladder. “B”: brain; “M”: muscle; “I”: intestine.

3.4 Sequence modification of mutated LH gene

Several types of mutations were observed from the multiple alignment result: 1 bp insertion (5 of 13 mutated individuals), 2 bp insertion (3 of 13), 1 bp substitution (1 of 13), 2 bp deletion (1 of 13) and complex type with both insertion and substitution (3 of 13) (Fig.7). However, these mutations were not located at the expected ZFNs targeting position. The mutation sites were all located at around 60bp upstream of the proposed target site, but were still within the ORF of LH β subunit gene.

```
TTTTCTTCTCCTG-TG--TTTCTTGATGAAC     wt
TTTTCTTCTCCTGGTG--TTTCTTGATGAAC
TTTTCTTCTCCTG-TGG-TTTCTTGATGAAC     1bp insertion
TTTTCTTCTCCTG-TGT-TTTCTTGATGAAC
TTTTCTTCTCCTGATG--TTTCTTGATGAAC
TTTTCTTCTCCTGGTGG-TTTCTTGATGAAC
TTTTCTTCTCCTGATGG-TTTCTTGATGAAC     2bp insertion
TTTTCTTCTCCTG-TGTGTTTCTTGATGAAC
TTTTCTCCTCCTG-TG--TTTCTTGATGAAC     substitution
TTTTCTTCTCCTG-----TTTCTTGATGAAC     deletion
TTTTCTCCTCCTGGTG--TTTCTTGATGAAC     complex
```

Fig.7 Sequences of luteinizing hormone (LH) gene with zinc finger nuclease (ZFN) induced mutations. The wild-type (wt) channel catfish, *Ictalurus punctatus*, LH gene sequence is shown on the top Red letters/dashes indicate the modified nucleotides of LH gene.

3.5 ZFN plasmids integration detection

Three pairs of ZFN plasmid specific primers (Table 2), amplifying vector backbone, ZFN domain and CMV promoter region, were used to detect potential plasmid integration or prevailing concatamers in the cytoplasm of these 3 groups of LH mutated channel catfish. Gel electrophoresis showed no band from all these individuals (Fig.8), indicating the genome of them did not contain exogenous DNA fragments.

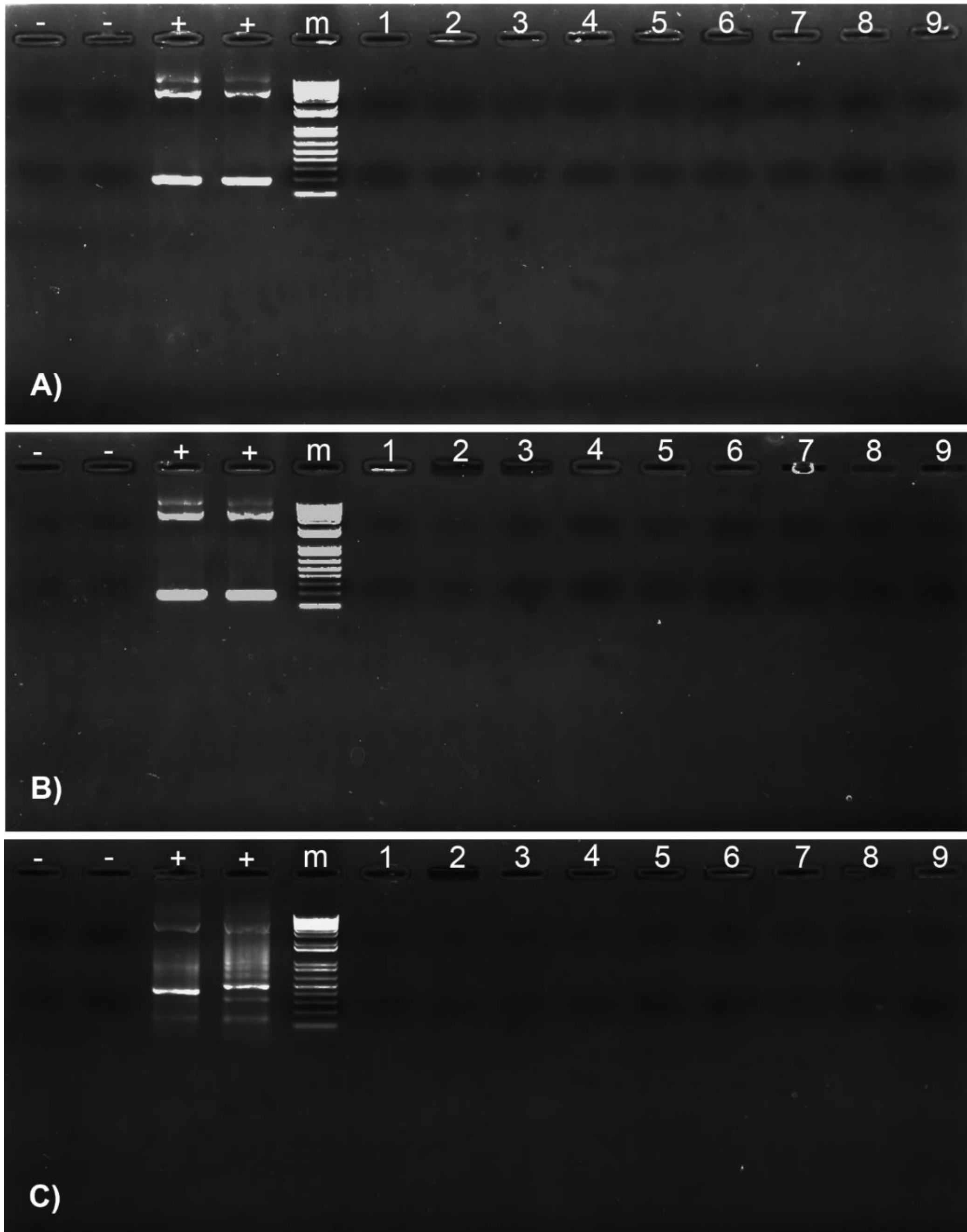

Fig.8 PCR inspection of zinc finger nuclease (ZFN) plasmids integration. "-" indicate the negative controls without template (1st lane) and with wild-type channel catfish (2nd lane), *Ictalurus punctatus*, as template, respectively; "+" indicates the positive controls with ZFN left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated LH gene; the same number indicates the same individual. A), B) & C) represent the PCR detection with the specific primers designed for integration detection.

3.6 Fertility restoration evaluation

Four pairs of two-year-old catfish carrying the mutated LH gene were introduced to aquaria for natural spawning, but none of these catfish successfully spawned. In contrast, three of four pairs of catfish from the control group spawned naturally with eggs hatched.

At three years old, eleven pairs of putative LH mutated channel catfish did not spawn when given a two-week opportunity to mate. No courtship behavior was exhibited although they had outstanding secondary sexual characteristics. A series of CPE injections did not induce any spawning. Meanwhile, eleven out of fifteen control pairs produced fertile egg masses.

4 Discussion

Three different sets of ZFNs were designed to target the channel catfish LH gene and different concentrations of each set were tested. ZNF set 1 gave the best results with a 31.3% mutation rate, and ZFN set 2 did not generate mutants. ZFN set 1 was predicted to be the most effective based upon MEL-I assay, validating this assay for ZFN design and evaluation for catfish research. ZFN set 2 also had the lowest survival. One possibility is that this set generated more off-target mutations resulting in the mortality of any mutated individuals for ZFN set 2. The hatch rate and fry survival of the control electroporated with buffer were both significantly higher than all of the ZFN treated embryos and fry suggesting that all 3 ZFN plasmid sets caused some level of lethal off-target effects during these early life stages. Obviously, these individuals were or would be quickly selected out of the population.

Different concentrations of ZFN plasmids used during the experiment also had distinct

outcomes. For the two successful ZFNs sets, 25 μg/ml of plasmids showed the best results, combining for a 33.3% (10 of 30) mutation rate and accounting for 76.9% (10 of 13) of all mutated fry. When the plasmid concentration was increased to 50 μg/ ml, no mutants were generated in all three sets of ZFNs. Taking the eggs hatch rate and fry survival rate into consideration, the best outcome was generated with ZFN set 1 with a plasmids concentration of 25 μg/ml.

ZFNs have been applied to target and manipulate genes in different animals such as zebrafish (*Danio rerio*) (Doyon et al., 2008; Meng et al., 2008; Foley et al., 2009), rainbow trout (*Oncorhynchus mykiss*) (Yano et al., 2012), frog (*Xenopus tropicalis*) (Young et al., 2011; Nakajima et al., 2012), mice (*Mus musculus*) (Carbery et al., 2010) and rat (*Rattus norvegicus*) (Geurts et al., 2009; Mashimo et al., 2010; Cui et al., 2011). Generally, ZFN technology results in the low mutation rates in fish (mostly zebrafish and medaka), often approximately 1% (Gupta et al., 2011; Gupta et al., 2012; Moore et al., 2012; Chen et al., 2013), sometimes 4-30% (Doyon et al., 2008; Foley et al., 2009; Gupta et al., 2011; Ansai et al., 2012; Taibi et al., 2013; Yano et al., 2014; Zhang et al., 2014a) and on rare occasion 30-100% (Zhang et al., 2014a). The overall mutation rate (19.7%, and 25.0 % if the failed 50 μg/ml treatment is discounted) and the highest mutation rate (38.9%) found in the current study are relatively high compared to most ZFN studies.

Microinjection was used to transfer the ZFNs mRNA into cells in previous studies. In our study, we transferred ZFNs plasmids instead of mRNA into the embryo with electroporation and successfully mutated the channel catfish LH gene. Introducing plasmids has advantages compared to introducing mRNA to induce mutations. Plasmids are easier to use and avoid degradation problems, require less time, effort and money and electroporation is technically easier than

microinjection. Additionally, plasmids can persist in the embryo for an extended time during the development allowing greater opportunity for mutation.

Electroporation allows transfer of ZNFs to a large number of embryos in a short time. Two hundred channel catfish embryos can be electroporated at one time. Electroporation was successfully used for gene knock-in at the ROSA26 locus in mouse cell lines (Perez-Pinera et al., 2012) using ZFNs, but until our research, ZFN plasmids had not been introduced to a whole embryo. One criticism of electroporating plasmids is the possibility of their integration into the catfish genome, producing a transgenic as well as gene edited organism. However, ZFN plasmids were not detected in the LH mutated channel catfish, indicating that this technique can be used to generate gene edited individuals without the unwanted outcome of transgenesis.

Sequencing results showed a variety of LH knockouts were induced, including insertions, deletions, substitutions and a complex of multiple types. These mutations were not located at the ZFN targeted position, but were all located approximately 60 bp upstream of the target site. However, they were still located in the ORF of channel catfish LH gene.

Mosaicism is a common phenomenon in ZFNs mediated gene knockout animals (Carbery et al., 2010; Nakajima et al., 2012; Yano et al., 2012) as apparently ZFNs rarely induce mutations at the one-cell stage. In our study, barbel, brain, fin, muscle and intestine were among the tissues examined for LH mutation with upwards to 3 tissues tested per mutated fish. If a mutation was detected in one tissue, it was detected for every tissue tested. To our knowledge, this is the first report that ZFNs produced a homogenous and uniform mutation in a teleost. The widespread incidence of mutation across multiple tissues is important as it would increases the probability of

the mutation being found in the germline, and it may allow loss of function studies in the initial P1 generation rather than having to wait for inheritance of the mutation in F1 or F2 fish.

ZFN off-target effects have been observed in zebrafish (Meng et al., 2008; Gupta et al., 2011) and human cells (Porteus and Baltimore, 2003; Cathomen and Joung, 2008; Cornu et al., 2008; Pattanayak et al., 2011) with the DNA sequencing of the targeted genes. ZFN-induced cytotoxicity is an issue reported in several studies, and are most likely the result of excessive cleavage at off-target sites (Cathomen and Joung, 2008). The only circumstantial evidence for off-target effects in the current study were the lower survival of fry from ZFN set 2 for which no survivors were edited, and the significantly higher hatch and fry survival of the buffer electroporated control.

Four pairs of channel catfish carrying the mutated LH gene did not spawn at two years of age, in contrast to the three of four pairs of control catfish that successfully spawned under the same conditions. In the following year, 11 pairs of LH edited channel catfish did not show courtship behavior and did not spawn despite having excellent gravidness and other secondary sexual characteristics. Recent studies indicated LH gene is indispensable in zebrafish (*Danio rerio*) reproduction especially for females (Chu et al., 2014, Zhang et al., 2014b). Surprisingly, LH appears less important for fertility in zebrafish males. This phenomenon needs to be explored and confirmed in other species. However, our study strongly suggests that desired loss of function, sterilization, was successfully achieved in both females and males. Further morphological and physiological research are needed to compare LH knockout channel catfish testis development and spermatogenesis with that of zebrafish and to validate the difference. These 11 pairs of three-year-old fish did not spawn after hormone therapy with CPE. CPE is the universal ovulater and should

have contained sufficient LH to allow final maturation of ova and sperm, but this hypothesis was apparently incorrect. Hormone therapy will need to be developed by evaluating different dose applications of purified LH to allow restoration of fertility and spawning of the LH mutated channel catfish.

5 Conclusion

An efficient approach for targeted gene mutagenesis with zinc finger nucleases in channel catfish *I. punctatus* is achieved. ZFNs targeting LH β subunit were used to induce LH gene mutations in the catfish genome, and LH inactivated channel catfish were generated. This is the first gene edited aquaculture species in the US using ZFNs.

Additionally, this study described an important approach that could be used to directly manipulate the genome of non-traditional animal models, which only requires the delivery of ZFN plasmids into the embryos with electroporation rather than the more technically difficult microinjection of mRNA into embryos. The mutated channel catfish did not respond to the CPE hormone therapy, and the technology to restore fertility will need to be improved.

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III Gene Editing of Luteinizing Hormone, Follicle-stimulating Hormone and Gonadotropin-releasing Hormone Genes to Sterilize Channel Catfish, *Ictalurus punctatus,* using Transcription Activator-like Effector Nuclease Technology

Abstract

Transcription activator-like effector nuclease (TALEN) is a powerful new research tool that has broad applications in genetic manipulation. In the present study we demonstrate the targeted mutation of channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH), follicle-stimulating hormone (FSH) and catfish type gonadotropin-releasing hormone (cfGnRH) genes with TALENs with the future goal of sterilization. TALEN plasmids were electroporated into embryos and successfully produced mutations confirmed with Cel-I assay and DNA sequencing. The mutation rates of LH, FSH and cfGnRH genes were 44.7%, 63.2% and 52.9% respectively, and mutations were detected at the expected TALEN cutting sites. Embryo hatch rates and fry survival rates were not different among either TALEN groups or among the TALEN and control groups, indicating low cell toxicity of TALENs. The electroporated plasmids were eventually degraded without integration based upon PCR. If the editing of these genes results in sterilization, it could be used in a variety of fish to minimize impacts on the natural environment, protect genetic biodiversity and ecosystems and increase environmental friendliness of aquaculture, interspecific hybrids, exotic species and transgenic fish.

1 Introduction

Transcription activator-like effector nucleases (TALENs) is a site-specific gene editing technology that has generated much interest as a broadly applicable technology with high efficiency (Baker, 2012; DeFrancesco, 2012). TALEN is engineered by a fusion of a *Fok*I endonuclease domain with a transcription activator-like (TAL) effector domain. The TAL effectors are a newly described class of specific DNA binding protein that produced by plant pathogenic bacteria *Xanthamonas*, and can directly modulate the host gene expression (Aigner et al., 2010).

Similar to zinc finger nucleases (ZFNs), the TAL effectors were engineered together to compose the DNA binding domain of TALEN. Each TAL effector consists of highly conserved 33-35 amino acid sequence repeats that could recognize and interact with a specific target nucleotide. The amino acids in position 12 and 13 of each amino acid repeat, referred to as "repeat variable di-residue" (RVD), vary and are what convey the specific DNA-binding properties. The four most common RVDs each preferentially associate with one of the four bases: NI binds to A, HD binds to C, NG binds to T and NN binds to G (Boch et al., 2009; Moscou and Bogdanove, 2009). Thus based on these straightforward sequence relationships, the TAL effector repeats can be joined together to form arrays binding to specific DNA sequence in the genome. The nuclease domain is the same as that of ZFNs with *Fok*I endonuclease fused following the DNA binding domain and works obligately as a heterodimer (Ansai et al., 2013).

After both the left and right TALENs bind to the target site, *Fok*I endonuclease will form a dimer and create a DNA double-strand break (DSB). This type of lesion is often repaired by non-homologous end-joining (NHEJ), which is an error-prone process and typically results in small

insertions or deletions (Stoddard, 2011). The gene will be disabled because of the change or shift of the translational reading frame.

Compared to ZFNs, TALENs have some significant advantages. ZFN DNA binding module recognizes and binds three nucleotides at a time. Therefore the target sequences are limited by the combination of zinc finger proteins and much of the genome cannot be recognized by the ZFN binding domain. In contrast, the TAL effector of TALEN only associates with one nucleotide and can be assembled based on the target sequence information, and thus can be designed to target nearly any site in any genome (Miller et al., 2011). ZFN binding units typically bind only 9-18 base pairs on each side of the target site, while TALEN can be engineered to recognize longer DNA sequences, and may confer increased specificity, thus reduce the incidence of off-target editing. Another important advantage is that TALENs are simple and straightforward in design and assembly strategy (Cermak et al., 2011), such that manufacture of effective TALENs is significantly cheaper and faster than that for ZFNs.

TALEN as a new and powerful genome editing technology has been successfully conducted in a number of animal models, including worm (*Bombyx mori*) (Ma et al., 2012), fruit fly (*Drosophila*) (Liu et al., 2012), ascidian (*Ciona intestinalis*) (Treen et al., 2014), zebrafish (*Danio rerio*) (Huang et al., 2011; Sander et al., 2011; Cade et al., 2012; Moore et al., 2012), medaka (*Oryzias latipes*) (Ansai et al., 2013), yellow catfish (*Tachysurus fulvidraco*) (Dong et al., 2014), frog (*Xenopus tropicalis*) (Lei et al., 2012), rat (*Rattus norvegicus*) (Tesson et al., 2011; Mashimo et al., 2013) and livestock (Carlson et al., 2012).

Catfish, especially channel catfish (*Ictalurus punctatus*) and its hybrid, channel catfish ♀ X

blue catfish (*Ictalurus furcatus)* ♂, are the most important aquaculture organism in the US. However, catfish culture is plagued by production problems such as relatively slow growth rates, disease problems and control of reproduction, bringing the US aquaculture industry is into crisis (Hanson and Sites, 2012). Exploiting fish genetics can greatly contribute to production and efficiency, and gene editing is a powerful technology which has high potential to increase production of aquaculture and profit to catfish industry (Dunham et al., 2000; Dunham, 2011). Nevertheless, sterilization is needed to dispel public concern regarding the potential environment and ecological risk of transgenic, as well as hybrid, exotic and invasive fish.

Gonadotropin-releasing hormone (GnRH) is known for its role as the final common signaling molecule used by the brain to regulate reproduction in all vertebrates (Fernald and White, 1999), and stimulated the synthesis and release of hypophysial gonadotropin, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then stimulates the secretion of steroid hormone from the gonads (Amano et al., 2002). In teleost fish, two or three forms of GnRH exist (Amano et al., 2002), while in *Siluriformes* one form named catfish type GnRH (cfGnRH) and considered to play the key role in sexual maturation in catfish (Zandbergen et al., 1995; Zohar et al., 2010). The gonadotropins, LH and FSH, are heterodimers, sharing a common α-subunit and differing in their β-subunits. They coordinate for the fine-tuning of ovarian and testicular function. FSH in certain fish has a dominant role in the initiation of gametogenesis and regulation of gonadal growth, whereas, LH is dominant mainly during gonadal maturation and spermiation/ovulation (Rosenfeld et al., 2007; Levavi-Sivan et al., 2010). Hypothetically, the mutations of LH, FSH and cfGnRH genes prevent transcription and lead to sterilization of fish.

In this experiment, we delivered TALEN plasmids targeting the channel catfish LH gene, FSH gene and cfGnRH gene, respectively, into fertilized eggs with electroporation and propose to create the gene edited sterile channel catfish by TALEN mediated targeting mutagenesis on genomic level. Our objectives include accomplishing knockout of channel catfish LH, FSH and cfGnRH genes by TALENs, compare the mutation efficiency and embryo hatch rate to ZFNs, and evaluate potential TALEN plasmids integration.

2 Material and Methods

2.1 Construction of the transcription activator-like effector nuclease

The design and assembly of TALENs plasmids, "XTN TALENs Site-Specific Nucleases" plasmids, were provided by Transposagen Company (Lexington, KY). All the plasmids were driven by the cytomegalovirus (CMV) promoter and the T7 promoter, followed by the site-specific TAL repeats, *Fok*I domain and the ampicillin resistant element (Fig.9).

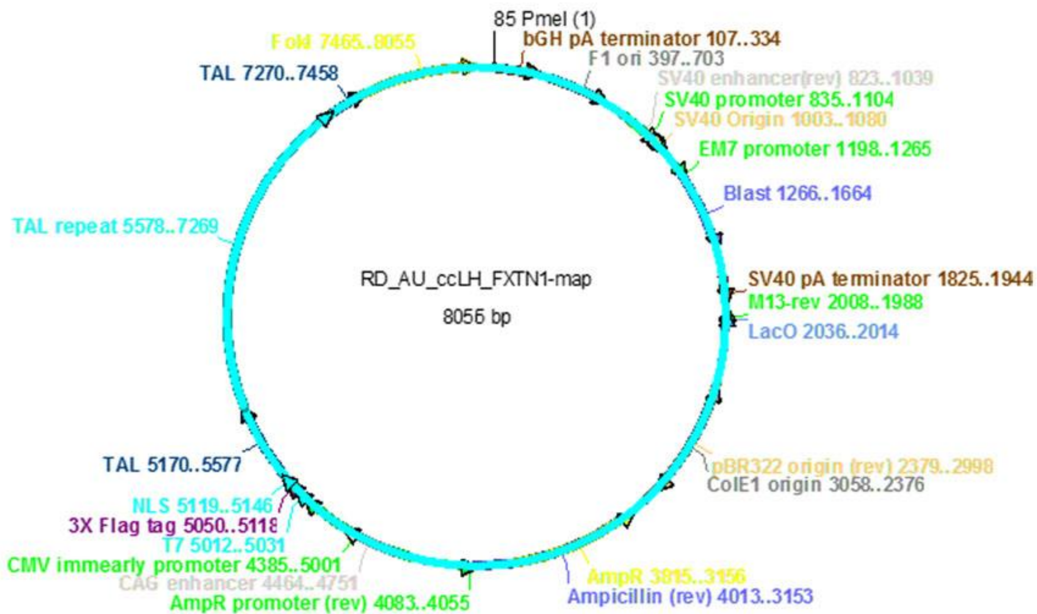

Fig.9 Schematic representation of transcription activator-like effector nuclease (TALEN) plasmid structure targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene.

The channel catfish (*I. punctatus*) LH gene β subunit (AF112192) (Liu et al., 2001), FSH gene β subunit (AF112191) (Liu et al., 2001) and the cfGnRH gene (data unpublished) were chosen as the target genes and one target site of each gene was selected (Fig.10) for gene editing to sterilize catfish.

1) LH_XTN

TCTTGATGAACTCCTTCTCCCCCGCTCAAAGCTACATTCTGCCACACTGCGA

2) FSH_XTN

TACCAACATCTCCATCACCGTGGAGAGCGACGAGTGTGGCAGCTGCATCA

3) GnRH_XTN

TTCACCTCGGAATAAACTCTACAGGCTGAAAGATCTGCTGGTGCACAGCTCA

Fig.10 Transcription activator-like effector nuclease (TALEN) plasmid sets targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene, follicle-stimulating hormone (FSH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene. TALEN binding sites are underlined and cutting site is in grey.

A set of TALENs was designed to individually target the channel catfish LH gene, FSH gene and cfGnRH gene, respectively. The targeting sites of these TALENs were all located within the ORF, in order to disturb their function (Fig.11).

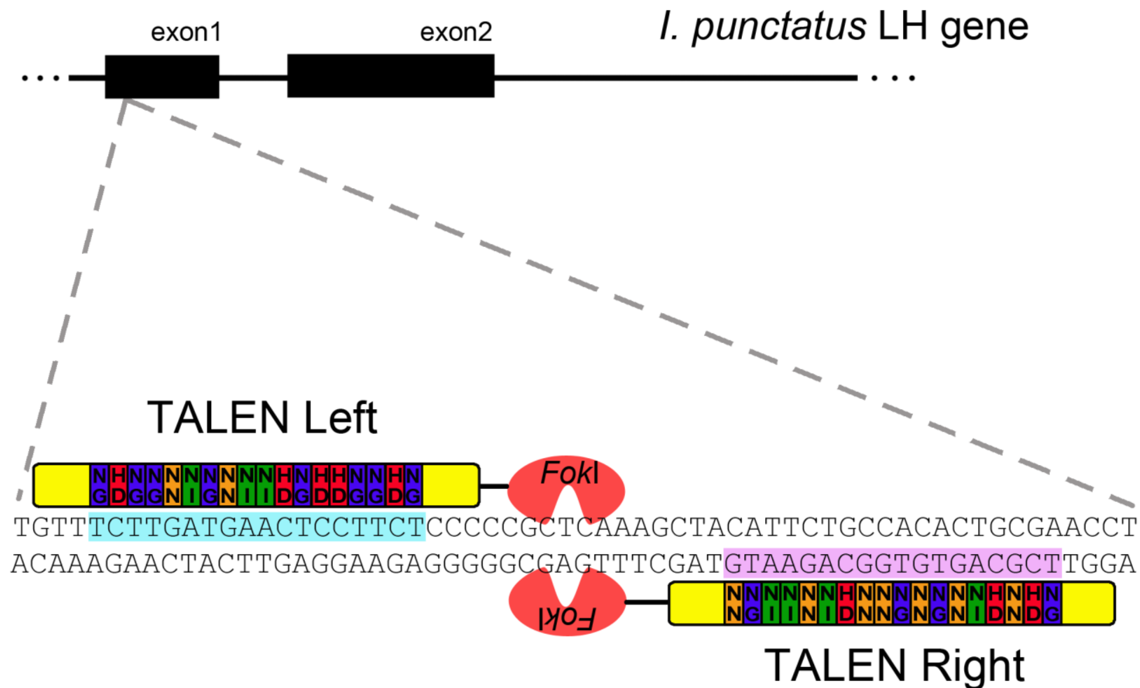

Fig.11 The location of the transcription activator-like effector nuclease (TALEN) target site in the channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene. Exons and introns of LH gene are indicated by black boxes and lines, respectively. Small boxes with two letters (NG, NI, HD and NN) inside indicate TAL repeats in the TALEN DNA binding domain that specifically recognize nucleotide bases.

2.2 Plasmid preparation

The TALEN plasmids were transformed into One Shot Top 10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY) following the manufacturer's instruction and cultured in LB broth. A 100 µl transformation mix of each plasmid was used to spread on the LB agar plate with 100 µg/ml ampicillin. A single colony was picked up from each plate and cultured in 400 ml LB broth with 100 µg/ml ampicillin. Plasmids were then extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville, Holliston, MA) and their quantity and quality were inspected by gel electrophoresis and spectrophotometry.

Each TALEN plasmid set was prepared for the purpose of double electroporation (Su, 2012; Dunham and Winn, 2014). Equal amounts of both left and right TALENs were mixed together and diluted with 2 ml saline (0.9% NaCl) to the final concentration of 25μg/ml each for the first electroporation of sperm. The same concentration of TALENs was diluted with 5 ml TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0), for the second electroporation of embryos.

2.3 Brood stock spawning

Sexually mature Kansas random channel catfish females were implanted with luteinizing hormone releasing hormone analog (LHRHa) at 90 μg/kg body weight to facilitate ovulation. Eggs were stripped from two ovulating females into metal pie pans coated with grease (Crisco, Orrville, OH). Two male channel catfish (Kansas random and AR) (Dunham and Smitherman, 1984) were euthanized and the testes were macerated into saline (0.9 % NaCl) to release sperm and produce a sperm homogenate.

2.4 Fertilization, electroporation and incubation

Double electroporation was performed for each of the TALEN groups with a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters set at 6 kV, 2^7 pulses, 0.8s burst, 4 cycles, 160 μs (Powers et al., 1991). Briefly, channel catfish sperm were electroporated with one of the three TALEN plasmid sets, then two hundred eggs were fertilized with the electroporated sperm. Sixty minutes later, embryos were collected and incubated with the same TALEN plasmid set for 10 minutes, followed with one more electroporation. The same

procedure was performed on the control group, but without plasmids.

Then embryos were moved into separate 10L tubs filled with Holtfreter's solution (Bart and Dunham, 1996) containing 10ppm doxycycline and incubated at 27℃ until hatch. Dead embryos were removed and water was changed daily. Channel catfish fry were then transferred into a recirculating system.

2.5 Sample collection, DNA extraction and mutation analysis

The pelvic fin and barbel of 6-month-old fingerlings were sampled for DNA analysis. Samples were digested with 100 μg/ml proteinase K followed by protein precipitation and DNA ethanol precipitation as described by Kurita et al. (2004). DNA quantity and quality were determined with gel electrophoresis and spectrophotometry.

Channel catfish LH gene β subunit specific primer, FSH gene β subunit specific primer and cfGnRH gene specific primers (Table 5) were designed and Roche Expand High Fidelity^{Plus} PCR System (Roche, Indianapolis, IN) was used to amplify these DNA samples. The PCR amplification procedure was as follows: initial denaturation for 2min at 94℃; followed by 35 cycles of 94℃ for 30s, 60℃ for 30s and 72℃ for 1min; and a final elongation for 10min at 72℃. PCR products were examined by gel electrophoresis.

Table 5 Primer sequences used for the amplification of luteinizing hormone (LH) β subunit, follicle-stimulating hormone (FSH) β subunit and catfish type gonadotropin-releasing hormone (cfGnRH) in channel catfish (*Ictalurus punctatus*).

Primer	Sequence (5'-3')	Product size (bp)	Description
LH-F LH-R	AGGATGTCAGTGCCAGCTTC CTTGGAGTAAATGGACTCGTTG	572	LH gene amplification and mutation analysis
FSH-F FSH-R	CACAACTCCAGCTGTGACAA CAGAATTCCGTGGCCATTTA	511	FSH gene amplification and mutation analysis
GnRH-F GnRH-R	ATGGATGCTGTCTTTGTTTTCC CCACACGAAATAAAGGCAAAG	550	cfGnRH gene amplification and mutation analysis

Gene mutations were detected through the Cel-I mutation detection assay with SURVEYOR Mutation Detection Kit (Integrated DNA Technologies, Coralville, IA) as described by Miller et al. (2007). Briefly, PCR products were denatured and re-annealed as follows: 94℃ 10min; 94℃ to 85℃ -2℃/s; 85℃ to 25℃ -0.1℃/s; cooling to 4℃; then 1 µl Enhancer S and 1 µl Nuclease S were added into 5 µl of the products above and incubated at 42℃ for 30min. The digested PCR products were resolved on 2% UltraPure Agrose-1000 high resolution agarose gel (Invitrogen, Grand Island, NY).

2.6 TA clone and sequencing

LH, FSH and cfGnRH genes from the mutated fingerlings were amplified and were purified with IsoPure DNA Purification Kit (Denville, Holliston, MA), and then inserted into the vector of TOPO TA Cloning Kit for Sequencing (Invitrogen, Grand Island, NY) and transformed into the One Shot TOP10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY). Colonies were

selected from a LB agar plate containing 100 μg/ml ampicillin.

Ten colonies corresponding to each individual carrying mutated genes were picked and amplified. The bacteria glycerol stock were added into 96-well plates and sent to Eurofins Genomics Company (Louisville, KY) for sequencing.

Upon receiving the results, the quality was checked and the sequences were aligned with the wild type channel catfish LH, FSH and cfGnRH genes using the online multiple sequence alignment tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), respectively.

2.7 Plasmid integration inspection

To determine the presence of plasmids integrated into the channel catfish genome or persisting in the cytoplasm, two pairs of specific primers (Table 6) for each set of the TALEN plasmids targeting LH, FSH and cfGnRH genes were designed to detect the plasmid DNA in corresponding mutated channel catfish. The amplification regions of these two primers were the CMV promoter region and TAL repeats region, respectively. The PCR procedure was the same as above of amplifying LH, FSH and cfGnRH genes and products were inspected with electrophoresis.

Table 6 Primer sequences used for the detection of transcription activator-like effector nuclease (TALEN) plasmids targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene, follicle-stimulating hormone (FSH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene.

Primer	Sequence (5'-3')	Product size (bp)	Description
LI1-F LI1-R	AACAACAACGGCGGTAAG TTCCCTCCATTGTTATTCGC	323	TALEN LH plasmids integration detection
LI2-F LI2-R	GCGAATAACAATGGAGGGAA GTCGTGGGATGCAATGG	318	TALEN LH plasmids integration detection
FI1-F FI1-R	GCAAATAATAACGGTGGCAA GTTTCCCTCCGTCATGCG	124	TALEN FSH plasmids integration detection
FI2-F FI2-R	GCGAATAACAATGGAGGGA GCCACCGTTATTATTTGCAA	324	TALEN FSH plasmids integration detection
GI1-F GI1-R	AACAACAACGGCGGTAAG CCCATTATTGTTCGCGATTG	114	TALEN cfGnRH plasmids integration detection
GI2-F GI2-R	GCATGACGGAGGGAAAC CCATTATTGTTCGCGATTGA	215	TALEN cfGnRH plasmids integration detection

2.8 Statistical analysis

Mutation rates, hatch rates and survival rates from TALEN sets targeting different genes were analyzed utilizing Fisher's Exact Test and Pearson's Chi-square Test (McDonald, 2014). All analysis were performed with statistical software R (version 3.1.3).

3 Results

3.1 Hatch rate and survival rate

Two hundred eggs were double electroporated for the three TALEN treatment groups and the one control group. For the TALEN-LH group, 67 hatched with a hatch rate of 33.5% (Table 7). Eighty of 200 eggs hatched for the TALEN-FSH group (40%), while 76 hatched in TALEN-GnRH group (36.5%). No significant difference exited among different groups (Pearson's Chi-square Test,

p=0.387). At 6 months, the fry survival of the TALEN-LH, TALEN-FSH and TALEN-GnRH groups was 56.7%, 71.3% and 67.1%, respectively, which was not different among groups (Fisher's Exact Test, *p*=0.182).

Two hundred eggs in control group has a hatch rate of 44.0% (84 of 200) and a survival rate of 75.0% (63 of 84). When comparing with the treatment groups, there is no significant difference exists on both hatch rate (Pearson's Chi-square Test, *p*=0.340) and survival rate (Fisher's Exact Test, *p*=0.107).

Table 7 Comparison of the embryo hatch rate and fry survival rate for channel catfish (*Ictalurus punctatus*) treated with transcription activator-like effector nucleases (TALENs) targeting luteinizing hormone (LH) gene, follicle-stimulating hormone (FSH) gene or catfish type gonadotropin-releasing hormone (cfGnRH) gene.

Constructs	Eggs	Fry hatched	Hatch rate (%)*	Fry survival	Survival rate (%)†
TALEN-LH	200	67	33.5	38	56.7
TALEN-FSH	200	80	40.0	57	71.3
TALEN-GnRH	200	76	36.5	51	67.1
Control	200	84	44.0	63	75.0

* No significant difference exits either among different treatment groups (*p*=0.387), or between treatment groups and the control group (*p*=0.340).

† No significant difference exits either among different treatment groups (*p*=0.182), or between treatment groups and the control group (*p*=0.107).

3.2 Mutation rate

As indicated by the three bands evident for some individual on the gel shown in Fig.12, individuals exposed to TALENs targeting channel catfish LH gene had a 44.7% mutation rate (Table 8). None of the individuals in the control group were mutated.

Table 8 Mutation rate of channel catfish (*Ictalurus punctatus*) electroporated with transcription activator-like nuclease (TALEN) plasmids targeting luteinizing hormone (LH) gene, follicle-stimulating hormone (FSH) gene or catfish type gonadotropin-releasing hormone (cfGnRH) gene.

TALEN sets	Concentration (μg/ml)	N fry	N mutated fry	Mutation rate (%)
LH	25	38	17	44.7
FSH	25	57	31	63.2
cfGnRH	25	51	27	52.9
Control	0	63	0	0

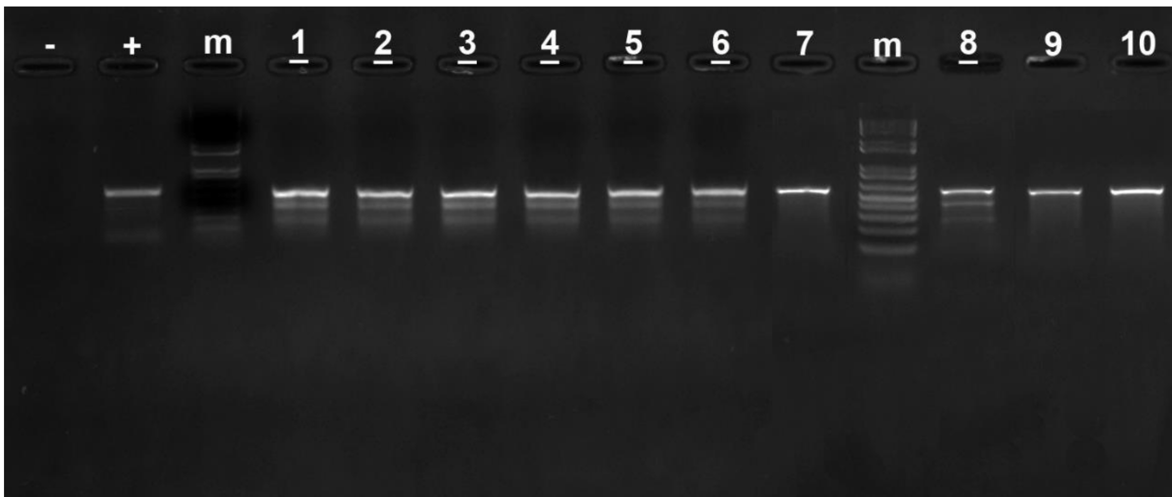

Fig.12 Identification of edited luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*) using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Three bands indicate mutations occurred and the mutated channel catfish are underlined.

The FSH gene was successfully mutated in 31 of 57 channel catfish fingerlings which had a 63.2% mutation rate (Table 8, Fig.13). In the group electroporated with TALEN plasmids targeting the cfGnRH gene, the mutation rate was 52.9% (Table 8). Two patterns of DNA bands, both of which indicated the cfGnRH gene mutation, could be found on the gel, one with three bands and

the other with five bands (Fig.14).

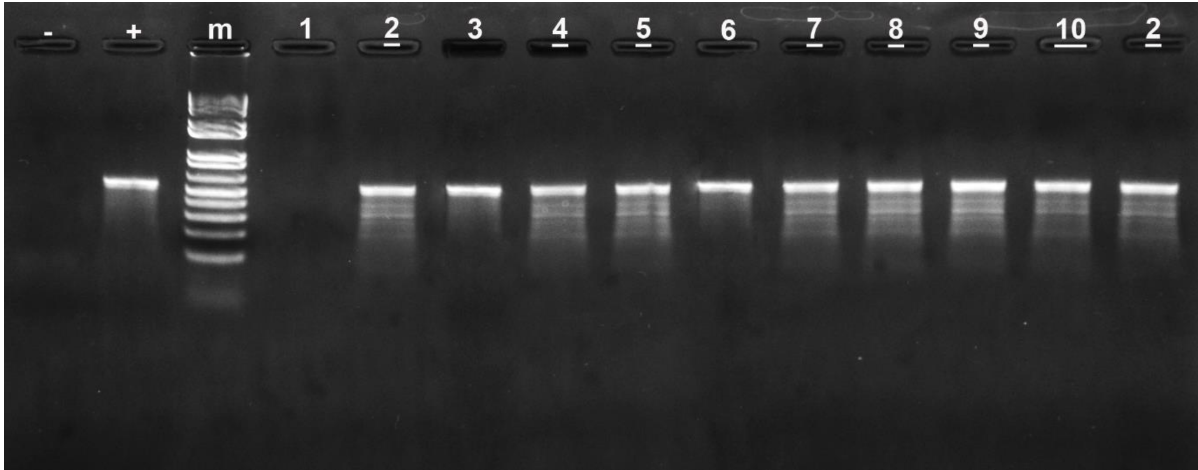

Fig.13 Identification of edited follicle-stimulating hormone (FSH) gene in channel catfish (*Ictalurus punctatus*) using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Multiple bands indicate mutations occurred and the mutated channel catfish are underlined.

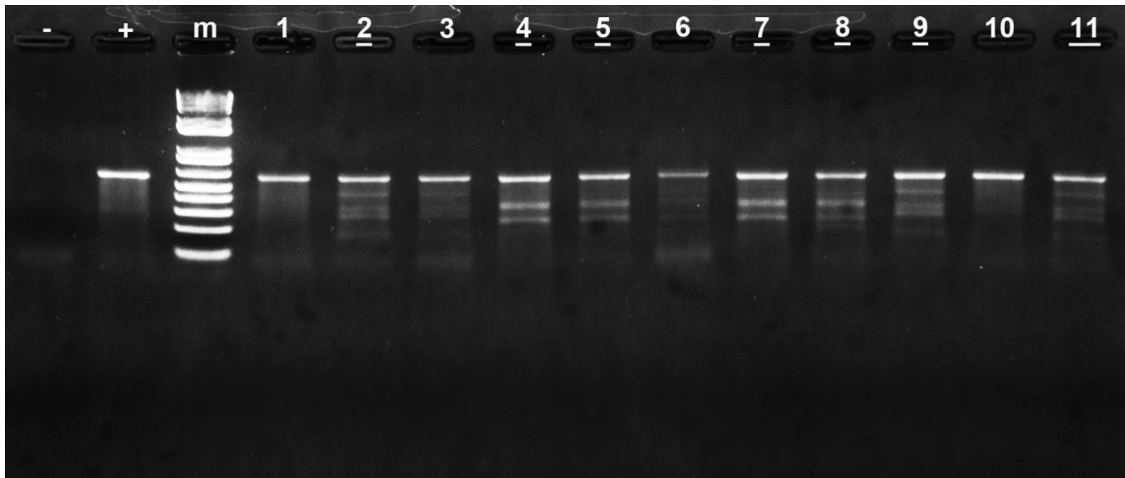

Fig.14 Identification of edited catfish type gonadotropin-releasing hormone (cfGnRH) gene in channel catfish (*Ictalurus punctatus*) using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Multiple bands indicate mutations occurred and the mutated channel catfish are underlined.

3.3 Sequence modification of the mutated LH, FSH and cfGnRH genes

Sequencing and alignment results confirmed that channel catfish were successfully mutated for the LH gene, multiple types of mutations were generated. Most of the mutations occurred as one to five base deletions within the TALEN target cutting site; some changes were base substitutions and the remainder were in the form of base insertions (Fig.15). Unlike the ZFN experiment, the TALEN technology introduced mutations located within the expected cutting site of the LH gene, and thus showed higher specificity in the gene editing process. The mutation should lead to a frame-shift or early termination in transcription and the LH amino acid sequences will be altered, disrupting normal LH function.

```
GTTTCTTGATGAACTCCTTCTCCCCCGCTCAAAGCTACATTCTGCCACACTGCGAACCT      wt
GTTTCTTGATGAACTCCTTCTCCCCCG-TCAAAGCTACATTCTGCCACACTGCGAACCT
GTTTCTTGATGAACTCCTTCTCC---GCTCAAAGCTACATTCTGCCACACTGCGAACCT
GTTTCTTGATGAACTCCTTCTCCCC---TCAAAGCTACATTCTGCCACACTGCGAACCT      deletion
GTTTCTTGATGAACTCCTTCTC----GCTCAAAGCTACATTCTGCCACACTGCGAACCT
GTTTCTTGATGAACTCCTTCTCC-----TCAAAGCTACATTCTGCCACACTGCGAACCT
GTTTCTTGATGAACTCCTTCTCCCCCGCTCTAAGCTACATTCTGCCACACTGCGAACCT      substitution
GTTTCTTGATGAACTCCTTCTCCCCCGCATCAAAGCTACATTCTGCCACACTGCGAACCT
GTTTCTTGATGAACTCCTTCTCCCCCCGCTCCAAAGCTACATTCTGCCACACTGCGAACCT    insertion
```

Fig.15 Sequences of channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene with transcription activator-like effector nuclease (TALEN) induced mutations. The wild-type (wt) channel catfish LH gene sequence is shown on the top. Sequences underlined are the TALEN binding sites; red letters/dashes indicate the modified nucleotides of LH gene.

Analysis of the sequence information from the FSH gene mutated individuals and cfGnRH gene mutated individuals generated similar results as for the LH gene. All the mutations occurred

within the TALEN cutting site, and base deletion, substitution and insertion were found for both genes (Fig.16, Fig.17). All of these mutations were introduced in the ORF region, which should disrupt the corresponding normal gene functions.

```
GTCTTACCAACATCTCCATCACCGTGGAGAGCGACGAGTGTGGCAGCTGCATCACTG          wt
GTCTTACCAACATCTCCATCACCGTGGA--GCGACGAGTGTGGCAGCTGCATCACTG
GTCTTACCAACATCTCCATCACCGTG---AGCGACGAGTGTGGCAGCTGCATCACTG          deletion
GTCTTACCAACATCTCCATCACCG----GAGCGACGAGTGTGGCAGCTGCATCACTG
GTCTTACCAACATCTCCATCACCGTGGA----GACGAGTGTGGCAGCTGCATCACTG
GTCTTACCAACATCTCCATCACCGTGGAGAGCGCCGAGTGTGGCAGCTGCATCACTG          substitution
GTCTTACCAACATCTCCATCACCGTGGAGAGCGGCGAGTGTGGCAGCTGCATCACTG
GTCTTACCAACATCTCCATCACCGTGGACTCGGAGCGACGAGTGTGGCAGCTGCATCACTG      insertion
GTCTTACCAACATCTCCATCACCGTGGAGAGCGACGAGTGCGGTGTGGCAGCTGCATCACTG
```

Fig.16 Sequences of channel catfish (*Ictalurus punctatus*) follicle-stimulating hormone (FSH) gene with transcription activator-like effector nuclease (TALEN) induced mutations. The wild-type (wt) channel catfish FSH gene sequence is shown on the top. Sequences underlined are the TALEN binding sites; red letters/dashes indicate the modified nucleotides of FSH gene.

```
TGTTTCACCTCGGAATAAACTCTACAGGCTGAAAGATCTGCTGGTGCACAGCTCATAAT      wt
TGTTTCACCTCGGAATAAACTCT--AGGCTGAAAGATCTGCTGGTGCACAGCTCATAAT
TGTTTCACCTCGGAATAAACTCT---GGCTGAAAGATCTGCTGGTGCACAGCTCATAAT      deletion
TGTTTCACCTCGGAATAAACTCTACAG---GAAAGATCTGCTGGTGCACAGCTCATAAT
TGTTTCACCTCGGAATAAACTCTA-----TGAAAGATCTGCTGGTGCACAGCTCATAAT
TGTTTCACCTCGGAATAAACTCTACAAGCTGAAAGATCTGCTGGTGCACAGCTCATAAT      substitution
TGTTTCACCTCGGAATAAACTCTACAGCCTGATAGATCTGCTGGTGCACAGCTCATAAT
TGTTTCACCTCGGAATAAACTCTACAGGGGCTGAAAGATCTGCTGGTGCACAGCTCATAAT    insertion
```

Fig.17 Sequences of channel catfish (*Ictalurus punctatus*) catfish type gonadotropin-releasing hormone (cfGnRH) gene with transcription activator-like effector nuclease (TALEN) induced mutations. The wild-type (wt) channel catfish cfGnRH gene sequence is shown on the top. Sequences underlined are the TALEN binding sites; red letters/dashes indicate the modified nucleotides of cfGnRH gene.

3.4 TALEN plasmids integration evaluation

Two pairs of TALEN plasmids specific primers (Table 6), which amplify the CMV promoter or TAL repeats region of each TALEN sets, were used to detect the presence of plasmids DNA in all mutated individuals. Similar to the results of ZFNs experiment, no plasmid DNA was detected with PCR for all TALEN-LH (Fig.18), TALEN-FSH (Fig.19) and TALEN-GnRH (Fig.20) mutated fish. None of the channel catfish fingerlings carried the exogenous DNA.

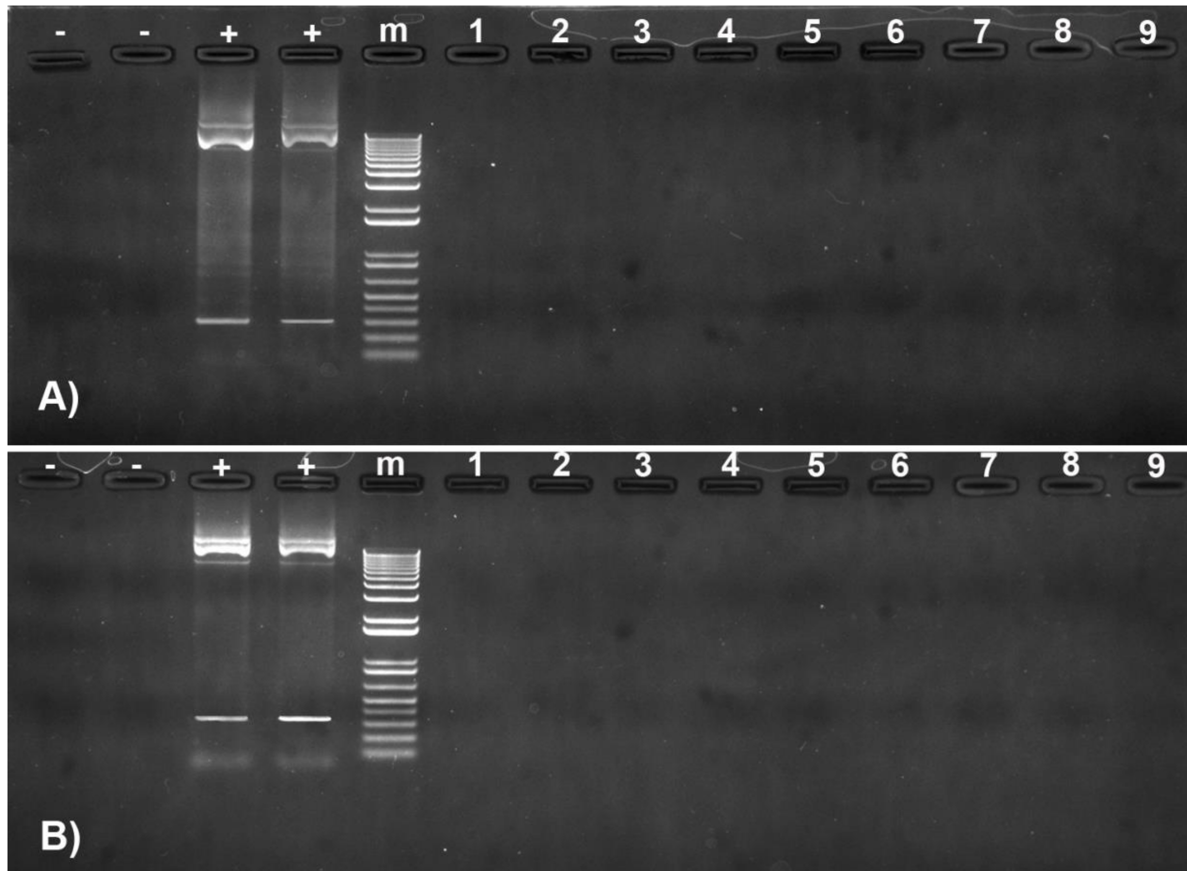

Fig.18 PCR inspection of transcription activator-like effector nuclease (TALEN) plasmids [targeting the luteinizing hormone (LH) gene] integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st and 2nd lanes) and with wild-type channel catfish (3rd and 4th lanes) as template, respectively; "+" indicates the positive controls with TALEN left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated LH gene; the same number indicates the same individual. A) & B) represent the PCR detection with different specific primers designed to amplify CMV promoter region and TAL repeats region, respectively, for integration detection or presence of the plasmids in the cytoplasm.

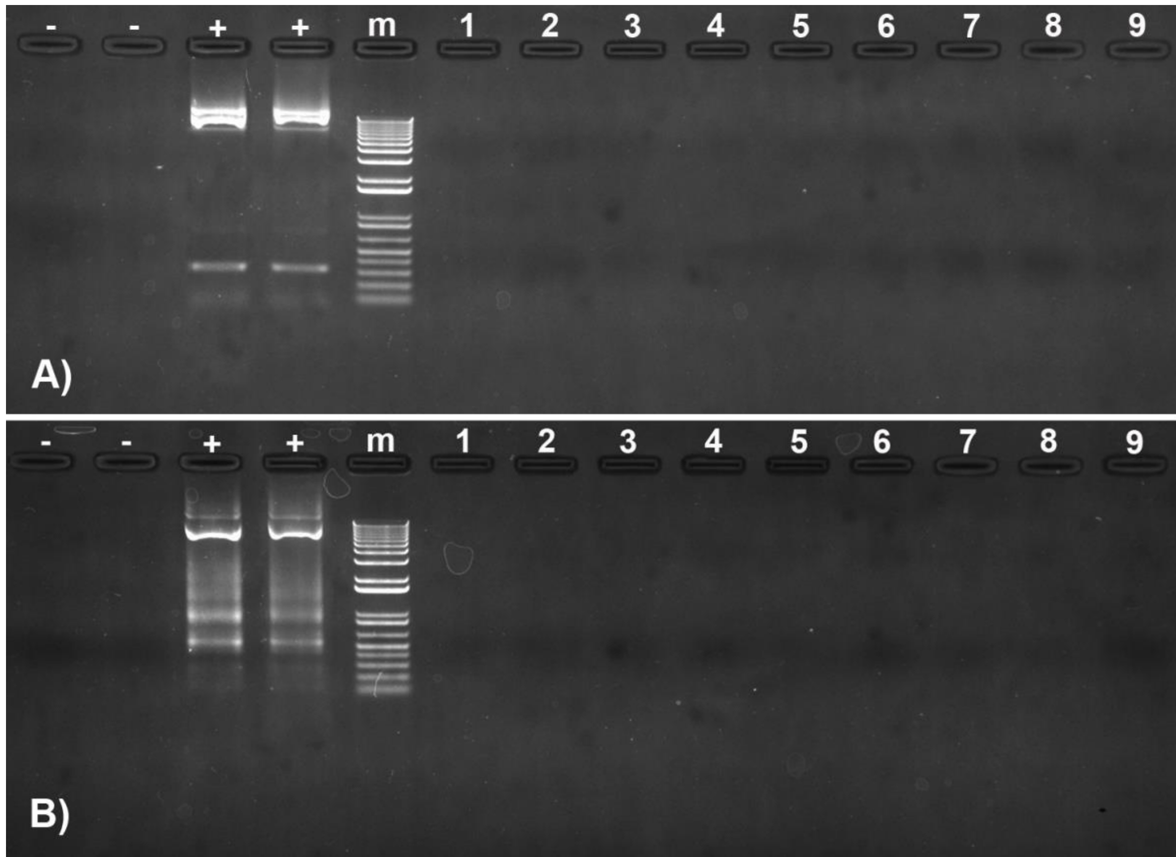

Fig.19 PCR inspection of transcription activator-like effector nuclease (TALEN) plasmids [targeting the follicle-stimulating hormone (FSH) gene] integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st and 2nd lanes) and with wild-type channel catfish (3rd and 4th lanes) as template, respectively; "+" indicates the positive controls with TALEN left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated FSH gene; the same number indicates the same individual. A) & B) represent the PCR detection with different specific primers designed to amplify CMV promoter region and TAL repeats region, respectively, for integration detection or presence of the plasmids in the cytoplasm.

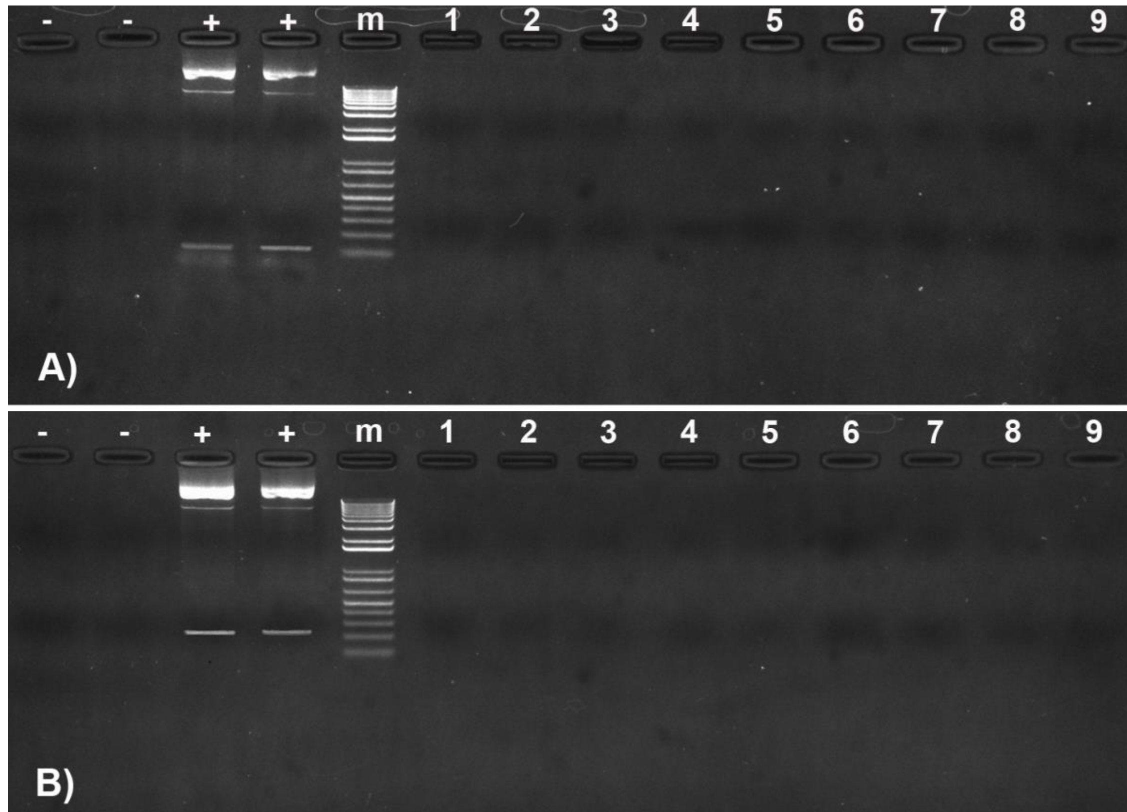

Fig.20 PCR inspection of transcription activator-like effector nuclease (TALEN) plasmids [targeting the catfish type gonadotropin-releasing hormone (cfGnRH) gene] integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st and 2nd lanes) and with wild-type channel catfish (3rd and 4th lanes) as template, respectively; "+" indicates the positive controls with TALEN left and right plasmids as template, respectively; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated cfGnRH gene; the same number indicates the same individual. A) & B) represent the PCR detection with different specific primers designed to amplify CMV promoter region and TAL repeats region, respectively, for integration detection or presence of the plasmids in the cytoplasm.

4 Discussion

Gene mutation targeting channel catfish LH gene, FSH gene and cfGnRH gene, using the engineered TALEN technology with electroporation was accomplished. The mutation rate varied among groups targeted for the different genes, ranging from 44.7% to 63.2%. The hatch rate and survival rate of these treatment groups were not different from the control group. Apparently, the

TALEN technology did not have any negative effects on the development of embryos and fry. Thus, off-target mutations did not occur or did not affect genes important for development and early survival. A variety of mutations were induced including deletions, additions and substitutions. This is the first report of using TALENs to induce mutations in a major US aquaculture species.

TALEN has been proved a powerful approach to introduce DNA mutations in a number of animal models, including worm (*Bombyx mori*) (Ma et al., 2012), fruit fly (*Drosophila*) (Liu et al., 2012), ascidian (*Ciona intestinalis*) (Treen et al., 2014), frog (*Xenopus tropicalis*) (Lei et al., 2012), rat (*Rattus norvegicus*) (Tesson et al., 2011; Mashimo et al., 2013) and livestock (Carlson et al., 2012). In teleost, TALENs have been utilized to investigate gene function in medaka (*Oryzias latipes*) (Ansai et al., 2013), yellow catfish (*Tachysurus fulvidraco*) (Dong et al., 2014), and mostly in zebrafish (*Danio rerio*) (Huang et al., 2011; Sander et al., 2011; Cade et al., 2012; Moore et al., 2012). The mutation rates of TALEN varies in different species. In zebrafish the mutation rate ranged from 2.0% to 76.8% (Bedell et al., 2012; Cade et al., 2012; Dahlem et al., 2012; Moore et al., 2012), and in medaka the mutation rate could be as high as 100% in some cases (Ansai et al., 2013). In the current experiment, the mutation rate for channel catfish LH gene, FSH gene and cfGnRH gene were 44.7%, 63.2% and 52.9%, respectively, which was relatively high compared to rates in other species.

TALEN mutation rates are generally higher compared to ZFN, and as much as10 times higher in some cases (Chen et al., 2013). In the current experiment, the mutation rate of TALEN exposed embryos, 44.7%, 63.2% and 52.9% for TALEN-LH, TALEN-FSH and TALEN-GnRH, respectively, were all significantly higher than that of ZFNs group (19.7%, see Chapter II) when

analyzing with Fisher's Exact Test (p=0.0125, p=0.0001, p=0.0003). Meanwhile band intensity in Cel-I assay results were darker and more distinct in the TALEN groups (Fig.12, Fig.13, Fig.14) than that in the ZFN groups (Fig.5), which means higher mutation efficiency was achieved using TALEN (Qiu et al., 2004). More bands were obtained in some of cfGnRH mutated individuals, indicating more mutations may exist. Multiple mutations were not found in a single individual and a potential reason could be we only picked up 10 colonies of each mutated individual for sequencing. It may be possible to discover more mutations with further more sequencing and alignment analysis. All the evidence indicated TALEN technology has better efficiency than ZFN when performing targeted gene manipulation.

The sequences of the detected mutations in this experiment were all located within the designed TALEN cutting sequence for all three TALENs targeting channel catfish LH gene, FSH gene and cfGnRH gene. Nucleotide deletion, insertion and substitution were all observed. All these modifications were in the ORF of the corresponding genes, and will lead to a change of amino acid sequence, which likely will disable gene function. In contrast, in the ZFN experiment (Chapter II), off-target mutations occurred outside the supposed site, but within the targeted gene ORF. This phenomenon was also observed in other species, such as zebrafish (Meng et al., 2008; Gupta et al., 2011), when using ZFN technology. TALEN had higher specificity than ZFN when utilized in the current targeted gene editing studies on channel catfish.

Almost all previous targeted gene editing work using TALEN or ZFN technology was conducted with microinjection of mRNA into cells (Doyon et al., 2008; Ansai et al., 2012; Cade et al., 2012; Moore et al., 2012). Although mutation rates from microinjection of TALEN mRNA is

high in these studies (Cade et al., 2012; Moore et al., 2012), this technique is a time and labor consuming process and it is difficult to produce a large number of mutated individuals in a short time. Electroporation is an alternative method to introduce exogenous nucleic acid into cells, but has been rarely utilized in gene editing research. Only a few studies were conducted with electroporation and most of them performed on cultured cells. But there is a research that TALEN plasmids were electroporated into ascidian (*Ciona intestinalis*) embryos and successfully mutated target genes (Treen et al., 2014). Utilizing electroporation we successfully mutated channel catfish LH gene, FSH gene and cfGnRH gene, by delivering TALEN plasmids instead of mRNA into channel catfish embryos in this study. The mutation rate from electroporation of TALEN plasmids was as high as 63.2%, which was a higher mutation rate than what was obtained in other studies that utilized microinjection of TALEN mRNA. This process is much easier than microinjection, avoids the degradation problems, requires less time and effort and could produce large amount of mutated fish in a short time. Additionally, plasmid DNA did not integrate into channel catfish genome (Fig.18, Fig.19, Fig.20), resulting in mutated non-transgenic channel catfish. Based upon the results of both the ZFN and TALEN experiments with channel catfish, electroporation is a good technique for targeted gene editing without inducing transgenesis.

Cell toxicity is an issue in the gene targeting mutation studies. Cell death and apoptosis are most likely associated with off-target effects created by these technologies. ZFNs are reported to have relatively high off-target effects in previous studies (Gupta et al., 2011). In contrast, TALENs generally have low incidence of off-target effects and thus low cell toxicity (Mussolino et al., 2011; Zhu et al., 2013; Liu et al., 2014). Our experimental results showed that both the embryo hatch

rate and fry survival rate of TALEN treatment groups was not different than that of the control group, indicating that introduction of TALEN plasmids and the resulting mutations did not increase the embryo and fry mortality, and were likely on target. Thus, the results with channel catfish are consistent with previous results, indicating than TALEN technology gives higher mutation rate and lower cell toxicity and is a better method to induce gene editing on the genomic level compared to ZFN technology and even when electroporation of plasmids is utilized.

TALENs with low off-target effect have multiple benefits on commercial application. The low cost of design and assembly of TALENs enables large scale application in aquaculture industry. Low off-target effect reduces the cell toxicity of gene editing and does not influence embryo hatching and fry survival, will not dramatically increase the initial investment. In addition, TALENs decrease other unwanted gene modifications and thus will not change the good commercial trials and not affect production and profit. However, pleiotropic effects of the mutant allele will need evaluation to ensure no adverse effects on economically important traits.

If the editing of LH gene, FSH gene or cfGnRH gene using TALENs results in sterilization, it could be applied to overcome potential environmental risk, as well as combining with other fish genetic enhancement programs to profit aquaculture industry.

5 Conclusion

A simple, convenient and efficient method of targeted gene editing with TALEN technology in channel catfish *I. punctatus* was demonstrated. TALEN plasmids targeting channel catfish LH β subunit, FSH β subunit and cfGnRH gene were introduced into fertilized eggs with

electroporation and these reproductive related genes were successfully mutated. This is the first time that an aquaculture species in the US was gene edited at the genomic level with TALENs.

Further study is needed to evaluate the reproductive status of LH, FSH and cfGnRH mutated individuals when they reach sexual maturity. The restoration of fertility with hormone therapy will also need to be developed to allow production of fertile brood stock to complete the reversible sterilization in channel catfish.

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IV Gene Editing of Luteinizing Hormone and Gonadotropin-releasing Hormone Genes to Sterilize Channel Catfish, *Ictalurus punctatus,* using Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Technology

Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is a powerful new research tool that enables targeted gene editing in a wide variety of animals. Here we demonstrate efficient targeted mutagenesis in channel catfish (*Ictalurus punctatus*), the most important freshwater aquaculture species in the US. CRISPR/Cas9 was utilized targeting channel catfish luteinizing hormone (LH) and catfish type gonadotropin-releasing hormone (cfGnRH) genes to generate sterile channel catfish. CRISPR/Ca9 plasmids were electroporated into embryos to mutate LH and cfGnRH genes, while CRISPR/Cas9 RNAs were microinjected into embryos to target cfGnRH gene. Both methods successfully produced mutations that were confirmed with Cel-I assay and sequencing. The mutation rates for the electroporation were 37.5% and 38.5%, while it is 100% when using microinjection. However, the egg hatch rate was only 9.0% using microinjection and lower than that of electroporation, 23.0% and 21.0% (Fisher's Exact Test, p =0.008). The electroporated plasmids were eventually degraded without integration as they were not detectable in mutated individuals using PCR. If the editing of these genes results in sterilization,

several applications could be explored to profit the catfish industry and overcome potential environmental risk of various genetic manipulations.

1 Introduction

Recent advances in the study of prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system provide an alternative genome editing approach. The CRISPR/CRISPR-associated (Cas) system is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements that protects bacteria and archaea from invading viruses and plasmids (Deveau et al., 2010; Bhaya et al., 2011; Makarova et al., 2011; Chang et al., 2013). Three major types of CRISPR (types I-III) have been categorized on the basis of locus organization and conservation (Makarova et al., 2011), wherein each system comprises a cluster of Cas genes, noncoding RNAs and a distinctive array of repetitive elements (direct repeats). These repeats are interspaced by short variable sequences derived from exogenous DNA targets known as protospacers, and together they constitute the CRISPR RNA (crRNA) array (Makarova et al., 2011). Within the DNA target, each protospacer is always associated with a protospacer adjacent motif (PAM), which can vary depending on the specific CRISPR system (Ran et al., 2013).

The type II CRISPR system is one of the best characterized (Gasiunas et al., 2012; Jinek et al., 2012), which consists of the nuclease Cas9, the crRNA array that encodes the guide RNAs and a required auxiliary trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units. Each crRNA unit then contains a 20-nt guide sequence and a partial direct repeat, where the former direct Cas9 to a 20-bp DNA target. The crRNA and

tracrRNA can be fused together to create a chimeric, single-guide RNA (sgRNA) (Ran et al., 2013). Cas9 can thus be re-directed toward almost any target of interest in immediate vicinity of the PAM sequence by altering the 20-nt guide sequence within the sgRNA. Similarly to zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), Cas9 promotes genome editing by stimulating a double-strand break (DSB) at target locus. Upon cleavage by Cas9, the target locus typically undergoes the NHEJ and HDR. In the absence of a repair template, DSBs are re-ligated by the NHEJ process, which leaves scars in the form of insertion/deletion (indel) mutations, which can lead to frameshift and premature stop codons.

Cas9 offers several potential advantages, including the ease of customization, higher targeting efficiency and the ability to facilitate multiplex genome editing. Cas9 can be easily retargeted to new DNA sequences by simply change the 20-nt guide sequence. The Cas9 derived from *Streptococcus pyogenes* is known to make a DSB between the 17th and 18th bases in the target sequences (Jinek et al., 2012), whereas TALENs cleave nonspecifically in the 12-24bp linker between the pair of binding sites. There is a requirement for the Cas9 target sites, which is the presence of a PAM sequence directly 3' of the 20-bp target sequence. Also Cas9 can be used to target multiple genomic loci simultaneously by co-delivering a combination of sgRNAs (Jao et al., 2013).

CRISPR/Cas9 system has been successfully utilized in many animals for the gene function disruption, such as nematode (*Caenorhabditis elegans*) (Friedland et al., 2013; Ward, 2015), fruit fly (*Drosophila*) (Gratz et al., 2013), frog (*Xenopus tropicalis*) (Nakayama et al., 2013), zebrafish (*Danio rerio*) (Chang et al., 2013; Hwang et al., 2013; Jao et al., 2013; Xiao et al., 2013), mice

(Wang et al., 2013; Shen et al., 2013; Wu et al., 2015) and human cells (Cong et al., 2013; Hwang et al., 2013; Mali et al., 2013). Higher efficiency and lower cell toxicity have been reported.

Channel catfish (*Ictalurus punctatus*) and its hybrid, channel catfish ♀ X blue catfish (*Ictalurus furcatus)* ♂, are the most important aquaculture organism in the US, however the catfish farming is in crisis recently (Hanson and Sites, 2012). A effective method to improve the production and efficiency is exploring fish genetics (Dunham, 2011) but sterilization is needed to prevent potential environmental and ecological risk for certain genetic manipulations.

Gonadotropin-releasing hormone (GnRH) regulates reproduction (Fernald and White, 1999) and stimulates the synthesis of gonadotropin. Luteinizing hormone (LH) stimulates the steroid hormone secretion, boosts ovarian and testicular function and plays a key role during gonadal maturation and spermiation/ovulation (Levavi-Sivan et al., 2010). Deactivating LH and GnRH genes function has the potential possibility of inducing fish sterilization.

In this study, we utilize CRISPR/Cas9 technology for the targeted gene disruption of LH gene and cfGnRH gene, to sterilize channel catfish. Both electroporation and microinjection approaches were performed and evaluated, and we aim to produce sterile channel catfish with CRISPR/Cas9 targeted gene editing.

2 Material and Methods

2.1 Construction of the CRISPR sgRNA and Cas9 nuclease plasmids

The CRISPR/Cas9 system was utilized in this project to mutate channel catfish (*I. punctatus*) LH gene β subunit (AF112192) (Liu et al., 2001) and the cfGnRH gene (data unpublished) for

sterilization. To mutate these two genes using electroporation, CRISPR sgRNA plasmids were obtained from the Transposagen Company (Lexington, KY). These plasmids (CRISPR/LH-U6 and CRISPR/GnRH-U6) were driven by the U6 promoter, and the sgRNA scaffold was fused following the promoter (Fig.21). Ampicillin resistance element was also inserted to assist selection.

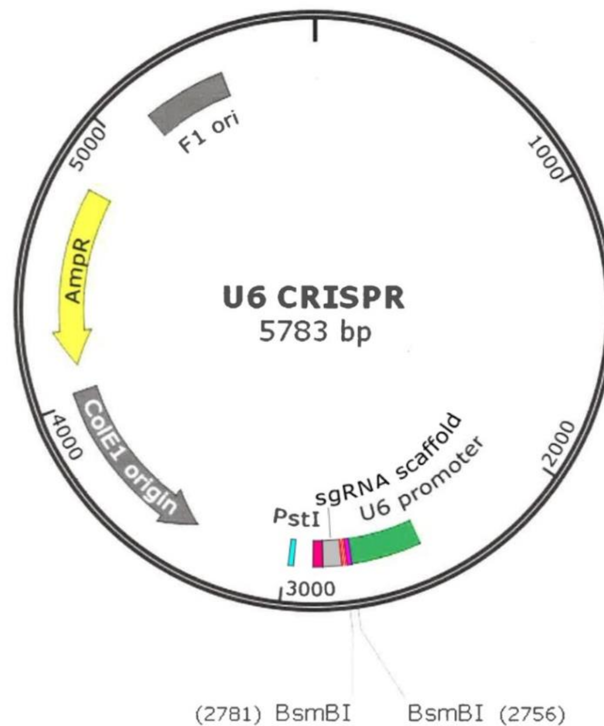

Fig.21 Schematic representation of clustered regularly interspaced short palindromic repeats (CRISPR) sgRNA plasmid used for targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene with electroporation.

The Cas9 plasmid (pCS2-nCas9n), which was electroporated together with the two CRISP-U6 sgRNA plasmids, was obtained from Addgene (Cambridge, MA). pCS2-nCas9n plasmid was driven by the CMV promoter and contains the ampicillin selection element (Fig.22).

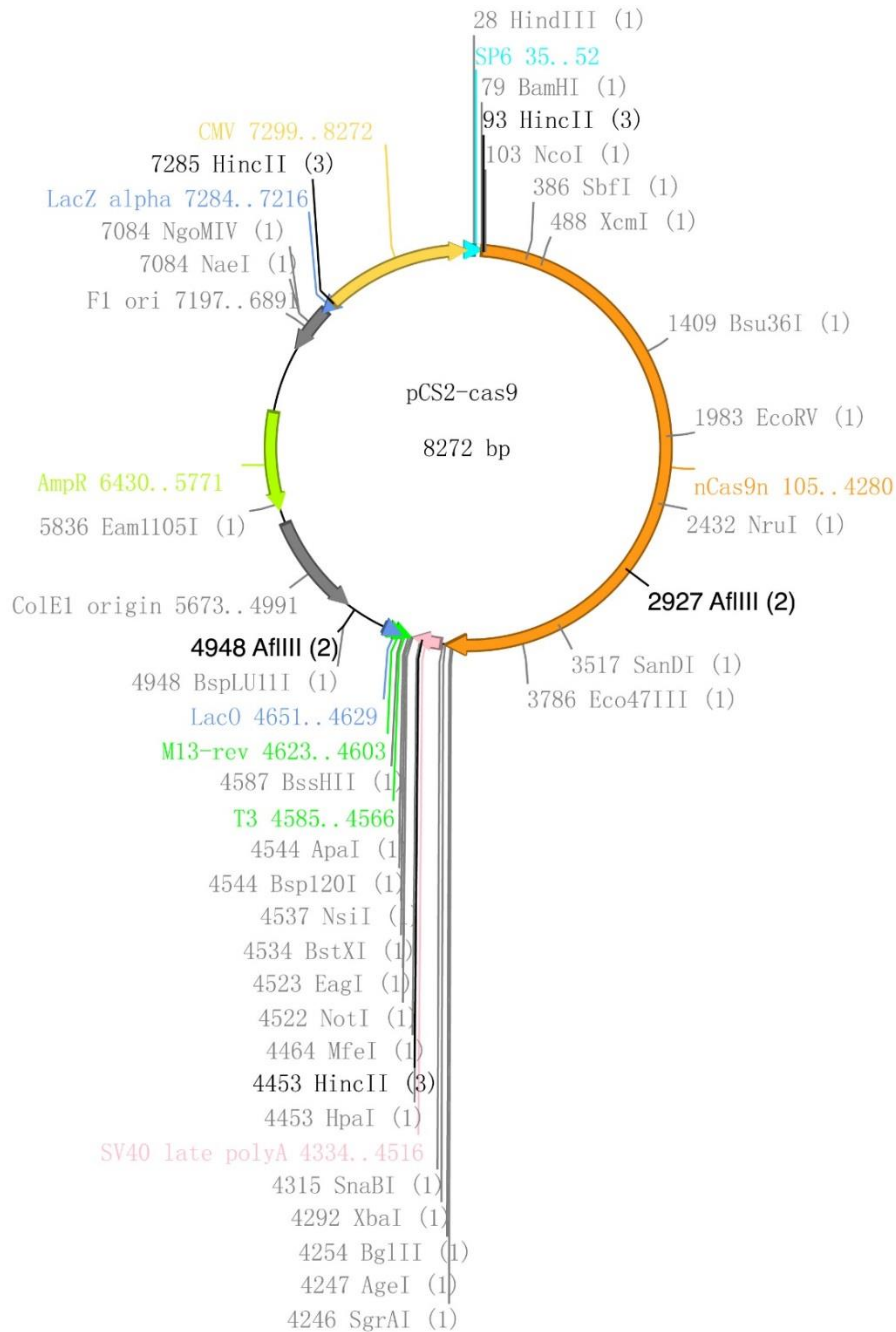

Fig.22 Schematic representation of Cas9 endonuclease plasmid used for electroporation targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) and catfish type gonadotropin-releasing hormone (cfGnRH).

The CRISPR plasmid (CRISPR/GnRH-T7) and Cas9 plasmid (Cas9-T7) utilized to mutate cfGnRH gene with microinjection technique were obtained from Transposagen Company (Lexington, KY). CRISPR/GnRH-T7 plasmid was driven by T7 promoter and contains the kanamycin resistance element (Fig.23), while the Cas9-T7 plasmid was driven by both CMV and T7 promoters (Fig.24).

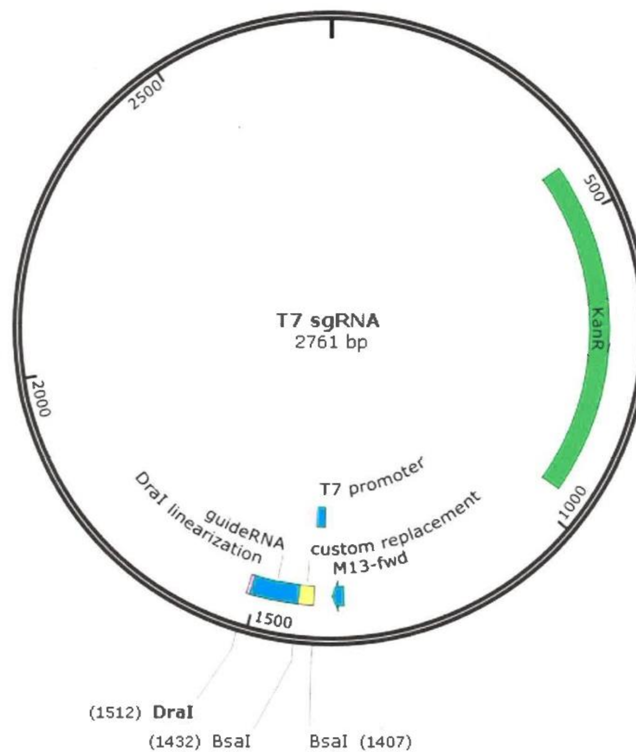

Fig.23 Schematic representation of clustered regularly interspaced short palindromic repeats (CRISPR) sgRNA plasmid used for in vitro RNA synthesis and targeting channel catfish (*Ictalurus punctatus*) catfish type gonadotropin-releasing hormone (cfGnRH) gene.

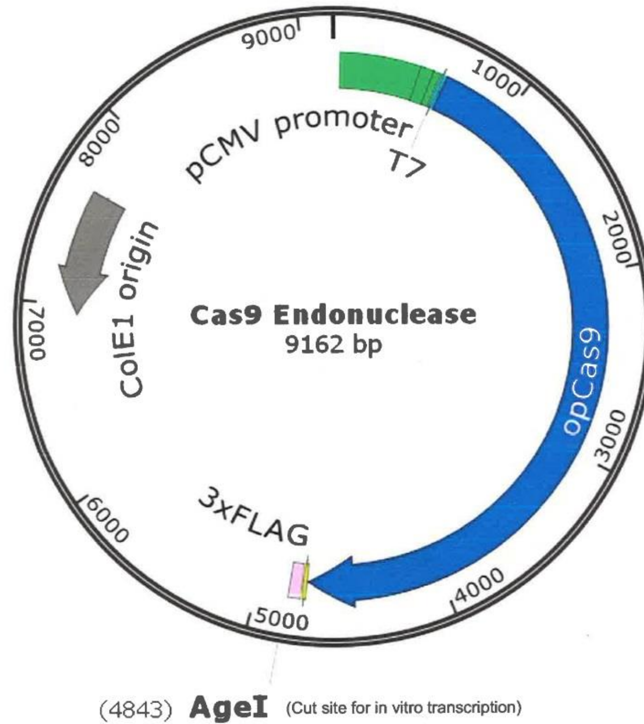

Fig.24 Schematic representation of Cas9 endonuclease plasmid used for in vitro mRNA synthesis and microinjection targeting channel catfish (*Ictalurus punctatus*) catfish type gonadotropin-releasing hormone (cfGnRH) gene.

The target sequences of sgRNA plasmids in channel catfish LH gene and cfGnRH gene are shown in Fig.25.

1) LH sgRNA target sequence

CCTTCTCCCCCGCTCAAAGCTACATTCTGCCACACTGCGAACCT

2) GnRH sgRNA target sequence

TGCCGAGGACCTCCGGCTACGTGTGTGATTACGTAGATGTTTCA

Fig.25 Clustered regularly interspaced short palindromic repeats (CRISPR) plasmid sets targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene. sgRNA recognition and binding sites are in grey.

The targeting site of LH gene was located in the first exon (Fig.26) and the targeting sire of cfGnRH was in the third exon. Upon successful mutation, both of them should interrupt the normal gene function.

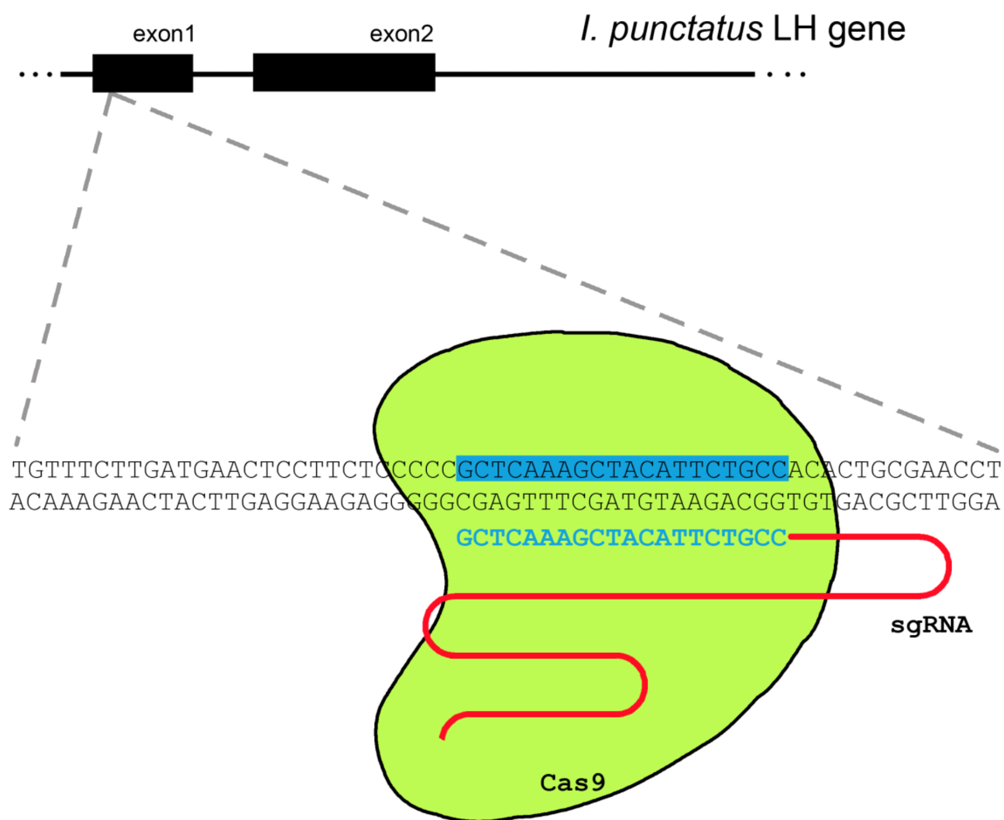

Fig.26 The location of the clustered regularly interspaced short palindromic repeats (CRISPR) target site in the channel catfish, *Ictalurus punctatus*, luteinizing hormone (LH) gene. Exons and introns of LH gene are indicated by black boxes and lines, respectively. sgRNA targeting sequences were in blue color.

2.2 Plasmid preparation

The CRISPR/LH-U6, CRISPR/GnRH-U6 and pCS2-nCas9n plasmids were transformed into One Shot Top 10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY) and 100 µl transformation mix of each plasmid was used to spread on the LB agar plate with 100 µg/ml ampicillin. Single colonies were pick up and cultured in 400 ml LB broth with 100 µg/ml ampicillin and plasmids were then extracted with the IsoPure Plasmid Maxi II Prep Kit (Denville,

Holliston, MA). The quality and quantity were examined with gel electrophoresis and spectrophotometry. The same procedure was performed to amplify CRISPR/GnRH-T7 plasmids with the only modification of using 50 μg/ml kanamycin.

CRISPR/LH-U6 plasmids and CRISPR/GnRH-U6 plasmids were mixed together with pCS2-nCas9n plasmids, respectively, and diluted with 2 ml saline (0.9% NaCl) to the final concentration of 25μg/ml each for the first electroporation of sperm. Additionally, plasmids were diluted with 5ml TE buffer (5mM Tris-HCl, 0.5M EDTA, pH=8.0), for the second electroporation of embryos.

2.3 mRNA preparation

The CRISPR/GnRH-T7 plasmid and Cas9-T7 plasmid were utilized for in vitro transcription of sgRNA and Cas9 mRNA, used for the targeted mutation of channel catfish cfGnRH gene by microinjection. Plasmids were first linearized with restricted endonuclease digestion. Ten μg of CRISPR/GnRH-T7 was mixed with 4 μl *Dra*I (20U/μl) (NEB, Ipswich, MA), 5 μl NEBuffer (10x) and diluted with water to 50 μl. 10 μg Cas9-T7 plasmids was mixed with 16 μl *Age*I (5U/μl) (NEB, Ipswich, MA), 5μl NEBuffer (10x) and diluted to 50μl. Digestion systems were incubated 37℃ for 2 hours. Linearized plasmids were inspected with gel electrophoresis and purified with MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA).

The linearized plasmids were then in vitro transcribed to sgRNA and Cas9 mRNA with MessageMAX T7 ARCA-Capped Message Transcription Kit (CellScript, Madison, WI) following the manufacturer's instruction. Synthesized Cas9 mRNA was added with the PolyA tail by Poly(A) Polymerase Tailing Kit (Epicentre, Madison, WI).

sgRNA was purified with mirVana miRNA Isolation Kit (Ambion, Grand Island, NY) and Cas9 mRNA was purified by MEGAclear Kit (Ambion, Grand Island, NY) following the instructions. RNA quality and quantity were inspected with spectrophotometry, and the RNA stored at -80℃ until use.

2.4 Brood stock spawning

Sexually mature Kansas random channel catfish females were implanted with luteinizing hormone releasing hormone analog (LHRHa) at 90 μg/kg body weight to facilitate ovulation. Eggs were stripped from two ovulating females into metal pie pans coated with grease (Crisco, Orrville, OH). Two male channel catfish (Kansas random and AR) (Dunham and Smitherman, 1984) were euthanized and the testes were macerated into saline (0.9 % NaCl) to release sperm and produce a sperm homogenate.

2.5 Fertilization, electroporation and microinjection

Double electroporation was performed for each of the CRSPR/Cas9 plasmids groups that targeting the channel catfish LH gene and cfGnRH gene described above with a Baekon 2000 macromolecule transfer system (Baekon, Inc., Saratoga, CA) with parameters set at 6 kV, 2^7 pulses, 0.8s burst, 4 cycles, 160 μs (Powers et al., 1991). Briefly, channel catfish sperm were electroporated with one of the CRISPR/Cas9 plasmids sets, then two hundred eggs were fertilized with the electroporated sperm. Sixty minutes later, embryos were collected and incubated in plasmid solution for 10 minutes, followed by the second electroporation. The same procedure was

performed on the control group, but without plasmids.

sgRNA that targeted cfGnRH gene was also microinjected together with the Gas9 nuclease mRNA. Channel catfish eggs were fertilized with normal sperm, and incubated in fresh water for 50min. One hundred eggs were picked and placed on a 10ml petri dish. One hundred picogram sgRNA and three hundred picogram Cas9 mRNA mixture were injected into the blastodisc using the Eppendorf Microinjector 5242 system (Hamburg, Germany). The control group was microinjected with the same solution without RNAs.

Then embryos were moved into 10L tubs filled with Holtfreter's solution (Bart and Dunham, 1996) containing 10ppm doxycycline and incubated at 27℃ until hatch. Dead embryos were removed and water was changed daily. Channel catfish fry were then transferred into a recirculating system.

2.6 Sample collection, DNA extraction and mutation analysis

The pelvic fin and barbel of 6-month-old fingerlings were sampled for DNA analysis. Samples were digested with 100 μg/ml proteinase K followed by protein precipitation and DNA ethanol precipitation as described by Kurita et al. (2004). DNA quantity and quality were determined with gel electrophoresis and spectrophotometry.

Channel catfish LH gene β subunit specific primer and cfGnRH gene specific primers (Table 9) were designed and Roche Expand High Fidelity^{Plus} PCR System (Roche, Indianapolis, IN) was used to amplify these DNA samples. The PCR amplification procedure was as follows: initial denaturation for 2min at 94℃; followed by 35 cycles of 94℃ for 30s, 60℃ for 30s and 72℃ for

1min; and a final elongation for 10min at 72 ℃. PCR products were examined by gel electrophoresis.

Table 9 Primer sequences used for the amplification of luteinizing hormone (LH) β subunit and catfish type gonadotropin-releasing hormone (cfGnRH) in channel catfish (*Ictalurus punctatus*).

Primer	Sequence (5'-3')	Product size (bp)	Description
LH-F LH-R	AGGATGTCAGTGCCAGCTTC CTTGGAGTAAATGGACTCGTTG	572	LH gene amplification and mutation analysis
GnRH-F GnRH-R	ATGGATGCTGTCTTTGTTTTCC CCACACGAAATAAAGGCAAAG	550	cfGnRH gene amplification and mutation analysis

Gene mutations were detected through the Cel-I mutation detection assay with SURVEYOR Mutation Detection Kit (Integrated DNA Technologies, Coralville, IA) as described by Miller et al. (2007). Briefly, PCR products were denatured and re-annealed as follows: 94℃ 10min; 94℃ to 85℃ -2℃/s; 85℃ to 25℃ -0.1℃/s; cooling to 4℃; then 1 µl Enhancer S and 1 µl Nuclease S was added into 5 µl of the products above and incubated at 42℃ for 30min; The digested PCR products were resolved on 2% UltraPure Agrose-1000 high resolution agarose gel (Invitrogen, Grand Island, NY).

2.7 TA clone and sequencing

To identify the exact modification of each gene, PCR products amplified from each individual of different groups were purified with IsoPure DNA Purification Kit (Denville, Holliston, MA), then inserted into the vector of TOPO TA Cloning Kit for Sequencing (Invitrogen, Grand Island,

NY) and transformed into the One Shot TOP10F' Chemically Competent *E.coli* (Invitrogen, Grand Island, NY). Colonies were selected from a LB agar plate containing 100 μg/ml ampicillin.

Ten colonies corresponding to each individual carrying mutated genes were picked and amplified. The bacteria glycerol stock were added into 96-well plates and sent to Eurofins Genomics Company (Louisville, KY) for sequencing.

Upon receiving the results, the quality was checked and the sequences were aligned with the wild type channel catfish LH and cfGnRH genes using the online multiple sequence alignment tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), respectively.

2.8 Plasmid integration inspection

To determine the presence of plasmids integrated into the channel catfish genome or persisting in the cytoplasm, two pairs of specific primers (Table 10) for each of the CRISPR/LH-U6, CRISPR/GnRH-U6 and pCS2-nCas9n plasmids were designed to detect the plasmid DNA in corresponding mutated channel catfish. The amplification regions of primers were the CMV promoter region, U6 promoter region and the backbone of plasmids, respectively. The PCR procedure was the same as of amplifying LH and cfGnRH genes and products were inspected with electrophoresis.

Table 10 Primer sequences used for the detection of clustered regularly interspaced short palindromic repeats (CRISPR) and Cas9 plasmids targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene.

Primer	Sequence (5'-3')	Product size (bp)	Description
LI1-F LI1-R	GCCTATTTCCCATGATTCCT ACTGCAAACTACCCAAGAAA	145	CRISPR/LH-U6 plasmids integration detection
LI2-F LI2-R	ATTTCTTGGGTAGTTTGCAG CTTTCAAGTTACGGTAAGCA	173	CRISPR/LH-U6 plasmids integration detection
GI1-F GI1-R	TTCATCCATAGTTGCCTGAC ATAAAGTTGCAGGACCACTT	176	CRISPR/GnRH-U6 plasmids integration detection
GI2-F GI2-R	ATCTTACCGCTGTTGAGATC ACGCTGGTGAAAGTAAAAGA	183	CRISPR/GnRH-U6 plasmids integration detection
CI1-F CI1-R	CTTCCTAATACCGCCCATAG AACGGATATGAATGGGCAAT	222	pCS2-nCas9n plasmids integration detection
CI2-F CI2-R	AAACCAACAGGAAAGTGACT CATTCCTCTGTCCTCAAACA	233	pCS2-nCas9n plasmids integration detection

2.9 Statistical analysis

Mutation rates and survival rates from different CRISPR/Cas9 sets were analyzed utilizing Fisher's Exact Test and Pearson's Chi-square Test (McDonald, 2014). All analyses were performed with statistical software R (version 3.1.3).

3 Results

3.1 Hatch rate and survival rate

Two hundred eggs were double electroporated with the CRISPR/Cas9 plasmids targeting channel catfish LH gene, cfGnRH gene groups or with buffer only. In the LH mutation group, 46 eggs hatched (hatch rate 23.0%) (Table 11) and after 6 months, only 13 were still alive (survival rate 28.3%). A similar result was for the cfGnRH mutation group, with 42 eggs hatched (21.0%)

and only 8 fingerlings were alive (19.1%) after 6 months. For the control group, 55 of 200 eggs hatched in the control group (27.5%) and 14 of them were alive (25.5%). There was no significant difference between the treatment and control groups for both embryo hatch rate (Pearson's Chi-square Test, p=0.295) and fry survival rate (Fisher's Exact Test, p=0.610).

One hundred eggs were microinjected with sgRNA and Cas9 mRNA targeting the cfGnRH gene. Only 9% hatched and after 6 months only 4 were alive (survival rate 44.4%). For the control group, 11% hatched and 5 survived (45.5%). There was no difference between these two groups for mutation or survival rate (Fisher's Exact Test, p=0.814).When comparing hatch rate and survival rate between electroporated and microinjected eggs and fry, embryo hatch rates were found to be different between these two techniques (Pearson's Chi-square Test, p=0.0004), however, there was no difference between the fry survival rates (Fisher's Exact Test, p=0.301).

Table 11 Comparison of the embryo hatch rate and fry survival rate for clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 targeting channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene or catfish type gonadotropin-releasing hormone (cfGnRH) gene.

Constructs and method	Eggs N	Fry N	Hatch rate (%) [†]	Fingerlings N	Survival rate (%) [*]
Electroporation					
CRISPR-LH	200	46	23.0[a]	13	28.3
CRISPR-GnRH	200	42	21.0[a]	8	19.1
Control	200	55	27.5[a]	14	25.5
Microinjection					
CRISPR-GnRH	100	9	9.0[b]	4	44.4
Control	100	11	11.0[b]	5	45.5

[†] Different letters indicate significant difference between electroporation and microinjection (p=0.0004).

[*] No significant difference between electroporation and microinjection (p=0.301).

3.2 Mutation rate

Five of 13 individuals electroporated with CRISPR/Cas9 plasmids targeting channel catfish LH gene group and 3 of 8 individuals electroporated with CRISPR/Cas9 plasmids targeting channel catfish cfGnRH gene group (Fig.27) carried the mutated gene (Table 12), as indicated by the clear three-banded pattern produced gel electrophoresis (Fig.27), with mutation rates of 38.5% and 37.5%, respectively. No control fish were mutated.

Table 12 Comparison of luteinizing hormone (LH) gene and catfish type gonadotropin-releasing hormone (cfGnRH) gene mutation rate with electroporation and microinjection of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system in channel catfish (*Ictalurus punctatus*).

CRISPR	Procedure	N	N mutated	Mutation rate (%)
LH	Electroporation	13	5	38.5
GnRH	Electroporation	8	3	37.5
Control	Electroporation	14	0	0
GnRH	Microinjection	7	7	100
Control	Microinjection	5	0	0

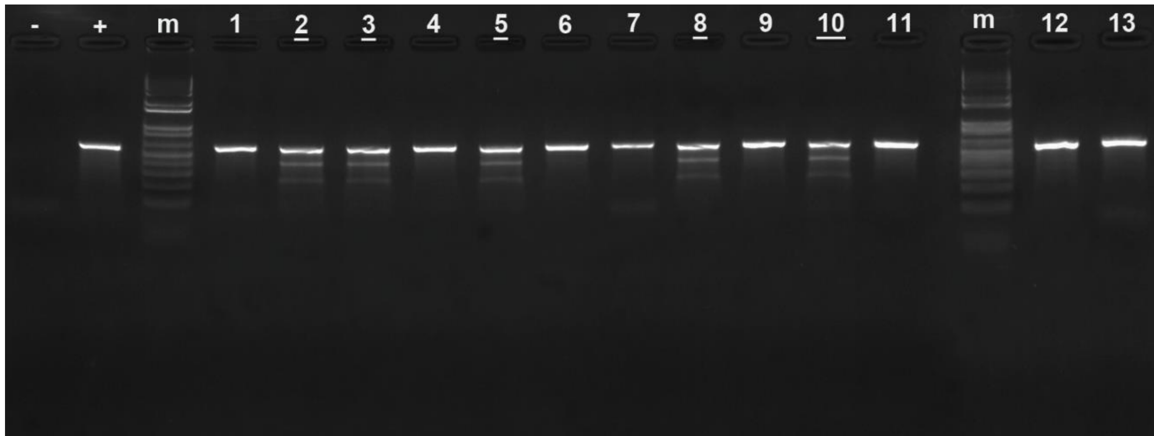

Fig.27 Identification of edited luteinizing hormone (LH) gene in channel catfish (*Ictalurus punctatus*) electroporated with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 plasmids using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Three bands indicate mutations occurred and the mutated channel catfish are underlined.

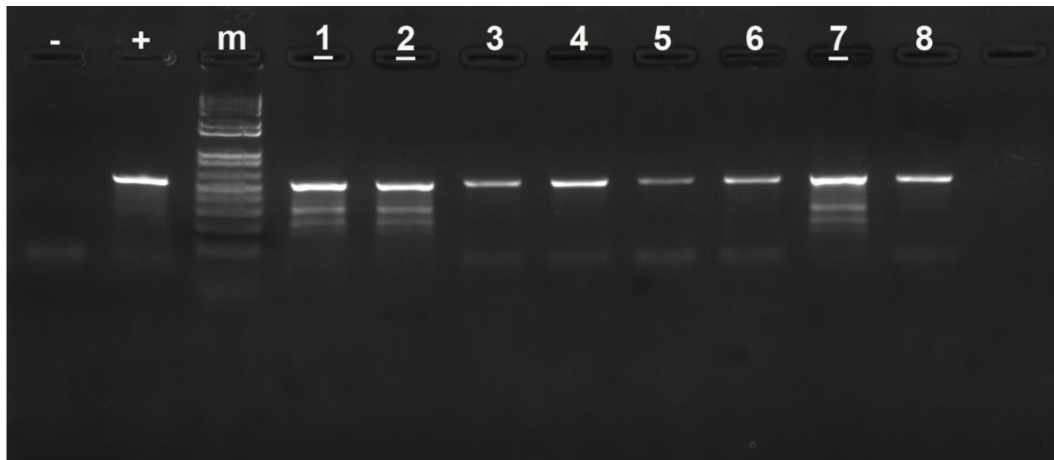

Fig.28 Identification of edited catfish type gonadotropin-releasing hormone (cfGnRH) gene in channel catfish (*Ictalurus punctatus*) electroporated with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 plasmids using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Three bands indicate mutations occurred and the mutated channel catfish are underlined.

When sgRNA and Cas9 mRNA was delivered via microinjection to target cfGnRH gene, 7 of 7 individuals were mutated (Table 12). The DNA band pattern was similar as that found in the TALEN experiment (Fig.14, Chapter III). There were multiple bands on the gel, indicating cfGnRH gene was edited when compared with the control (Fig.29). The mutation rate in this group was 100%.

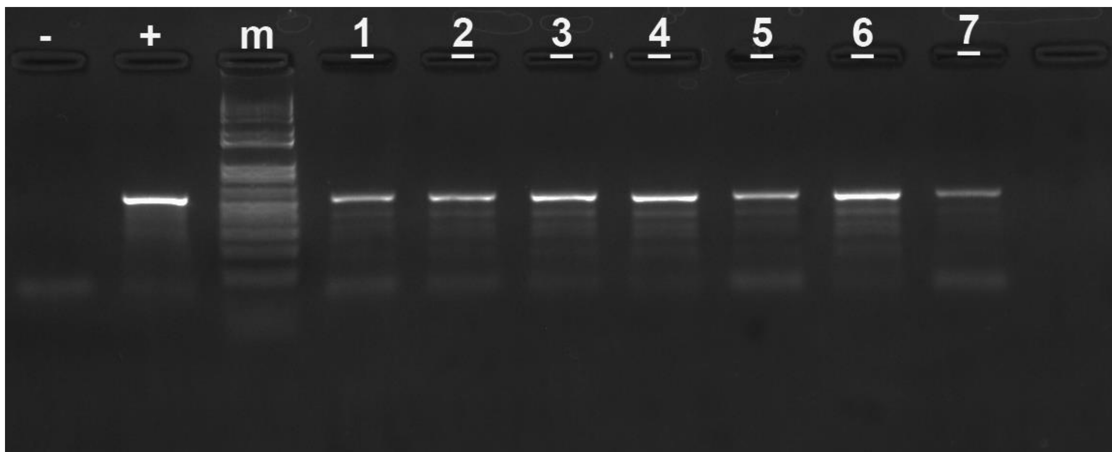

Fig.29 Identification of edited catfish type gonadotropin-releasing hormone (cfGnRH) gene in channel catfish (*Ictalurus punctatus*) microinjected with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 RNAs using Cel-Ⅰ mutation detection assay. "-" indicates the negative control without template; "+" indicates the control with wild-type channel catfish DNA as template; "m" indicates 1 kb DNA ladder. Numbers represent different channel catfish tested. Multiple bands indicate mutations occurred and the mutated channel catfish are underlined.

3.3 Sequence modification of the mutated genes

In the LH gene mutated fingerlings, mutations occurred within the CRISPR targeting site. For all 5 individuals evaluated, only deletions existed based on the alignment result (Fig.30).

```
TGAACTCCTTCTCCCCCGCTCAAAGCTACATTCTGCCACACTGCGAACC     wt
TGAACTCCTTCTCCCCCGCTCAAAGCTACATTC-GCCACACTGCGAACC
TGAACTCCTTCTCCCCCGCTCAAAGCTACATTCT--CACACTGCGAACC
TGAACTCCTTCTCCCCCGCTCAAAGCTACATT----CACACTGCGAACC     deletion
TGAACTCCTTCTCCCCCGCTCAAAGCTACATT------CACTGCGAACC
TGAACTCCTTCTCCCCCGCTCAAAGC--------GCCACACTGCGAACC
```

Fig.30 Sequences of channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH) gene with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 plasmids electroporation induced mutations. The wild-type (wt) channel catfish LH gene sequence is shown on the top. Sequences underlined are the CRISPR sgRNA binding sites; red dashes indicate the modified nucleotides of LH gene.

The mutated cfGnRH gene generated by the CRISPR/Cas9 plasmids electroporation also showed only deletions based upon the results found from 3 individuals (Fig.31).

```
GAAATGCCGAGGACCTCCGGCTACGTGTGTGATTACGTAGATGTTTCAC     wt
GAAATGCCGAGGACCTCCGGCTAGTGTGTGG--TACGTAGATGTTTCAC
GAAATGCCGAGGACCTCCGGCTAGTTGTGTGA--ACGTAGATGTTTCAC     deletion
GAAATGCCGAGGACCTCCGGCTAGTTGTG-----ACGTAGATGTTTCAC
```

Fig.31 Sequences of channel catfish (*Ictalurus punctatus*) catfish type gonadotropin-releasing hormone (cfGnRH) gene with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 plasmids electroporation induced mutations. The wild-type (wt) channel catfish cfGnRH gene sequence is shown on the top. Sequences underlined is the CRISPR sgRNA binding sites; red dashes indicate the modified nucleotides of cfGnRH gene.

In the microinjection experiment with CRISPR/Cas9 mRNA targeting cfGnRH gene, multiple types of mutations were generated. Four fish lost nucleotides at the cutting site, 1 had a one base substitution and the remainder had nucleotides insertions (Fig.32).

Similar to the TALEN experiment, CRISPR/Cas9 showed high gene targeting specificity as all these mutations located within the expected targeting sites in the ORF. These mutations should lead to a frame-shift or early termination in transcription and disrupt normal gene functions.

```
GAAATGCCGAGGACCTCCGGCTACGTGTGTGATTACGTAGATGTTTCAC      wt
GAAATGCCGAGGACCTCCGGCTACGTGTGTG-TTACGTAGATGTTTCAC      deletion
GAAATGCCGAGGACCTCCGGCTACGTGTG--ATTACGTAGATGTTTCAC      deletion
GAAATGCCGAGGACCTCCGGCTACGTGTGTG--TACGTAGATGTTTCAC      deletion
GAAATGCCGAGGACCTCCGGCTACGTGTGTGTTTACGTAGATGTTTCAC      substitution
GAAATGCCGAGGACCTCCGGCTACGTGTGTGATATTACGTAGATGTTTCAC    insertion
GAAATGCCGAGGACCTCCGGCTACGTGTGTGATCCTTACGTAGATGTTTCAC   insertion
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Fig.32 Sequences of channel catfish (*Ictalurus punctatus*) catfish type gonadotropin-releasing hormone (cfGnRH) gene with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 RNAs microinjection induced mutations. The wild-type (wt) channel catfish cfGnRH gene sequence is shown on the top. Sequences underlined is the CRISPR sgRNA binding sites; red letters/dashes indicate the modified nucleotides of cfGnRH gene.

3.5 CRISPR and Cas9 plasmids integration evaluation

Two pairs of specific primers for each of the CRISPR/LH-U6, CRISPR/GnRH-U6 and pCS2-nCas9n plasmids (Table 10) were used to detect the presence of plasmids DNA in the electroporation experiment in all of the mutated individuals. Similar to the results of the other

experiments, no DNA of the CRISPR/LH-U6 (Fig.33), CRISPR/GnRH-U6 (Fig.34) and pCS2-nCas9n plasmid (Fig.35) was detected with PCR. All these results indicated neither the CRISPR plasmids nor the Cas9 plasmid was present in mutated channel catfish.

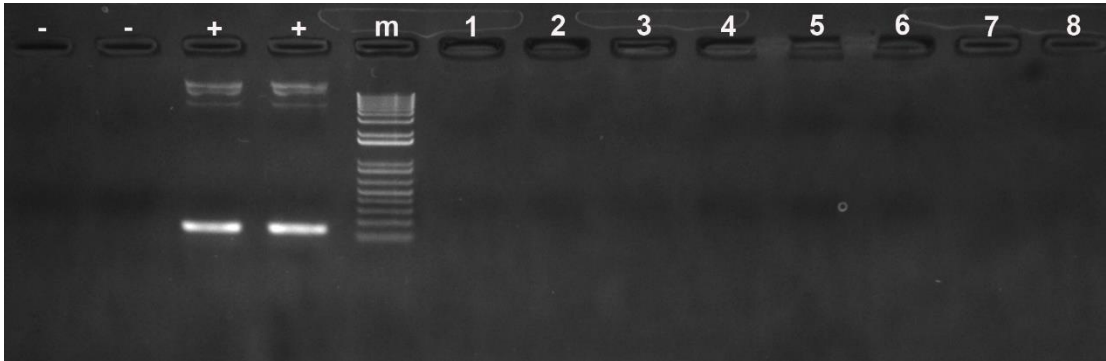

Fig.33 PCR inspection of clustered regularly interspaced short palindromic repeats (CRISPR) plasmid (targeting the luteinizing hormone (LH) gene) integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st lane) and with wild-type channel catfish (2nd lane) as template, respectively; "+" indicates the positive controls with CRISPR/LH-U6 plasmid as template; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated LH gene.

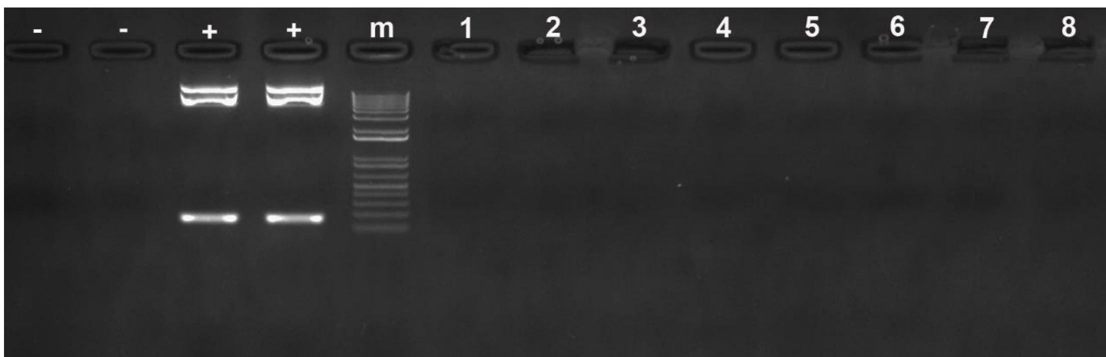

Fig.34 PCR inspection of clustered regularly interspaced short palindromic repeats (CRISPR) plasmid (targeting the catfish type gonadotropin-releasing hormone (cfGnRH) gene) integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st lane) and with wild-type channel catfish (2nd lane) as template, respectively; "+" indicates the positive controls with CRISPR/GnRH-U6 plasmid as template; "m" indicates 1 kb DNA ladders. Numbers represent channel catfish individuals carrying mutated cfGnRH gene.

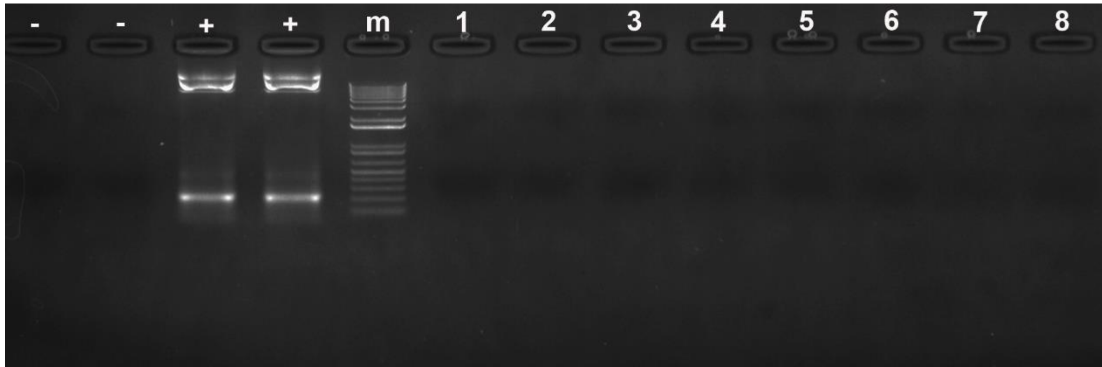

Fig.35 PCR inspection of Cas9 plasmid integration into channel catfish (*Ictalurus punctatus*) genome. "-" indicate the negative controls without template (1st lane) and with wild-type channel catfish (2nd lane) as template, respectively; "+" indicates the positive controls with pCS2-nCas9n plasmid as template; "m" indicates 1 kb DNA ladders. Numbers represent gene mutated channel catfish individuals.

4 Discussion

Gene editing of channel catfish LH gene and cfGnRH gene, using the CRISPR/Cas9 technology with both electroporation and microinjection was accomplished. The mutation rate varied from ~38% with electroporation to 100% with microinjection. Additionally, embryo hatch rate and fry survival rate of the treatment groups and control groups did not differ. Thus, CRISPR/Cas9 technology did not have negative effects on the development of embryos and fry, and off-target mutations did not occur or were minimal and did not affect embryonic development and early survival. Several types of mutations were induced at the targeting sites with sequencing confirmation. This is the first time of using CRISPR/Cas9 to perform mutagenesis on genomic level in a major US aquaculture species.

CRISPR/Cas9 system is a newly developed technology but has been successfully utilized for targeted gene disruption in lots of animal models, such as nematode (*Caenorhabditis elegans*)

(Friedland et al., 2013; Ward, 2015), fruit fly (*Drosophila*) (Gratz et al., 2013), frog (*Xenopus tropicalis*) (Nakayama et al., 2013), zebrafish (*Danio rerio*) (Chang et al., 2013; Hwang et al., 2013; Jao et al., 2013; Xiao et al., 2013), mice (Wang et al., 2013; Shen et al., 2013; Wu et al., 2015) and human cells (Cong et al., 2013; Hwang et al., 2013; Mali et al., 2013). The mutation rate varies in different studies which was as low as 2%-4% (Mali et al., 2013) to as high as 75%-99% (Jao et al., 2013). In the present experiment, the mutation rate for LH gene and cfGnRH gene were 38.5% and 37.5%, respectively, when using electroporation method, while the cfGnRH gene mutation rate was 100% when using microinjection. All these mutation rates are comparable with other studies and we successfully utilized the CRISPR/Cas9 technology in channel catfish gene mutagenesis.

Similar to the TALEN experiments, the sequences of the detected mutations in channel catfish DNA were all located in the CRISPR sgRNA binding sites within the gene ORF. The mutations should disrupt normal gene expression and likely will cause loss of gene function. Therefore, CRISPR/Cas9 system also has higher specificity in the targeted gene editing experiments, less off-target effect, and thus low cell toxicity, which is similar to what was achieved in zebrafish (Jao et al., 2013).

Up to now, overwhelming majority of works using CRISPR/Cas9 system for targeted gene mutagenesis was done by microinjection (Chang et al., 2013; Jao et al., 2013). Recently, a few researchers edited genes mice by delivering CRISRP/Cas9 RNAs into zygotes with electroporation (Hashimoto and Takemoto, 2015; Qin et al., 2015), indicating electroporation is a good delivery approach of CRISPR/Cas9. Additionally, our studies using ZFN and TALEN successfully mutated

channel catfish genes indicated electroporation with plasmids is also a promising method for gene editing.

In the present study, electroporation and microinjection with CRIPR/Cas9 system were compared. With electroporation of plasmids, channel catfish LH gene and cfGnRH gene mutation rate were 38.5% and 37.5%, respectively. When microinjection of mRNA was performed, the cfGnRH gene mutation rate rose to 100%, which is significant higher than that of electroporation (Fisher's Exact Test, p=0.0159). A potential explanation is that when using microinjection, it is assured each egg was successfully injected with CRISPR sgRNA and Cas9 mRNA, as the whole procedure was monitored under the microscope. But when using electroporation, it is possible that some eggs were not punched with micro holes in the egg shell or the plasmids did not reach the embryo proper. These eggs will not be affected by CRISPR/Cas9 resulting in microinjection providing a higher success rate.

However when comparing the egg hatch rate, there is significant difference between these two techniques. The egg hatch rates were 23.0% and 21.0% for LH and cfGnRH mutation groups with electroporation, respectively. This rate dropped to only 9.0% when using microinjection. Physical damage from the microneedle during microinjection is the likely cause of the reduced hatch.

CRISPR/Cas9 has proved to have a low incidence of off-target effects and a high gene mutation rate. If the editing of LH gene and cfGnRH gene of channel catfish realizes sterilization, it could be applied to overcome potential environmental and ecosystem risk, and profit the catfish farming industry.

5 Conclusion

We successfully performed CRISPR/Cas9 technology for targeted gene editing in channel catfish *I. punctatus* in this study. Both electroporation and microinjection approaches were applied and channel catfish reproductive related LH β subunit as well as cfGnRH gene were successfully mutated. This is the first time that CRISPR/Cas9 system has been used to edit genes of an aquaculture species in the US.

CRISPR/Cas9 system is a good technology for targeted gene manipulation and both electroporation and microinjection could induce high success rates. Whether to choose electroporation or microinjection could be decided by the aim of the study, and for large scale application, electroporation should be superior because of its simplicity and convenience.

Further study is needed to evaluate the fertility status of LH and cfGnRH mutated individuals when they reach sexual maturity. The restoration of fertility with hormone therapy will also need to be developed and evaluated to allow production of fertile brood stock to complete the reversible sterilization in channel catfish.

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V Conclusion

In the present study, channel catfish (*Ictalurus punctatus*) luteinizing hormone (LH), follicle-stimulating hormone (FSH) and catfish type gonadotropin-releasing hormone (cfGnRH) genes were mutated using the targeting gene editing technologies zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. ZFN, TALEN and CRISPR/Cas9 plasmids were delivered into channel catfish embryos through electroporation, and CRISPR/Cas9 RNAs were also introduced with microinjection. All approaches achieved success in gene mutagenesis, and LH, FSH and cfGnRH gene sequences were altered that should lead to lose of function. This is the first time that the three targeted gene editing technologies have been compared to induce mutations in the major US aquaculture species using plasmids, the first time that they have used to mutate a major US aquaculture species and the first time that mRNA microinjection has been directly compared to plasmid electroporation for efficacy.

Three sets of ZFN plasmids with different concentrations were electroporated to target LH gene and the overall mutation rate was 19.7%, among which ZFN set 1 with concentration 25 μg/ml was the best treatment. TALEN plasmids targeting LH, FSH and cfGnRH genes were electroporated into embryos and the mutation rates of were higher than that of ZFNs, 44.7%, 63.2%

and 52.9%, respectively. For the CRISPR/Cas9 study when plasmids were transferred targeting LH and cfGnRH genes through electroporation, 38.5% and 37.5% mutation rates were obtained, respectively. When CRISPR/Cas9 RNAs were microinjected into embryos, the mutation rate rose to 100%. The mutation rates of TALEN exposed embryos were significant higher than that of ZFN groups (p=0.0125, p=0.0001, p=0.0003), but there was no difference among ZFN and CRISPR/Cas9 groups when using electroporation (p=0.159, p=0.595). However, when performing microinjection using CRISPR/Cas9 RNAs, the mutation rate was much higher (p<0.0001) than ZFN exposed embryos.

The embryo hatch rates of three ZFNs groups were 23.5%, 19.0% and 21.0%, respectively, and there was no difference among them (p=0.544), and they were lower than the control group, 48.0% (p<0.0001). In TALEN experiments, embryos exposed to LH, FSH and cfGnRH plasmids had hatch rates of 33.5%, 40.0% and 36.5%, respectively, and did not differ from the control group, 44.0% (p=0.340). Electroporating CRISPR/Cas9 plasmids into embryos did not alter the hatch rates, which were 23.0%, 21.0% and 27.5% in LH, cfGnRH and control groups. Meanwhile, no difference existed between CRISPR/Cas9 RNA microinjected and control groups hatch rates, 9.0% and 11.0%, respectively. However, comparing electroporation with microinjection in CRISRP/Cas9 experiment, we found the hatch rate of embryos under microinjection was much lower than that of electroporation (p=0.0004). The only treatment that decreased embryos hatch rate when compared to the control group was ZFN technology. A possible explanation is that ZFN has higher off-target effects during embryos development, and thus induces higher cytotoxicity. Microinjection led to much lower embryo hatch rate, which may result from physical damage from

the microneedle during the microinjection process.

Similar to embryos hate rates, the fry survival rates from different technologies varied. In ZFN treatment groups, the survival rates were different among different ZFN sets (68.1%, 31.6% and 52.4%, p=0.004), and were also different from the control group (82.3%, p=0.033). In contrast, fry survival rates in TALEN experiment did not differ either among plasmids exposed groups (56.7%, 71.3% and 67.1%, p=0.182) or between exposed and control groups (75.0%, p=0.107). In CRISPR/Cas9 experiments, fry survival rates were 28.3%, 19.1% and 25.5% for LH, cfGnRH and control groups with electroporation, and 44.5%, 45.5% for GnRH and control groups with microinjection. No differences existed (p=0.301). Apparently, the off-target effects of ZFN technology, presumably semi-lethal and lethal mutations at other genomic locations, that occurred during embryonic development continued during the fry and fingerling life stages.

Sequencing results from the individuals which carried mutated genes indicated several types of mutations occurred, including nucleotides deletions, insertions and substitutions, in all three targeted gene editing technologies ZFN, TALEN and CRISPR/Cas9. For TALEN and CRISPR/Cas9 experiments, these mutations occurred within the expected cutting site of LH, FSH and cfGnRH genes. However, the LH mutations induced by ZFN did not occur at the targeting sites but were all located approximately 60 bp upstream. Nevertheless, all the mutations induced by these three technologies were located within the ORF of corresponding genes and should affect gene translation and normal gene function.

Four pairs of LH gene mutated channel catfish from ZFN experiment were paired but did not spawn at two years of age. Eleven pairs of LH edited channel catfish still did not spawn when

mated as three-year old despite having excellent gravidness and other secondary sexual characteristics. The desired loss of function, sterilization, was successfully achieved by ZFN targeted LH mutation, which was similar to studies conducted by knockout of LH gene in zebrafish and demonstrated LH is essential for fish reproduction. However, these fish did not spawn after hormone therapy with CPE, and hormone therapy will need to be developed by evaluating different dose applications of purified LH. Further work is needed for evaluation of fertility status of LH, FSH and cfGnRH mutated individuals, induced by TALEN and CRISPR/Cas9 technologies, when they reach sexual maturity, as well as the restoration of their fertility after hormone therapy.

Our studies with ZFN, TALEN and CRISPR/Cas9 technologies all indicated delivery of plasmids into embryos using electroporation is a promising approach to create mutagenesis. Electroporation allows transfer of plasmids to a large number of embryos in a short time, requires less effort and is applicable for large scale utilization. ZFN, TALEN and CRISPR/Cas9 plasmids were not detected in mutated channel catfish, demonstrating electroporation could be used to generate gene edited animals without the unexpected transgenesis. Microinjection of mRNA was the most effective procedure for gene editing, however, it was tedious and had low yield of surviving hatchlings. Additionally, this technology would require more effort to generate multiple lines to counteract the inbreeding and random genetic drift associated with modern biotechnologies that initially generate low numbers of founders.

This research could serve as a model to be utilized for other aquaculture species. Applications could include sterilization of domestic, interspecific hybrid, exotic or transgenic fish to minimize environmental and ecological risks, protect genetic biodiversity and increase environmental

friendliness of aquaculture and transgenic fish. The procedures developed in this study would also greatly facilitate knockout studies for functional genomic experimentation.